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The 4th Annual Symposium of the Protein Society of Thailand
Protein Research: from Basic Studies to Applications in Health Sciences

Chulabhorn Research Institute Conference Center
Vibhavadee Rangsit Highway, Bangkok 10210, Thailand
August 26-28, 2009

Organizing Committee

The 4th Annual Symposium of the Protein Society of Thailand
Protein Research: from Basic Studies to Applications in Health Sciences

Prof. M.R. Jisnuson Svasti
Prof. Worachart Sirawaraporn
Assoc. Prof. James Ketudat-Cairns
Assoc. Prof. Pimchai Chaiyen
Dr. Visith Thongboonkerd
Dr. Chantragan Srisomsap Phiphobmongkol
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Editorial Board

Proceedings of the 4th Annual Symposium of the Protein Society of Thailand
Protein Research: from Basic Studies to Applications in Health Sciences.

Preface

This Fourth Annual Symposium of Protein Society of Thailand: "Protein Research: from Basic Studies to Applications in Health Sciences" is jointly hosted by the Protein Society of Thailand, the Faculty of Science, Mahidol University, the Chulabhorn Research Institute, and the Biochemistry and Molecular Biology Section of the Science Society of Thailand under the Patronage of H.M. The King.

These Annual Meetings of the Protein Society of Thailand have become important occasions, because they have brought together protein scientists from all over Thailand to exchange knowledge, experience, and technical expertise. The present theme for this year "Protein Research: from Basic Studies to Applications in Health Sciences" has been chosen because research on proteins and enzymes continues to provide improved understanding of molecular basis of disease, novel therapeutic strategies, as well as to improve the quality of life and the environment. Many of the invited lectures are either in forefront areas of research, or are at the interface with other research areas, or use novel techniques of protein research. We have an interesting program, covering a broad range of topics in protein research. This includes 3 Invited Lectures from Overseas Speakers, 10 Invited Lectures from Local Speakers. All together we have 59 Abstracts submitted, 7 of which are selected for Oral Presentations. There will also be a Poster Competition. This year we also have 12 Proceedings in addition to regular Oral and Poster presentations. An important feature of our annual meetings is that they place emphasis on providing opportunities for young scientists, at postdoctoral and pre-doctoral level, to make oral presentations, and become better known in the field in Thailand. In addition, we try to work with companies who provide equipment and supplies for protein research, and this year apart from equipment exhibitions, we also have 2 Luncheon Lectures and 5 Company-sponsored Lectures.

On behalf of the Organizing Committee, I would like to thank all speakers and poster presenters, as well as more than 200 registered participants for their interest. We are very grateful to the Commission for Higher Education, the Chulabhorn Research Institute, Mahidol University, and Science Society of Thailand, as well as various companies for their support. I also wish like to thank the members of the Organizing Committee, and those who have helped to arrange this meeting for their assistance. I sincerely hope that you will find this meeting enjoyable and beneficial to your research.

Professor M.R. Jisnuson Svasti
Chairman, Organizing Committee,
24 July 2009

Symposium Program

The 4th Annual Symposium of Protein Society of Thailand
Protein Research: From Basic Studies to Applications In Health Sciences

26th August 2009

08.00 – 08.30 Registration & Posters Set up

08.30 – 08.40 Opening Ceremony

Proteins and Diseases

Chairperson: Prof. M.R. Jisnuson Svasti

08.40 – 09.20 **Invention of broad spectrum influenza vaccines**
Prof. Wanpen Chaicumpa (Mahidol University)

09.20 – 10.00 **Tracking for cholangiocarcinoma associated marker using monoclonal antibody approach**
Assoc. Prof. Sopit Wongkham (Khon Kaen University)

10.00 – 10.10 **Coffee/tea break**

Protein Function and Technology

Chairperson: Assoc. Prof. James Ketudat-Cairns

10.10 – 10.50 **Tight junction associated proteins: possible role in intestinal absorption of calcium**
Prof. Nateetip Krishnamra (Mahidol University)

10.50 - 11.30 **Monoclonal antibody: production and its applications on characterization of leukocyte surface molecules**
Prof. Watchara Kasinrerk (Chiang Mai University)

11.30 – 12.00 **Taiwan synchrotron facilities for bio structure and imaging research**
Dr. Keng Liang (National Synchrotron Center, Taiwan)

12.00 – 13.00 **Luncheon Lecture: Advance technology beyond proteomics information**
Zhu Hua (sponsored by GE Healthcare)

13.00 – 14.00 **PST Annual Meeting**

Selected Oral Presentation 1

Chairperson: Assoc. Prof. Pimchai Chaiyen

14.00 – 14.20 **Avian influenza A/H5N1 neuraminidase expressed in yeast with a functional head domain**
Dr. Suganya Yongkiettrakul (BIOTEC)

14.20 – 14.40 **Structural studies reveal diverse chondroitin sulphate binding sites in DB1 domains important in malaria during pregnancy**
Mr. Pongsak Khunrae (Cambridge University, UK)

14.40 – 15.00 **Altered glycoproteins in distal renal tubular cells upon calcium oxalate dihydrate crystal adhesion**
Ms. Wararat Chiangjong (Mahidol University)

15.00 – 15.30 **Coffee/tea break**

15.30 – 16.00 **BIOTECHNOLOGY SESSION**

Top-down determination of PEGylation site using matrix assisted laser desorption/ionization in-source decay MS analysis and other means of detecting post-translational modifications.
Dr. Jaran Jainhuknan (sponsored by Bruker Biospin AG)

15.30 – 17.00 **POSTER SESSION**

17.00 – 18.30 **Welcome Reception**

27th August 2009

Protein Crystallization

Chairperson: Assoc. Prof. Palangpon Kongsaeree

08.30 – 09.10 **Collaboratory program for structural biology in Southern Taiwan by protein crystallography**
Dr. Chun-Jung Chen (National Synchrotron Center, Taiwan)

09.10 – 09.40 **Roles of Trefoil Factor 1 in calcium oxalate crystal growth, aggregation, and transformation**
Dr. Somchai Chutipongtanate (Mahidol University)

09.40 – 10.10 **Membrane protein crystallography: lessons from MhpI, the nucleobase-cation-symport-1 family transporter**
Dr. Kuakarun Krusong (Chulalongkorn University)

10.10 – 10.30 **Coffee/tea break**

Applications in Protein studies

Chairperson: Assist. Prof. Kittisak Yokthongwattana

10.30 – 11.10 **Cellular and molecular tools for apoptosis study**
Prof. Kovit Pattanapunyasat (Mahidol University)

11.10 – 12.00 **Plasmodial serine hydroxymethyltransferase –A potential drug target**
Assoc. Prof. Pimchai Chaiyen (Mahidol University)

Applications in Protein studies (cont'n)

Chairperson: Assoc. Prof. Rudee Surarit

12.00 – 13.00 **Luncheon Lecture: New tools and techniques for proteomics research**
Dr. Anita Wan (sponsored by Merck Ltd)

13.00 – 13.30 **Synthesis of phosphatidylinositol mannoside (PIM) glycans from *Mycobacterium tuberculosis* and their biological properties: protein-carbohydrate interactions**
Dr. Siwarutt Boonyarattanakalin (Thammasart University)

13.30 – 13.50 **Soluble form of a tumor mediator, CD147 transmembrane protein, was detected in normal and patient sera**
Dr. Seangdeun Moonsom (Chiang Mai Univesity)

13.50 – 14.10 **Human liver cancer cell line for study an alternative tumor vascularization – vasculogenic mimicry**

Dr. Kriengsak Lirdprapamongkol (CRI)

14.10 – 14.30 **Structure and dynamics of anti-p17 single chain Fv**
Dr. Vannajan S. Lee (Chiang Mai University)

14.30 – 14.50 **Fine-epitope mapping of therapeutic HuScFv antibody on the H5 hemagglutinin**
Dr. Santi Maneewatch (Mahidol University)

15.00 – 15.30 **Coffee/tea break**

15.30 - 16.30 **BIOTECHNOLOGY SESSION**

15.30 – 16.00 **HaloTag™– A novel protein labeling technology for cell imaging and protein analysis**
Ms. Nantarudee Juabsamai, Regional Marketing Manager, Promega Singapore (sponsored by Bio-Active)

16.00 – 16.30 **Biomarker discovery: progression of a mouse model of lung fibrosis using LCMALDI multiplex proteomics**
Mr. Jason Neo (sponsored by Gene System)

15.30 – 17.30 **POSTER SESSION**

28th August 2009

Microarrays

Chairperson: Dr. Chantragan Srisomsap

09.00 – 09.40 **Proteomics identification of proteins involved in colorectal cancer metastasis**
Prof. Maxey Chung (NUS, Singapore)

09.40 – 10.10 **Microarray platform and its applications**
Dr. Nitsara Karoonuthaisiri (BIOTEC)

10.10 – 10.30 **Coffee/tea break**

10.30 – 11.30 **Biotechnology Session**

10.30 – 11.00 **IFNγ ELISpot - Assessing immunogenicity in clinical HIV-1 vaccine trials and tuberculosis studies in eastern Africa**
Dr. Alexandra Schuetz (sponsored by Biomed-Diagnostics)

11.00 – 11.30 **Proteomics identification of serological biomarkers for preeclampsia**
Prof. Maxey Chung (sponsored by Bio-Rad)

10.30 – 11.30 **Poster Session**

11.30 – 12.00 **Protein research, academic excellence, and networking**
Prof. M.R. Jisnuson Svasti (Mahidol University)

12.00 - 12.30 **Poster award and closing ceremony (lunch box provided)**

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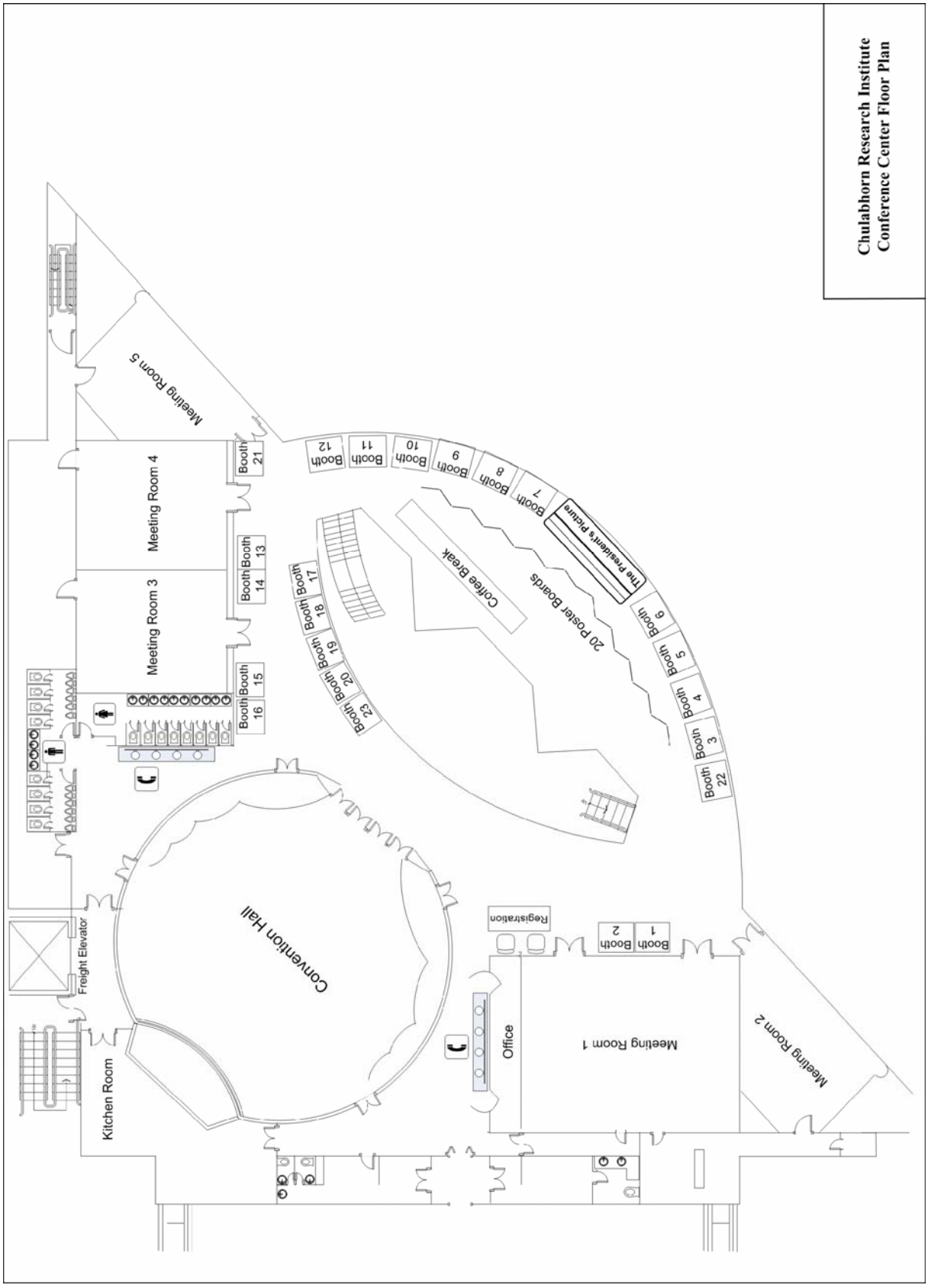
Chulabhorn Research Institute
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Invited Lectures

Luncheon Lectures

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ABSTRACTS

Invited Lectures

Invited Lecture 1

INVENTION OF BROAD SPECTRUM INFLUENZA VACCINES

Kanyarat Thueng-in, Potjanee Srimanote, Thaweesak Songserm, Nitat Sookrung, Santi Maneewatch, Jeeraphong Thanongsaksrikul, Pongsri Tongtawe, Pramuan Tapchaisri, and Wanpen Chaicumpa

Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University
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Background: A non-egg, non-cell culture based-influenza vaccine that cross protects against infection caused by several influenza A virus strains and subtypes is ideal against a new influenza pandemic.

Objective: To develop prototype broad spectrum influenza vaccines

Methods: Full length recombinant NP and M2 which are highly conserved proteins of influenza A viruses were produced by using cDNA of the H5N1 avian influenza A virus as template. The recombinant proteins were purified and used as influenza vaccine components. They were entrapped either alone or together into multilamellar liposomes (L) made of phosphatidylcholine and cholesterol. The vaccines, designated L-NP, L.M2 and L-NP+M2, were injected intramuscularly either one dose or two doses at 14 day interval into mice (C57BL/6 or BALB/c). Empty liposomes and vaccine diluent served as mocks. Innocuity (safety, tolerability) and immunogenicity (HIR, CMIR and cytokine profile) of the vaccines and placebos were studied. For protective efficacy testing, vaccinated and control mice were challenged intranasally with heterologous influenza A viruses (different strain/clade/subclade of avian A/H5N1 or swine subtype A/H1N1). Morbidity, virus recovery, mortality, and histopathology of mice were investigated.

Results: None of the vaccines or mocks caused any adverse reaction in the injected mice. The vaccines were highly immunogenic. Serum antibody responses indicated that they induced both Th1 and Th2 responses (L-NP and L-NP+M2 vaccines elicited NP-specific IgG1 and IgG2b; L-M2 and L-NP+M2 vaccines induced all M2-specific IgG subclasses). Nevertheless, cytokine studies indicated that the vaccines were biased towards a cell-mediated immune response, *i.e.* a dominant TNFγ, IFNγ, IL-12 and IL-18 cytokine expression in the immune splenocytes. Mice vaccinated with NP containing vaccines also had a marked increase of cytotoxic lymphocytes ($CD8^+$i-$IFNg^+$ spleen cells). The vaccines reduced virus recovery and protected vaccinated mice against challenges with different clade/subclade A/avian/H5N1 and A/swine/H1N1 subtypes.

Discussion and Conclusion: All liposome adjuvanted vaccines made up of influenza A conserved proteins: NP, M2 and NP+M2, were safe, highly immunogenic, and conferred cross-protective immunity to heterologous influenza A viruses in vaccinated mice. The vaccines warrant further developing, step by step (*i.e.* testing in other mammalian animal model such as ferrets and subjecting to clinical trial phases I, II and III in humans), for future use as non-egg, non-cell culture based-broad spectrum influenza vaccines in humans.

Invited Lecture 2

CONTRIBUTION OF MONOCLONAL ANTIBODY TO CANCER BIOMARKER DISCOVERY IN CHOLANGIOCARCINOMA

Sopit Wongkham[a], Atit Silsirivanit[a], Hisashi Narimatsu[b], Norie Araki[c], Kazuhioko Kuwahara[c], Nobuo Sakaguchi[c]

[a]Department of Biochemistry, and Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand
[b]Research Center For Medical Glycoscience, AIST, Tsukuba, Japan
[c]Graduate school of Medical Sciences, Kumamoto University, Kumamoto, Japan

Cholangiocarcinoma (CCA) is the second most common primary cancer of the liver. Its incidence is highest in the northeast of Thailand with increasing of incidence and mortality rates worldwide. The major challenges in CCA patients relate to lack of methods for early diagnosis and the absence of effective treatment. Serum tests to diagnose development of CCA are limiting due to its low sensitivity and specificity. This may due to the heterogeneity of CCA by nature. In this study, we used the monocloncal antibodies raised against variety of CCA tumor tissues to screen for CCA-associated antigens in patients' serum. Crude extracts from pooled CCA tissues were used as antigen to immunize the Ganp-Tg Balb/c mice for high affinity antibody production. Of 576 clones investigated, a mAb KKU-S27 was selected and characterized as IgM specifically to blood group antigen, Le^a. We have successfully developed a lectin captured ELISA to detect Le^a antigen in serum using KKU-S27 mAb. Le^a antigen was detected in serum from CCA patients with 60% sensitivity and 96% specificity. High level of serum Le^a antigen was related with poor prognosis and shorter survival of CCA patients. Immunohistochemistry using KKU-S27 as primary antibody indicated that Le^a antigen was produced mainly from CCA tissues. In addition, KKU-S27 immunohistochemistry study in animal model revealed that Le^a +ve cells were detected in bile duct epithelium of the liver fluke associated CCA hamster as early as precancerous development. None of Le^a +ve cells were observed in the control hamsters infected with liver flukes or treated with carcinogen NDMA. Overall data suggested that Le^a antigen, was markedly produced during CCA development and can be a potential tumor marker for diagnosis and prognosis of CCA.

Invited Lecture 3

TIGHT JUNCTION-ASSOCIATED PROTEINS : POSSIBLE ROLE IN INTESTINAL CALCIUM TRANSPORT

Prof. Nateetip Krishnamra, Ph.D.

Department of Physiology, Consortium for Calcium and bone Research, Faculty of Science, Mahidol University

Epithelial cells are joined together at the apical end by a belt-like structure called tight junction (TJ), that functions in cell polarity and as a paracellular barrier. TJs are composed of an interconnected network of transmembrane proteins, such as occludins, claudins, tricellulin, JAMs, and CAR, and cytoplasmic proteins that act as scaffolds, signaling and cytoskeletal attachment. A body of evidence has shown that claudins are the principle molecules responsible for barrier property of TJ. Claudin family in mammals consists of 24 members, each of which has four transmembrane domains of ~23 kDa. It has been suggested that the homotypic and heterotypic interactions of different claudins may define the permselectivities of the epithelia. It is hypothesized that particular combination of claudins could form pores or channels that have permselectivity for charged and non-charged solutes.

Generally, calcium can be absorbed across the intestinal epithelium via two routes, transcellular and paracellular. Calcium absorption can also be classified into electrochemical gradient-dependent passive paracellular transport and ATP-dependent active transport. We have shown that active transport further comprises transcellular and paracellular solvent drag-induced calcium transport. Paracellular ion movement is generally regulated by size-and charge-selective properties of the TJ. A number of claudins, ie., claudin-2, -3, -8, -10, -12, and -15 are found to be cation-selective. We recently showed that prolactin-induced increase in paracellular calcium transport was paralleled by serine phosphorylation of claudin -15, but not claudin -2, -3, -8, -10, or -12. Therefore, we hypothesize that claudin -15 may play a role in the regulation of paracellular active calcium transport.

The roles of claudins in permselective barrier-property of TJ may also be important in other aspects of cell functions, such as cell proliferation as well as in the development of pathologies like cancer.

Invited Lecture 4

MONOCLONAL ANTIBODY: PRODUCTION AND ITS APPLICATIONS ON CHARACTERIZATION OF LEUKOCYTE SURFACE MOLECULES

Watchara Kasinrerk

Biomedical Technology Research Center, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University

Monoclonal antibody has 2 special properties. First, monoclonal antibody can specifically react to its recognized antigen. Second, monoclonal antibody can be produced by hybridoma technique in any laboratory. Currently, monoclonal antibodies become an important tool for basic research in many areas and also are employed for development of various immunodiagnostics, prevention and treatment of various diseases. At the Biomedical Technology Research Center, Chiang Mai University, various efficient technologies for production of monoclonal antibodies have been established. The principle and detail procedures of the developed methods will be presented. The developed methods were used to generate monoclonal antibodies against various leukocyte surface molecules. The produced monoclonal antibodies could be employed for biochemical characterization and functional analysis of the corresponding cell surface molecules.

Invited Lecture 5

TAIWAN SYNCHROTRON FACILITIES FOR BIO STRUCTURE AND IMAGING RESEARCH

Keng S. Liang

National Synchrotron Radiation Research Center, Hsinchu, Taiwan

The history of synchrotron radiation research for Taiwan began in July 1983 when the national synchrotron center was established. At that time Taiwan had very limited expertise in accelerator-based technology. In late 1980s, the Center started the construction of a third generation 1.5 GeV synchrotron which was open to the users in October 1993. As the country has been transforming from a labor-intensive economy toward an economy based on high technology in the past twenty five years, NSRRC has been steadily gaining advanced accelerator technology as well as evolving in scientific research. As the users demands grow, a new synchrotron project was proposed in 2004, the Taiwan Photon Source, which is a low-emittance 3 GeV synchrotron designed for future X-ray research in nano and bio sciences. The project has obtained government approval in June 2007 with a total budget of ~ US$220M for civil and accelerator constructions. The commissioning of the TPS is aimed for late 2013.

NRSSC is a nonprofit institution for synchrotron research and is open to domestic and international users. In my talk, I will present the experimental stations available today for bio structure and medical imaging research, which includes x-ray protein crystallography, small angle X-ray scattering, fluorescence optical microscopes, and transmission X-ray microscope. The uses of these stations by Thai users are specially welcome.

Invited Lecture 6

COLLABORATORY PROGRAM FOR STRUCTURAL BIOLOGY IN SOUTHERN TAIWAN BY PROTEIN CRYSTALLOGRAPHY

Chun-Jung Chen[1], and Keng S. Liang[2]

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A new cooperative program originated from the promotion of the synchrotron radiation on protein structure applications at the southern regions in Taiwan has been initiated. Under this program, the essential facilities of protein production and protein crystallography are established. By combining the most advanced synchrotron radiation X-ray source from National Synchrotron Radiation Research Center (NSRRC) with the resources of life sciences from National Cheng Kung University (NCKU) and nearby research institutes and universities in the southern Taiwan, protein purification, characteristic study, crystal growth and structure analysis can be performed efficiently in considerable quantities.

By utilizing the three-dimensional structures of proteins, we can much clearly understand the function and mechanism of the protein molecules. Furthermore, by combining with the crystal structures of protein targets and the rational screening of drug databases, new medicine can be developed by the pharmaceutical companies. The establishment of this protein crystallography laboratory with advanced facilities not only enhances the competitiveness in the scientific research of the research institutions and scholars at the southern area of Taiwan, but also gives opportunities of research collaborations among international life sciences and medical related institutions. Here, we report the current status of the new established “protein crystallography laboratory” in Tainan, Taiwan, and the some recent research progresses at NSRRC.

Invited Lecture 7

ROLES OF TREFOIL FACTOR 1 IN CALCIUM OXALATE CRYSTAL GROWTH, AGGREGATION, AND TRANSFORMATION

Somchai Chutipongtanate[1], Prida Malasit[2], and Visith Thongboonkerd[1]
[1]Medical Proteomics Unit, and [2]Medical Molecular Biology Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand; Tel/Fax: +66-2-4184793; E-mail: s_keing@hotmail.com

Crystal growth and aggregation are the important mechanisms for calcium oxalate (CaOx) stone formation in the kidney. Modulation of these mechanisms by either inhibitory or promoting factors can affect the stone formation. We combined conventional biochemical methods with recent advances in mass spectrometry (MS) to identify a novel CaOx crystal growth inhibitor in normal human urine. Anionic proteins were isolated by DEAE adsorption and separated by HiLoad 16/60 Superdex 75 gel filtration. A fraction with potent inhibitory activity against CaOx crystal growth was isolated and purified by anion exchange chromatography (Resource Q). The protein in two subfractions that retained inhibitory activity was identified by MS and MS/MS analyses as human '*Trefoil Factor 1*' (TFF1), which was confirmed by western blot analysis. Functional studies revealed that its inhibitory potency was similar to that of nephrocalcin. The inhibitory activity of urinary TFF1 was dose-dependent and could be inhibited by TFF1 antisera. Anti-carboxy-terminal antibody was particular effective, consistent with our proposed model in which the four carboxy-terminal glutamic residues of TFF1 interact with calcium ions to prevent CaOx crystal growth. In addition, morphological studies provided direct evidence, which clearly indicated that urinary TFF1 and nephrocalcin at physiological levels could inhibit CaOx crystal growth and aggregation. At a supraphysiological concentration, both urinary TFF1 and nephrocalcin could transform crystalline CaOx monohydrate to its dihydrate form, which has much less adsorptive capability. Concentrations and relative amounts (normalized with urine creatinine and total protein) of TFF1 in the urine of patients with idiopathic CaOx kidney stone were significantly less (2.5-fold for the concentrations and 5- to 22-fold for the relative amounts) than those in the normal controls. In conclusion, we provide herein both indirect and direct evidences demonstrating that urinary TFF1 is a novel potent inhibitor against CaOx kidney stone formation.

Supported by The Thailand Research Fund (Senior Research Scholar Program, grant RTA4680017, to P. Malasit). S. Chutipongtanate was supported by Medical Scholars (PhD-MD) Program of Mahidol University

Invited Lecture 8

MEMBRANE PROTEIN CRYSTALLOGRAPHY: LESSONS FROM MHPI, THE NUCLEOBASE-CATION-SYMPORT-1 FAMILY TRANSPORTER

Kuakarun Krusong, Ph.D.

Department of Biochemistry, Faculty of Science, Chulalongkorn University, Phyathai Rd., Patumwan, Bangkok 10330 Thailand

Membrane protein crystallography remains one of the hardest challenges in structural biology. Even though membrane protein are involved in many functions essential for life, including membrane transport and energy conversion; and more than half of all current drugs target membrane proteins, there is only a modest number of membrane protein structures in the protein data bank. Here, we address the intrinsic problems that hinder crystallisation of this class of proteins and provide a successful example of structural studies of membrane proteins: Structure and Molecular Mechanism of a Nucleobase-Cation-Symport-1 Family Transporter (Weyand, S. *et al*, *Science* 322, 709 (2008)). The X-ray structures of the Mhp1 from *Microbacterium liquefaciens* reveal the possible transport process of this protein.

Invited Lecture 9

CELLULAR AND MOLECULAR TOOLS FOR APOPTOSIS STUDY

Kovit Pattanapanyasat Ph.D.

Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University

Apoptosis or program cell death is a highly regulated pathway. It plays a critical role in maintaining homeostasis in multicellular organism. Apoptosis can be initiated through two pathways. The extrinsic pathway is triggered through the Fas or death receptor. The second pathway is the intrinsic pathway which is imitated by intracellular damage leading to the release of cytochrome-c from the mitochondria to further activate the death signal. Both pathways converge to a final common pathway involving activation of caspases that cleave regulatory and structural molecules and culminate in the death of the cell. Inappropriate control of apoptosis results in pathogenesis of several diseases such as neurodegenerative diseases, heart disease, autoimmune disorders and cancer. In recent years, interest has emerged in employing various therapeutic approaches to target apoptosis signaling pathway and the understanding of the molecular mechanism of apoptosis is the basis for novel targeted therapies that can for example induce death in malignant cells or sensitize them to cytotoxic agents and radiation therapy. The cellular and molecular dissection of apoptosis is therefore one of the most important challenges from both mechanisms and therapeutic points of view. The goal of this presentation is to provide the current knowledge on the cellular and molecular tools for apoptosis study. For example, caspase-3 intracellular activity can be measured by flow cytometry or ELISA technique. The expression of apoptosis related protein such as Fas protein can also be detected by flow cytometry, western blot or immunochemistry technique. Early apoptotic signal such as externalization of phosphatidylserine from the cytoplasmic face of the plasma membrane to the outer leaflet of membrane can be detected by flow cytometer or fluorescent microscope technique. Another commonly used method to identify apoptotic cells is DNA fragmentation which can be measured by Terminal deoxynucleotidyl transferase dUTP Nick End-Labeling (TUNEL). Finally, some key molecular molecules in the induction of apoptosis such as those in signaling pathways will also be discussed.

Invited Lecture 10

PLASMODIAL SERINE HYDROXYMETHYLTRANSFERASE –A POTENTIAL DRUG TARGET

Kittipat Sopitthammakhun[1], Somchart Maenpuen[1], Yongyuth Yuthavong[2], Ubolsree Leartsakulpanich[2], and Pimchai Chaiyen[1]

[1] Department of Biochemistry and Center for Excellence in Protein Structure & Function, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok, 10400, Thailand
[2] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, 113 Paholyothin Road, Pathumthani 12120, Thailand

The putative gene of *Plasmodium vivax* serine hydroxymethyltransferase (PvSHMT) was cloned and expressed in *Escherichia coli*. The purified enzyme was shown to be a dimeric protein with a monomeric molecular mass of 49 kDa. PvSHMT has a maximum absorption peak at 422 nm with a molar absorption coefficient of 6,370 M-1cm-1. The K_d for binding of the enzyme and PLP was measured to be 0.14 + 0.01 μM. An alternative assay for measuring the tetrahydrofolate (THF)-dependent SHMT activity based on the coupled reaction with 5,10-methylenetetrahydrofolate reductase (MTHFR) from *E. coli* was developed. PvSHMT uses a ternary-complex mechanism with the k_{cat} value of 0.98 $\pm$ 0.06 s^{-1} and the K_m values of 0.18 $\pm$ 0.03 and 0.14 $\pm$ 0.02 mM for L-serine and THF, respectively. The optimum pH of the SHMT reaction was 8.0 and the Arrhenius's plot showed a transition temperature of 19°C. Besides L-serine, PvSHMT forms an external aldimine complex with D-serine, L-alanine, L-threonine, and glycine. PvSHMT also catalyzes the THF-independent retro-aldol cleavage of 3-hydroxy amino acids. Although L-serine is a physiological substrate for SHMT in the THF-dependent reaction, PvSHMT can also use D-serine. In the absence of THF at high pH, PvSHMT forms an enzyme-quinonoid complex (E-Q) with D-serine, not with L-serine, while SHMT from rabbit liver was reported to form E-Q complex with L-serine. Substrate specificity difference between PvSHMT and the mammalian enzyme indicates the dissimilarity between their active sites that could be exploited for the development of specific inhibitors against PvSHMT.

Invited Lecture 11

SYNTHESIS OF PHOSPHATIDYLINOSITOL MANNOSIDE (PIM) GLYCANS FROM *MYCOBACTERIUM TUBERCULOSIS* AND THEIR BIOLOGICAL PROPERTIES: PROTEIN – CARBOHYDRATE INTERACTIONS

Siwarutt Boonyarattanakalin[1], and P. H. Seeberger[2]

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Phosphatidylinositol mannosides (PIMs) are one of the most important cell wall components of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB). PIMs are common precursors of more complex mycobacterial cell wall glycolipids including lipomannan (LM), lipoarabinomannan (LAM), and mannan capped lipoarabinomannan (ManLAM). PIMs play crucial roles for *Mtb* by compromising host immune responses. An increasing number of PIMs' biological functions were recently revealed. Several immunological properties of PIMs are exerted by their interactions with proteins and receptors of mammalian host cells. However, the study of carbohydrate – protein interaction is usually limited by its weak interaction and accessibility to pure and sufficient carbohydrate compounds. In this presentation, we report a highly convergent and efficient synthesis of all PIM glycans. Bicyclic and tricyclic orthoesters were the key intermediates for rapid building block syntheses. The orthoesters were conveniently converted to mannosyl phosphates to serve as reliable glycosylating agents resulting in selective and high yielding glycosylation reactions. The synthetic carbohydrate compounds were equipped with a thiol-linker for immobilization on surfaces and carrier proteins for biological studies. The interactions of synthetic PIMs to the dendritic cell specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) receptor were elucidated on microarray slides. Immunization experiments in C57BL/6 mice showed that the synthetic PIMs served as immune stimulators when coupled to the model antigen keyholelimpet hemocyanin (KLH).

Invited Lecture 12

PROTEOMIC IDENTIFICATION OF PROTEINS INVOLVED IN COLORECTAL CANCER METASTASIS

Maxey C.M. Chung[1,2], Xuxiao Zhang[1], Yizhen Ng[1], Hwee Tong Tan[2], Sandra Tan[2], and Shing Chuan Hooi[3]

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Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer deaths in developed countries. About 50% of all patients with CRC die within 5 years after diagnosis. The high mortality of CRC is largely due to extensive metastatic spread of the disease. Acquisition of metastatic phenotype by the cancer cells thus represents one of the most lethal aspects of CRC progression. Patient survival in CRC will greatly benefit from earlier recognition (and intervention) of metastasis but as yet, proper markers indicating metastases are not available.

E1 is a highly metastatic cell line that had been derived from hepatic metastases following splenic injection of HCT-116 primary colon cancer cell line into nude mice. Using heparin affinity chromatography coupled to 2-D DIGE and tandem mass spectrometry (MS/MS), we carried out comparative proteomics analysis of HCT-116 and E1 cells to identify proteins associated with CRC metastasis. Eighty six proteins were identified to be differentially expressed between the 2 cell lines. A majority of these proteins are implicated in cell growth, survival, motility, invasiveness and angiogenesis, which are indispensable steps in the metastatic cascade. Our work has thus identified a set of proteins that may represent candidate biomarkers and therapeutic targets for metastatic colorectal cancer.

We proceeded to functionally validate stathmin which was the most up-regulated protein in the E1 as compared to HCT116. Our results showed that RNA interference knockdown of stathmin in E1 did not affect cell viability but decreased cell migration 41% in an *in vitro* assay. We also showed that stathmin expression levels correlate strongly with increasing metastatic potential in other paradigms/models of colorectal metastasis.

Invited Lecture 13

MICROARRAY PLATFORM AND ITS APPLICATIONS

Nitsara Karoonuthaisiri, Umaporn Uawisetwathana, Thidathip Wongsurawat, Rungnapa Leelatanawit, Ratthaphol Charlermroj, Plearnpis Luxananil, Oraprapai Gajanandana, Sirawut Klinbunga, and Kanyawim Kirtikara

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Microarray technology has enormous applications in research and development. Employing the same core platform technology, there are various types of arrays for different research purposes. Here, development and application of DNA microarray and antibody array will be presented. For DNA microarray, applications in gene expression analysis of reproductive maturation process in the black tiger shrimp (*Penaeus monodon*) will be presented. For antibody array platform, a proof-of-concept diagnostic system has been developed to simultaneously detect three highly-regulated foodborne pathogenic bacteria (*Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes*) using a chemiluminescent detector. The antibody array system is based on immunoassay similar to mini sandwich ELISA using nitrocellulose slides for antibody fabrication. The sensitivity and accuracy of this system are at least comparable to the conventional ELISA method, but with the improvement of shorter assay time and much lower amounts of antibodies required.

Supported by National Center for Genetic Engineering and Biotechnology and NanoAsia, Ltd.

Invited Lecture 14

PROTEIN RESEARCH, ACADEMIC EXCELLENCE, AND NETWORKING

Jisnuson Svasti

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I was interested in protein research since my undergraduate days, and was fortunate to have been trained in one of the best protein chemistry laboratories of the world during the 1960's-1970's. As a result, Protein Chemistry has been the focus of my research for 40 years. Upon returning to Bangkok, I continued with protein research, despite the lack of equipment, and helped to form the Reproductive Biology Group at the Faculty of Science, Mahidol University, which was probably the first protein research group in Thailand. Later, our interests turned to glycosidase enzymes, which catalyse similar reactions, but have different substrates, and thus serve as good examples for studying structure-function relationships in proteins. We also study abnormalities of proteins in disease, both in terms of genetic diseases and cancer, and our group at the Chulabhorn Research Institute was the first to successfully use proteomic techniques in Thailand. However, apart from research and teaching, I have been held several administrative positions, and tried to encourage academic excellence, while still continuing to be active in protein research. Another rewarding area has been my involvement with scientific societies and international activities to encourage cooperation between scientists for the betterment of mankind.

These various activities have helped me realize how fortunate I have been to have spent a life-time as a protein chemist and an academic.

Reference: *Svasti, J.* (2009) How I became a biochemist: what biochemistry has done for me? *IUBMB Life* **61**(4): 476–478.

ABSTRACTS

Luncheon Lectures

Luncheon Lecture 1 (sponsored by GE Healthcare Bio Sciences)

ADVANCE TECHNOLOGY BEYOND PROTEOMICS INFORMATION

Ms. Zhu Hua

Abstract to be delivered in the venue.

Luncheon Lecture 2

NEW TOOLS AND TECHNIQUES FOR PROTEOMICS RESEARCH

Anita Wan

Merck Biosciences, Application and sales manager, Asia Pacific Region

The sequencing of the human genome has not only created new opportunities for proteomics, but has also provided a sequence-based framework for mining the proteome of healthy and diseased tissues. Biological samples subjected to proteomic analysis are tissues, cell populations, and biological fluids. A common feature in these samples is their extraordinary complexity because of the high multidimensionality of their protein constituents, which differ in their cellular and subcellular distribution; their occurrence in complexes; their charge, molecular mass, hydrophobicity; their level of expression, and post-translational modifications. These complexities present a difficult challenge for analysis that no single analytical technique can overcome. The isolation of proteins starts with sample preparation, followed by protein fractionation, purification, and characterization. Maintaining proteins integrity during sample preparation has important advantages compared with strategies that digest proteins at an early step. The combined technologies of separation science and biological mass spectrometry are some of the most important techniques in proteomics, which continue to evolve to meet the increasing need for high sensitivity and high throughput. They are extensively used for protein quantification, identification, and analysis of post-translational modifications. EMD Chemicals has developed a series of ProteoExtract® Kits and other tools that cover various steps of the proteomics workflow, from protein extraction and abundant protein removal to concentration of protein mixtures and removal of interfering substances. Each kit works in concert with one another to provide samples that are directly compatible with downstream analytical applications, such as one-dimensional and two-dimensional gel electrophoresis, mass spectrometry, and other methods.

ABSTRACTS

Company Lectures

Company Lecture 1 (sponsored by Bruker Biospin AG)

TOP-DOWN DETERMINATION OF PEGYLATION SITE USING MATRIX ASSISTED LASER DESORPTION/IONIZATION IN-SOURCE DECAY MS ANALYSIS AND OTHER MEANS OF DETECTING POSTTRANSLATIONAL MODIFICATIONS

Dr. Jaran Jainhuknan

A novel matrix assisted laser desorption/ionization (MALDI)-based mass spectrometric approach has been evaluated to rapidly analyze a custom designed PEGylated peptide that is 31 residues long and conjugated with 20 kDa linear polyethylene glycol (PEG) at the side chain of Lys. MALDI-TOF MS provided sufficiently high resolution to allow observation of each of the oligomers of the heterogeneous PEGylated peptide (m/Deltam of ca. 500), while a typical ESI-MS spectrum of this molecule was extremely complex and unresolved. Reflector in-source decay (reISD) analysis using MALDI-TOF MS was attempted to identify the PEGylation site at intact molecular level without any sample treatment. The spectrum of the free peptide (reISD) was observed with abundant c-, y-, and [z+2]-fragment ion series, whereas, in the fragmented PEGylated peptide, the fragment ion series were truncated at the residue where PEG was attached. Therefore, a direct comparison of these top-down reISD spectra suggested the location of the PEGylation site. Results from this study demonstrate a clear analytical utility of the ISD technique to characterize structural aspects of heterogeneous biomolecules. Examples of top-down analysis of posttranslational modification using mass spectrometry are presented.

Company Lecture 2 (sponsored by Bio-Active)

HALOTAG™ A NOVEL PROTEIN LABELING TECHNOLOGY FOR CELL IMAGING AND PROTEIN ANALYSIS

Ms. Nantarudee Juabsamai

Regional Marketing Manager, Promega Singapore

Promega designed the HaloTag™ Technology – a modular protein tagging system that allowed different functionalities to be linked onto a single genetic fusion, either in solution, in living cells or in chemically fixed cells. The protein tag (HaloTag™) is a modified haloalkane dehydrogenase designed to bind to synthetic ligands (comprising a linker attached to an array of functionalities – fluorescent labels, affinity handles and solid surfaces). Covalent bond formation between the protein tag and the linker is highly specific, occurs rapidly under physiological conditions and is essentially irreversible.

This seminar explores exciting applications that has emerged rapidly based on key characteristics afforded by the technology. We present the utility of the system for cellular imaging and protein immobilization by analyzing multiple molecular processes associated with NFkB mediated cellular physiology, including subcellular translocation and capture of protein-protein complexes. A highlight of the talk will also focus on the Halolink Protein Array System that is used for multiplexed protein interaction studies.

Company Lecture 3 (sponsored by Gene System)

BIOMARKER DISCOVERY: PROGRESSION OF A MOUSE MODEL OF LUNG FIBROSIS USING LCMALDI MULTIPLEX PROTEOMICS STRATEGIES

Mr. Jason Neo

Applications Team Leader, Applied Biosystems BV, South East Asia

There are five million people worldwide that are affected by Pulmonary Fibrosis. In the United States there are over 200,000 patients with this disease. Of these more than 40,000 expire annually. There are currently no effective treatments or a cure for Pulmonary Fibrosis. Here, we present an iTRAQ 8-plex LC MALDI biomaker discovery workflow to identify the putative biomarkers confidently and also a methodology to validate these biomakers using MRM based approach.

Company Lecture 4 (sponsored by Biomed-Diagnostics)

IFN-γ ELISpot - ASSESSING IMMUNOGENICITY IN CLINICAL HIV-1 VACCINE TRIALS AND TUBERCULOSIS STUDIES IN EASTERN AFRICA

Alexandra Schuetz, PhD.

USAMC-AFRIMS, 315/6 Rajvithi Road, Bangkok 10400;
Mbeya Medical Research Programme, P.O. Box 2410, Mbeya, Tanzania

The Enzyme-linked Immuno Spot (ELISpot) assay measures the magnitude and quality of T cell immunity at a single cell resolution by detecting individual events of antigen-specific T cells that engage in secretion of cytokines such as IFN-gamma (IFN-γ) and effector molecules such as granzyme B and/or perforin. The new generations of ELISpot analyzers have transformed the ELISpot assay to a stable and high-throughout T cell assay with the potential of standardization and performing quality control. This makes the ELISpot assay a useful tool in assessing T cell immunity on a large-scale basis for a number of different applications such as immunogenicity monitoring in vaccine trials or new diagnostic tools. Furthermore the stability and the potential of standardization make the ELISpot assay feasible for application in developing countries.

The HIV epidemic continues to grow with 33 million people living with HIV/AIDS and an additional 7,400 new infections daily in 2007. While multiple HIV prevention strategies have been evaluated with some recent successes the development of a vaccine against HIV remains a global priority. Furthermore in individuals immunocompromised due to HIV infection the co-infection with *Mycobacterium tuberculosis*, the causing agent of tuberculosis (TB), represents a significant cause of morbidity and mortality. However TB control programmes are confronted with a number of complex problems. Major challenges are the diagnosis of TB especially in HIV infected patients and children.

In Mbeya, Southern Highlands, Tanzania, Eastern Africa the use of IFN-γ ELISpot was assessed in determine the immunogenicity of a multi-clade HIV-1 DNA plasmid/Adenovirus-5 vector (rAd5) vaccine trial as well as in serving as a tool for detecting Tuberculosis infection in HIV-positive and HIV-negative children, respectively. The collected data show that the vaccine regimen induced robust cellular immune responses at multiple time points in the majority of vaccinees by IFN-γ ELISpot. Furthermore the IFN-γ ELISpot has been proven to be a reliable and easy assay to determine TB-specific T-cell immunity and thereby contributes to the diagnosis of TB infection.

Company Lecture 5 (Sponsored by Bio-Rad)

PROTEOMICS IDENTIFICATION OF SEROLOGICAL BIOMARKERS FOR PREECLAMPSIA

Maxey C.M. Chung[1,3], Seow Chong Lee[1], and Annamalai Loganath[2]

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Preeclampsia is the leading cause of maternal and neonatal mortality and morbidity. Preeclampsia has been considered as a two-stage disorder, where impaired remodelling of the maternal vessels leads to the maternal syndrome, which is characterized by endothelial dysfunction, inflammation, dyslipidemia and increased oxidative stress. The etiology of the disease is still unknown and diagnosis of preeclampsia depends on the new onset of pregnancy-induced hypertension (PIH) and proteinuria after 20 weeks of gestation. We present here a proteomics analysis to compare the plasma proteome of women with normal pregnancy, pregnancy induced hypertension and preeclampsia. The pooled plasma samples from each subject group were fractionated using the equalizer beads to reduce the dynamic range of proteins in the samples. This ProteominerTM technology utilizes a combinatorial library of hexapeptides bound to beads to decrease high abundant proteins and enrich for middle and low abundant proteins in complex biological samples such as plasma and serum. The fractionated plasma samples were analyzed by 2-dimensional difference gel electrophoresis (2D-DIGE) coupled with MALDI-TOF/TOF mass spectrometry (MS). A total of 12 unique proteins from 26 differentially regulated spots were successfully identified, of which several were involved in inflammation and lipid metabolism.

ABSTRACTS

Oral Presentations

Oral Presentation 1

AVIAN INFLUENZA A/H5N1 NEURAMINIDASE EXPRESSED IN YEAST WITH A FUNCTIONAL HEAD DOMAIN

Suganya Yongkiettrakul[1], Katewadee Boonyapakron[2, 3], Anan Jongkaewwattana[4], Asawin Wanitchang[4], Ubolsree Leartsakulpanich[1], Penchit Chitnumsub[1], Lily Eurwilaichitr[2,3], and Yongyuth Yuthavong[1]

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The study reports heterologous expression in *Pichia pastoris* of active neuraminidase derived from avian influenza virus A/Viet Nam/DT-036/2005(H5N1). A gene encoding the neuraminidase N1 head domain (residues 63-449) was fused directly in-frame with the *Saccharomyces cerevisiae* alpha-factor secretion signal in pPICZaA vector. Recombinant N1 neuraminidase was expressed in *P. pastoris* as a 72-kDa secreted, soluble protein. Glycopeptidase F treatment generated a 45-kDa product, indicating that the secreted recombinant N1 neuraminidase is an N-linked glycoprotein. Kinetic studies and inhibition tests with oseltamivir carboxylate demonstrated that the recombinant N1 neuraminidase has similar K_m and K_i values to those of the viral N1 neuraminidase. This yeast-based heterologous expression system provided functionally active recombinant N1 neuraminidase should be useful in anti-influenza drug screening, and also as a potential protein-based vaccine.

Supported by BIOTEC grant: BT-B-02-MT-BC-5012.

Oral Presentation 2

STRUCTURAL STUDIES REVEAL DIVERSE CHONDROITIN SULPHATE BINDING SITES IN DBL DOMAINS IMPORTANT IN MALARIA DURING PREGNANCY

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Severe malaria during pregnancy is associated with accumulation of parasite-infected erythrocytes in the placenta through the interaction between VAR2CSA on the infected erythrocytes and placental chondroitin sulphate proteoglycans (CSPG). VAR2CSA protein contains multiple CSPG-binding domains, including the structurally characterised DBL3X domain. Here we present the structure of a second CSPG-binding domain from VAR2CSA, the DBL6ε domain, and the use of mutagenesis to map the CSPG binding sites of both domains. We confirmed that DBL3X binds CSPG through a positively charged patch and sulphate-binding pocket on the concave surface of the domain. This site is lacking in DBL6ε, which binds CSPG through a different positively charged patch, located to the side of subdomain 2. Finally, we showed that, unlike intact VAR2CSA, both domains could bind to various carbohydrates, with the greatest affinity for ligands with high sulphation and negative charge. This finding changes the way we view VAR2CSA by showing that the protein does not consist of a series of independent CSPG-specific binding domains that bind through a conserved mechanism. The information presented here about the surfaces contributed by DBL3X and DBL6ε to CSPG binding can provide valuable guidance for rational development of therapeutics to target placental malaria.

This work was supported by grants from the Royal Society and the Wellcome trust. P.K. is a KMUTT's academic member on study leave and a graduate student of the University of Cambridge. His Ph.D. scholarship is funded by the Ministry of Science, Thailand. PDB code of DBL6ε is 2wau.

Oral Presentation 3

ALTERED GLYCOPROTEINS IN DISTAL RENAL TUBULAR CELLS UPON CALCIUM OXALATE DIHYDRATE CRYSTAL ADHESION

Wararat Chiangjong[1], Supachok Sinchaikul[2], Shui-Tein Chen[2], and Visith Thongboonkerd[1]

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Calcium oxalate dihydrate (COD) crystals can adhere onto apical surface of renal tubular epithelial cells. This process is followed by crystal growth and aggregation, resulting in kidney stone formation. Glycoproteins have been thought to play roles in response to crystal adhesion. However, components of the glycoproteome that are involved in this cellular response remain largely unknown. Our present study was therefore aimed to identify altered glycoproteins upon adhesion of COD crystals onto tubular epithelial cells representing distal nephron, the initiating site of kidney stone formation. Madin-Darby Canine Kidney (MDCK) cells were maintained in culture medium with or without COD crystals (100 μg/mL) for 48 h (n=5 for each set). Thereafter, the cells were harvested into a tube containing 0.5 M EDTA in PBS to dissolve the adherent COD crystals and were washed with PBS. Cellular proteins were then extracted and resolved by 2-D gel electrophoresis. The separated protein spots were visualized by SYPRO Ruby total protein stain, whereas glycoproteins were visualized by Pro-Q Emerald 300 glycoprotein stain (Invitrogen – Molecular Probes). These spots were matched and analyzed by Image Master 2D Platinum software (GE Healthcare). Five gels were derived from five individual culture flasks for each condition (a total of 20 gels were analyzed). Approximately 850 total protein spots were detected by SYPRO Ruby stain, whereas about 240 glycoprotein spots were detected by Pro-Q Emerald dye. Quantitative intensity analysis revealed significantly altered levels of glycoprotein-staining for 16 protein spots, whose corresponding total protein levels did not change. These altered glycoproteins were then identified by Q-TOF MS and/or MS/MS analyses. Characterizations of these altered glycoproteome in distal renal tubular epithelial cells upon COD crystal adhesion may provide some novel insights into the pathogenic mechanisms of kidney stone disease.

Supported by The Thailand Research Fund and Commission on Higher Education

Oral Presentation 4

SOLUBLE FORM OF A TUMOR MEDIATOR, CD147 TRANSMEMBRANE PROTEIN, WAS DETECTED IN NORMAL AND PATIENT SERA

Seangdeun Moonsom[1], **Saichit Khummuang**[1], **Chatchai Tayapiwatana**[1], **Prachya Kongtawelert**[2], **Suchat Chaimuangrach**[3], **Sopit Wongkham**[4], **and Watchara Kasinrerk**[1]

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CD147 is a leukocyte surface glycoprotein which has a broad cellular distribution and plays a crucial role in immune regulation. Elevated expression of CD147 is always occurred in various cancers and associated with invasion, growth, angiogenesis and metastasis of cancers. CD147 was demonstrated to involve in tumor metastasis by stimulating production of matrix metalloproteinase and vascular endothelial growth factor. *In vitro*, the release of CD147 molecules has been found in culture medium of tumor cells. We hypothesized that soluble CD147 is present in sera and regulates tumor invasion. To prove this hypothesis, a competitive-Enzyme Linked Immunosorbent Assay (ELISA) was established in our laboratory. The developed ELISA has a sensitivity of 80 ng for detecting recombinant soluble CD147 molecules. The method was successfully applied for determination of CD147 from normal and cancerous sera. Here, it is the first report that the soluble CD147 was detected in human sera. Furthermore, the level of soluble CD147 was diverse which is highest in normal and hepatitic sera and was found to be slightly decreased in sera of hepatocellular carcinoma and cholangiocarcinoma then reached its minimum in sera of benign tumor and cancer of urinary tract, respectively. This finding prompted us to carry out future works on isolation of serum CD147, biochemical characterization and studies pfits role in tumor progression and invasion.

This work was supported by National Center for Genetics Engineering and Biotechnology

Oral Presentation 5

HUMAN LIVER CANCER CELL LINE FOR STUDY AN ALTERNATIVE TUMOR VASCULARIZATION – VASCULOGENIC MIMICRY

Kriengsak Lirdprapamongkol[1,3], Monnipa Sila-Asna[2], Khajeelak Chiablaem[1], Chantragan Srisomsap[1], Rudee Surarit[1], Ahnond Bunyaratvej[2], and Jisnuson Svasti[1,3]

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Recent evidences showed that cancer cells can form vascular channels without endothelial cell lining, which termed "vasculogenic mimicry" (VM). The VM functions similar to angiogenesis-derived blood vessels, however it resists to several angiogenesis inhibitors. Liver cancer is the top leading cause of death in Thailand. VM has been observed in human liver cancer tissues however there is still lack of cellular model for the study of VM in liver cancer. Here we used Matrigel-tube formation assay to evaluate *in vitro* VM capacity of human liver cancer cell lines was found to vary in differentiation status. Invasive phenotype of the cell lines was determined from cell migration and production of matrix metalloproteinase enzymes using Transwell assay and gelatin zymography, respectively. Expression pattern of endothelial-specific marker and liver-specific marker genes was determined by real-time RT-PCR. Among tested liver cancer cell lines, the VM formation was found only in the highly invasive cell line which did not express angiogenesis-related endothelial-specific genes, e.g. CD31 and VE-cadherin. The results suggest that the VM capacity of liver cancer cells is associated with invasive phenotype, and the mechanism of VM formation differs from that used by endothelial cells.

Supported by the Chulabhorn Research Institute and the Thailand Research Fund (RGJ-Ph.D. program)

Oral Presentation 6

STRUCTURE AND DYNAMICS OF ANTI-P17 SINGLE CHAIN FV

Vannajan S. Lee[1*], Panthip Tue-ngeun[1], Sawitree Nangola[2], Kuntida Kitidee[2], Jitrayut Jitonom[1], Piyarat Nimmanpipug[1], Supat Jiranusornkul[3], and Chatchai Tayapiwatana[2, 4*]

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The potential applications of antibody fragment ScFv have been reported in many fields including research tools, diagnostics and therapy. In this study, the model of ScFv anti-p17 scfv structure was built from its x-ray structure homolog and validated. The complex structures between the single chain Fv antibody and its natural peptide epitopes at C-terminal of p17 were then constructed by flexible docking algorithm using potential mean force (PMF) scoring. The selected complex models were further investigated through molecular dynamics simulations. Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) methodology and decomposition energy were calculated using the set of snapshots. The dynamics and the structural analysis of the antibody-antigen interface with the different functional behaviors between the wild-type and its mutants will be reported in details. These results may give useful implications for structure-based design of antibody combining site.

Supported by the Thailand Research Fund, the Thailand Research Fund, the National Center for Genetic Engineering and Biotechnology, Computational Nanoscience Consortium (CNC) Nanotechnology (NANOTEC), Biomedical Engineering (BME), Chiang Mai University, and the Center for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education of Thailand.

Oral Presentation 7

FINE-EPITOPE MAPPING OF THERAPEUTUC HuScFv ANTIBODY ON THE H5 HEMAGGLUTININ

Santi Maneewatch[1,4], Potjanee Srimanote[2], Thaweesak Songserm[3], and Wanpen Chaicumpa[4]

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Currently H5N1 viruses are resistant to anti-viral drugs and effective vaccines for influenza are being developed. Therefore, therapeutic human antibodies could play an attractive and important role for influenza prevention and treatment. Recently, a human single chain antibodies fragment (HuScFv) obtained from human antibody phage displayed library with neutralizing activity against both homologous and heterologous strains and clades of the H5N1 subtype were produced. HuScFv clone no. 13 with the highest cross neutralizing activity against different strains and clades of H5 influenza virus was characterized for biological and structural properties of immunoglobulin molecule, binding activity, and also insight the protective epitope specificity on H5 HA molecule through 12-mer random phage display peptide library, protein-protein interaction and bioinformatics. The results suggested that the protective HuScFv antibody recognized on HA1 portion which is important for viral binding to host receptor. The defined molecular determinant epitope on hemagglutinin molecule will be demonstrated. The HA epitope recognized by the protective HuScFv antibody will provide not only the mechanisms of neutralization but also new insight for the development of effective H5 influenza vaccine and diagnostic tools.

This work was supported by the National Center of Biotechnology and Genetic Engineering (BIOTEC), NSTDA

ABSTRACTS

Poster Presentations

Poster Presentation 1

THE EFFECT OF A HEAT STABLE HETEROMULTIMERIC GLYCOPROTEIN FROM *ARTOCARPUS HETEROPHYLLUS* LATEX ON HUMAN BLOOD COAGULATION TIME

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A heat stable heteromultimeric glycoprotein (HSHMG) from *Artocarpus heterophyllus* latex was purified by the combination of heat precipitation and ion-exchange chromatography. The size of this multimeric protein on SDS-PAGE was more than 97 kDa. The apparent molecular mass of each monomeric protein was 29, 31 and 33 kDa. The isoelectric points (pI) of the monomers were 6.93, 6.63 and 6.63, respectively. Prothrombin time (PT) and activated partial thrombin time (APTT) tests were used to investigate the effect of HSHMG on human blood coagulation time. The result of the APTT test showed that HSHMG had an effect on the intrinsic pathway of human blood coagulation system by significantly increasing the blood clotting time ($P < 0.05$) in parallel with increasing amounts of HSHMG. The PT test illustrated that HSHMG had no significant effect on the human extrinsic blood coagulation system.

Supported by the Thailand Research Fund (TRF) and the Commission on Higher Education (CHE), Ministry of Education.

Poster Presentation 2

VIMENTIN INTERACTS WITH hnRNPs AND DENGUE NS1 PROTEIN AND ITS ROLE IN VIRAL REPLICATION AND RELEASE

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Dengue virus (DENV) is a mosquito-borne pathogen causing a mild dengue fever to a life-threatening dengue haemorrhagic fever. There is no vaccine currently available. Better understanding of the virus-host interplay is therefore crucial for vaccine development. Our previous study using expression proteomics demonstrated that many proteins, particular five forms of heterogeneous nuclear ribonucleoproteins (hnRNPs) were up-regulated in DENV-infected human endothelial cells. To address the functional significance of these proteins upon infection, we identified more of hnRNPs-interacting proteins in DENV-infected endothelial cells (EA.hy926 cells). The EA.hy926 cells were infected with DENV serotype 2 at a multiplicity of infection of 10 and subjected to immunoprecipitation (IP) using antibody specific to hnRNP C1/C2 and K. Interacting partners were resolved by two-dimensional gel electrophoresis and then identified by Q-TOF MS and/or MS/MS analyses. The MS results revealed 18 and 13 partners of hnRNP C1/C2 and hnRNP K, respectively. Interestingly, vimentin was identified as a common partner for both hnRNP C1/C2 and K. The interaction was confirmed by reciprocal IP followed by immunoblotting and double immunofluorescence staining. Disruption of the vimentin network using 4 mM acrylamide resulted in dissociation of these complexes. We also demonstrated that vimentin associated with dengue nonstructural protein 1 (NS1). Vimentin disruption not only dissociated this complex but also reduced NS1 expression, as well as intracellular virus production and release. Our data have suggested a pivotal role of vimentin in dengue replication and release.

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Poster Presentation 3

INVESTIGATION THE ROLE OF THREONINE 169 IN THE REACTION CATALYZED BY PYRANOSE 2-OXIDASE

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Pyranose 2-oxidase (P2O; pyranose:oxygen 2-oxidoreductase; EC 1.1.3.10) is a covalently-linked flavoenzyme that catalyzes the oxidation of D-glucose and several aldopyranoses to 2-keto-aldoses and hydrogen peroxide by molecular oxygen. The wild-type P2O prefers D-glucose over D-galactose as an electron-donor substrate. Crystallographic studies of pyranose 2-oxidase to a resolution of 1.8 Å indicated that the active site residue, Thr169, may be important for the reductive and oxidative half-reactions. In this study, a series of mutants, Thr169Ser, Thr169Asn, Thr169Gly and Thr169Ala, have been constructed for investigating the role of this residue by pre-steady state and steady state kinetics. Pre-steady state kinetics indicated that Thr169 plays an important role in the reductive half-reaction, since the rate of flavin reduction by D-glucose in these mutants were decreased but those by D-galactose were increased when compared to the values of the wild-type. Mutation of Thr169 may improve the binding toward D-galactose, which differs from D-glucose in the axial orientation of its O4 hydroxyl group, by reducing the steric hindrance between the side chain of this residue and the O4 position of D-galactose. Studies of the oxidative half-reaction showed that this residue participates in the stabilization of a C4a-hydroperoxyflavin intermediate since intermediate formation was not observed in all mutants. Our findings show that, while the wild-type enzyme follows a Ping-Pong kinetic mechanism, the Thr169Ala mutant exhibited a ternary complex mechanism, indicating that the product may remain bound to the enzyme active site during the oxidative half-reaction. Replacement of Thr169 with alanine significantly affected the microenvironment of the flavin. In agreement with this conclusion, a midpoint reduction potential of -197 mv for the two-electron transfer was determined at pH 7.0 for Thr169Ala, which was ~92 mv more negative than that of the wild-type enzyme.

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Poster Presentation 4

STRUCTURE DETERMINATION OF RICE OS4BGLU12 β-GLUCOSIDASE WITH AND WITHOUT G2F INHIBITOR

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The crystals of native rice Os4BGlu12 β-glucosidase and an Os4BGlu12 complex with 2-deoxy-2-fluoroglucoside (G2F) diffracted X-rays to 2.50 and 2.45 Å resolution, respectively, and were determined to have tetragonal $P4_32_12$ space group symmetry. The structure of native Os4BGlu12 was solved by molecular replacement with the 1CBG structure of white clover cyanogenic β-glucosidase as a search model and had two molecules per asymmetric unit with a solvent content of 49.98% and V_M of 2.46 $Å^3$ Da^{-1}. The native Os4BGlu12 structure served as a template for rigid body refinement to solve the structure of Os4BGlu12 with G2F data set with corresponding V_M of 2.68 $Å^3$ Da^{-1} and 54.2% solvent content. The structures were similar to previously known GH1 enzymes, but the significant differences were seen in the main-chain trace of the loops surrounding the active site. The active site is located at the bottom of an approximately 20 Å deep slot-like pocket surrounding by large surface loops. In the innermost part of the active site in the crystal structure of the G2F complex, the surrounding amino acid residues, which are conserved in other GH1 enzymes, formed hydrogen bonds with the glucosyl unit. On the other hand, residues around the aglycone binding site, which likely determine the substrate specificity of the enzyme, are not conserved. There was no change in the conformation of Glu393, the catalytic nucleophile residue, in the glycosyl-enzyme intermediate complex when compared to the native Os4BGlu12 structure, in contrast to other known GH1 β-glucosidases, in which the nucleophile changes position upon glycosylation. In addition, the position of the nucleophilic Glu393 residue of Os4BGlu12-G2F has an angle and distance to anomeric carbon of G2F similar to those seen in an S-glycosidase, consistent with its ability in the hydrolysis of thioglycosides.

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Poster Presentation 5

STRUCTURAL ANALYSIS OF RICE BGlu1 GLYCOSYNTHASE WITH AND WITHOUT OLIGOSACCHARIDES BY X-RAY CRYSTALLOGRAPHY

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Rice BGlu1 β-glucosidase belongs to glycosyl hydrolase family 1 and hydrolyzes β-1,3- and β-1,4-linked oligosaccharides. Glycosynthases made by mutation of glycosidases allow synthesis of specific oligosaccharides and glycosides, which can be used for various applications. The rice BGlu1 mutant E386G is an efficient glycosynthase, which can synthesize long chain oligosaccharides, a property not previously seen in glycosynthases derived from exoglycanases like BGlu1. This property may be related to the long oligosaccharide binding site of BGlu1, which results in more efficient hydrolysis of longer cellooligosaccharides than shorter ones. To determine the basis of high efficiency synthesis of long oligosaccharide chains, the structure of the BGlu1 E386G glycosynthase with and without substrates are determined by X-ray crystallography. The BGlu1 E386G was co-crystallized with and without ligands, including *p*-nitrophenyl-β-D-cellobiose (*p*NPC2, acceptor for transglucosylation), α-glucosyl fluoride (α-GlcF, donor for transglucosylation), cellotetraose (C4), cellopentaose (C5) and α-GlcF with *p*NPC2 by the hanging drop vapor diffusion method with microseeding. The crystals diffracted X-rays to 2.20, 1.85, 1.90, 1.60, 1.58 and 1.75 Å resolution, for free enzyme, and enzyme with *p*NPC2, α-GlcF, C4, C5 and both α-GlcF and *p*NPC2, respectively. The BGlu1 E386G crystals with and without ligands were found to belong to space group $P2_12_12_1$, and were isomorphous with wild type BGlu1 crystals. So far, ligand electron densities were found only for α-GlcF, C4 and C5 in the active site of BGlu1 E386G. The structures of free BGlu1 E386G and BGlu1 E386G with α-GlcF, C4 and C5 were refined to *R*-factors of 18.16%, 16.91%, 17.89% and 16.70% and R_{free} of 21.26%, 20.33%, 20.63% and 18.95%, respectively.

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Poster Presentation 6

CONVERTING SUBSTRATE SPECIFICITY OF DALCOCHINASE FROM DALCOCHININ GLUCOSIDE TO LINAMARIN BY MULTIPLE MUTATIONS

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Dalcochinase (from Thai rosewood) and linamarase (from cassava) are β-glucosidase that share 60% sequence identity but their catalytic abilities are distinct. In hydrolysis, both enzymes show specificity for their natural substrates, dalcochinin-8'-O-β-D-glucoside and linamarin, respectively, but could not hydrolyze the natural substrate of the other enzyme. For transglucosylation, dalcochinase can synthesize alkyl glucoside using only primary and secondary alcohols, while linamarase can use primary, secondary and tertiary alcohols as acceptors. Previously, single mutations at ten residues in the substrate binding site of dalcochinase to the corresponding residues of linamarase decreased their specificities toward dalcochinin-8'-O-β-D-glucoside, but did not increase their activity toward linamarin. Interestingly, three mutants (I185A, N189F and V255F) showed improved transglucosylation activities using primary and secondary alcohols as acceptors compared with the wild-type enzyme. In this project, three double mutations (I185A-N189F, I185A-V255F and N189F-V255F) and one triple mutation (I185A-N189F-V255F) of dalcochinase were constructed to investigate the interactions of amino acid residues in substrate specificity and transglucosylation. Two double mutants, I185A-N189F and I185A-V255F, could hydrolyse linamarin, while the N189F-V255F and I185A-N189F-V255F mutants could not. So, it appeared that residue A185 could interact with either F189 or F255 to assist in hydrolysis of linamarin. However, their specificities toward dalcochinin-8'-O-β-D-glucoside decreased by 2-7 folds, compared with the wild-type enzyme. Thus, residues I185, N189 and V255 in dalcochinase played important roles in the hydrolysis of dalcochinin-8'-O-β-D-glucoside, with I185 being more dominant than the other two residues. In transglucosylation studies, three mutants, I185A-N189F, I185A-V255F and I185A-N189F-V255F, could improve transglucosylation efficiency using primary and secondary alcohols as acceptors, compared with the single mutant enzymes. In particular, the triple mutant I185A-N189F-V255F gave significantly high yield of alkyl glucoside from *iso*-propanol. However, none of our four dalcochinase mutants could catalyze transglucosylation using tertiary alcohols as acceptors.

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Poster Presentation 7

INVESTIGATION ON THE SUBSTRATE BINDING RESIDUES OF THE OXYGENASE COMPONENT OF *p*-HYDROXYPHENYLACETATE HYDROXYLASE

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p-hydroxyphenylacetate hydroxylase from *Acinetobacter baumannii* is a flavoprotein monooxygenase that catalyzes hydroxylation of *p*-hydroxyphenylacetate (HPA) to form 3,4-dihydroxyphenylacetate. This enzyme is a two-protein enzyme system containing a smaller reductase component (C_1) and a larger oxygenase component (C_2). From crystal structures of the oxygenase component (C_2), His120 and Ser146 were speculated to be residues important for binding of the substrate, HPA, since both residues can interact with the hydroxyl group of HPA via H-bonding. The interactions of His120 and Ser146 with HPA may facilitate the binding of HPA in the deprotonated form which is supposed to be the form supporting the hydroxyl group transfer via an electrophilic aromatic substitution mechanism. In our previous study with the wild-type enzyme, the hydroxylated product yield was found to be constant at pH 7-10 implying that the substrate may bound at the active site in the deprotonated form at this pH range. In this study, His120 and Ser146 were mutated into Asn (H120N) and Ala (S146A), respectively to investigate the role of both residues in the substrate binding. For the mutant H120N, stopped-flow experiments and single-turnover product analysis indicated that HPA was not bound to this mutant and the enzyme could not catalyze the hydroxylation reaction. This may be due to disruption of the H-bond between this residue and HPA. For the studies of S146A at pH 7, both stopped-flow experiments and rapid-quenched flow were carried out. The reaction of S146A:$FMNH^-$ with O_2 in the absence of HPA showed that formation a C(4a)-hydroperoxy-FMN intermediate occurred at a rate constant of 3.5×10^5 $M^{-1}s^{-1}$ (1.1×10^6 $M^{-1}s^{-1}$ for wild type). The rate of the FMN re-oxidation was 0.03 s^{-1}. This result is similar to the reaction of the WT in absence of HPA. In the presence of HPA, the reaction of S146A:$FMNH^-$ with O_2 indicated that formation of C(4a)-hydroperoxy-FMN-HPA intermediate occurred with a rate constant of 2.6×10^4 M^{-1} sec^{-1}(4.8×10^4 $M^{-1}s^{-1}$ for the wild type). The step of elimination of H_2O_2 without hydroxylation of the substrate HPA in the uncoupling pathway was observed at a rate constant of 4 s^{-1}. From rapid-quenched experiments, the rate constant of the hydroxylation step at pH 7 was 2 s^{-1}. The total DHPA formed were 55% at pH 7 and increased with pH rise. Results indicated that in this mutant, the uncoupling pathway with no hydroxylation occurred at a significant ratio at pH 7.0 while at higher pH, a higher yield of DHPA was found. All of the results suggest that the hydroxylation reaction of this mutant was increased with pH rise. This may be due to the increase of the deprotonated from of the substrate when pH is increased.

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Poster Presentation 8

GENETICALLY MODIFIED *ESCHERICHIA COLI* STRAIN BL21(DE3) AS A NOVEL BACTERIAL SURROGATE SYSTEM FOR STUDY OF MALARIA DIHYDROFOLATE-THYMIDYLATE SYNTHASE (DHFR-TS) ENZYME

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Escherichia coli strain BL21(DE3) is the most widely used bacterial host for efficient heterologous protein expression due to its lack of key proteases that would otherwise degrade the heterologous proteins being expressed. We therefore exploited this ideal protein expression characteristic and constructed a thyA (thymidylate synthase) knockout of BL21(DE3) for the complementation study of the malaria bifunctional enzyme DHFR-TS (dihydrofolate-thymidylate synthase), an important drug target in malaria and other infectious diseases. A modified methodology using the Red recombinase system of bacteriophage lambda was employed to inactivate the chromosomal thyA gene of the *E. coli* strain BL21(DE3) through homologous recombination with linear PCR products. The correct genotype of the thyA mutant was verified by PCR and Southern blot analysis. Like its thyA knockout counterpart *E. coli* γ2913, our thyA mutant's thymidine auxotrophy could be complemented with a number of variants of malaria DHFR-TS on minimal media lacking thymidine, indicating its usability as a host for studying functional TS enzyme of malaria. We propose that our thyA knockout of the *E. coli* strain BL21(DE3) may be a more attractive choice than the *E. coli* γ2913 strain as a bacterial host for TS enzyme complementation study, due to its advantageous genetic background that allows for better heterologous protein expression. In addition to malaria, we envisage that our thyA mutant may have beneficial applications in other important diseases, for example, screening TS inhibitors for treatment of cancer and tuberculosis.

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Poster Presentation 9

INVESTIGATION ON THE ROLE OF ASN593 IN THE CATALYTIC MECHANISM OF PYRANOSE 2-OXIDASE (P2O) FROM TRAMETES MULTICOLOR

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Pyranose 2-oxidase (P2O) is an enzyme that catalyzes oxidation of D-glucose and several aldopyranoses at the C2 position, resulting in the corresponding 2-keto sugars and H_2O_2. P2O belongs to an enzyme class of flavin-dependent oxidases in which their reactions can be divided into a reductive and an oxidative half-reaction. Recently, both reductive and oxidative half-reactions at pH 7.0 of the wild-type P2O have been studied using stopped-flow spectrophotometry. The crystal structure of P2O shows that the enzyme active site contains the catalytic residues His-Asn pair that are conserved in all enzymes in the GMC (Glucose-Methanol-Choline) oxidoreductase superfamily except in D-glucose-1-oxidase where the pair are shown as His-His. In this study, the role of the asparagine residue (N593) at the active site of P2O has been investigated. The mutants in which N593 has been changed to alanine (N593A) and histidine (N593H) were investigated their steady-state and pre-steady-state kinetics properties at pH 7.0 at 25C°. Steady-state kinetic analysis showed that K_m values of D-glucose for both N593A (90 mM) and N593H (95 mM) were significant higher than the value of the wild type enzyme (3.4 mM). Pre-steady state kinetics in reductive half-reaction showed 20-fold decrease in the rate of flavin reduction by D-glucose for N593A (k_{red} = 2.4 s^{-1}) and 50-fold decrease for N593H (k_{red} = 0.89 s^{-1}) when compared to that of the wild type enzyme (k_{red} = 48 s-1). This indicates that N593 plays a crucial role in flavin reduction. For the oxidative half-reaction, the C(4a)-hydroperoxy flavin which is detected as an intermediate during the reaction of the wild type enzyme was not observed in both mutants. This suggests that the Asn593 residue is important for the intermediate stabilization.

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Poster Presentation 10

SCREENING AND CHARACTERIZATION OF NOVEL HIGH GLUCOSE TOLERANT β-GLUCOSIDASE FROM *ASPERGILLUS* SPP.

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Nowadays every country all over the world is encountering to the oil crisis due to the depletion of natural fuel energy. Bioethanol production from cellulosic materials is a source of alternative energy to replace those from natural resources. Production of cellulosic ethanol requires degradation of cellulose by a concerted action of cellulase and β-glucosidase, which yields cellobiose and glucose, respectively. However, cellulase and β-glucosidase activities are inhibited by high concentration of their products. Therefore, it is necessary to search for high glucose-tolerance β-glucosidase (HGT-BG). This HGT-BG may help to increase efficiency of cellobiose hydrolysis to glucose, which will enhance the efficiency of cellulose hydrolysis to cellobiose. Fungal enzymes have been studied extensively due to the high cellulolytic activities. Among them, *Aspergillus* spp. is the important sources of endo β-glucanase and β-glucosidase. Therefore, 126 isolates of *Aspergillus* spp. from the existing stock at Kasetsart University were screened to select for isolates that produced high HGT-BG activity in minimal medium supplemented with various carbon sources. After that, the optimum concentrations of the selected carbon source were determined. From this study, 6 isolates of *Aspergillus* spp., which are ASKU18, ASKU22, ASKU28, ASKU62, ASKU129 and ASKU154, were selected due to its high expression level in different pattern. All 6 isolates produced highest HGT-BG activity in culture media containing beechwood xylan at concentrations ranging from 1.5-3.5% (w/v). Finally, HGT-BG from the selected isolates was purified and its properties characterized.

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Poster Presentation 11

PHOSPHOPROTEOME OF LUNG CANCER CELL LINE

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Lung cancer is the leading cause of cancer-related to death in both men and women. Protein biomarkers for lung cancer were investigated using the expression of proteins from lung cancer cell line (A549) and compared with those of normal lung fibroblast cell line (MRC-5). Two-dimensional gel electrophoresis of A549 and MRC-5 cells was carried out and followed by protein identification using nanoelectrospray tandem mass spectrometry. Most proteins overexpressed in A549 cells were phosphoproteins such as lamin AC 70 kDa, cyclophilin, α-enolase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, peroxiredoxin1 and peroxiredoxin-2. Moreover, some proteins were expressed only in A549 cells such as nuclear co-repressor KAP1, transketolase and cytokeratin 18. Furthermore, the phosphoprotein chaperonin 60 was highly expressed in A549 cells. It is known to function in protein interactions and protein conformation. The overexpression of this protein in the A549 cells may result in abnormalities of protein conformation and lead to early stage cancer. These proteins may be used as biomarkers of lung cancer for early detection and clinical prognosis.

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Poster Presentation 12

IMPROVEMENT OF RICE OS7BGLU26 β-MANNOSIDASE/β-GLUCOSIDASE PROTEIN EXPRESSION FOR PURIFICATION AND PRELIMINARY CRYSTALLIZATION

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β-glucosidases are enzymes that catalyze the hydrolysis of β-glycosidic bonds between the reducing side of a terminal glucosyl residue and an aryl or alkyl aglycone or oligosaccharide. These enzymes play important roles in many biological mechanisms in plants. Based on the protein sequence-based phylogenetic tree of rice glycosyl hydrolase family one (GH1), rice Os7βglu26 is closely related with rice BGlu1. However, rice Os7βglu26 hydrolyzes *p*NP-β-D-mannoside more efficiently than *p*NP-β-D-glucoside, while the rice BGlu1 shows the opposite preference. To understand this difference in substrate specificity and its catalytic mechanism, Os7βglu26 is being investigated at the structural level. The expression of Os7βglu26 His-tagged fusion protein in *E. coli* strain Origami(DE3) showed very low expression. Therefore, the expression host was changed to *E. coli* strain Rosetta-gami(DE3) and optimized by varying the IPTG concentration and induction time. The fusion protein was highly expressed in the conditions of 0.3 mM IPTG and 24 h induction time. Os7βglu26 was purified by immobilized metal affinity chromatography (IMAC). The 66 kDa thioredoxin and His-tagged Os7βglu26 fusion protein fractions contained many contaminating protein bands after the 1st IMAC column. It was then cleaved with enterokinase, and the 50 kDa tag-free protein was further purified by a 2nd round of IMAC. The purity of the protein increased to approximately 95%, so crystallization trials were begun. The purified Os7βglu26 was used for crystallization screening using the JY and Hampton 134 crystallization screens.

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Poster Presentation 13

SERUM PROTEIN PATTERN IN PATIENTS WITH NASOPHARYNGEAL CARCINOMA

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Nasopharyngeal carcinoma (NPC) is a serious head and neck cancer in southern China and Southeast Asia. Epidemiologically, Epstein-Barr virus, is an environmental carcinogens and genetic traits may contribute to NPC development. NPC is one of the leading causes of cancer death in Thai male and its incidence rate still increasing yearly. The feasible strategy to fight against NPC is early stage detection in high-risk people without NPC-related symptoms followed by earliest curative therapy. The most effective way in early detection of NPC is to search for high-risk people by using genetic biomarkers and subsequently screen for NPC early stages by using protein biomarkers. Recently, MALDI-TOF/TOF MS has been claimed as a powerful tool for the detection of new protein biomarkers in the serum of cancer patients with the potential to be used for a mass screening. The purpose of our study was to detect new NPC biomarkers based on a serum protein pattern for discriminating NPC patients from healthy controls by using MALDI-TOF/TOF MS. Serum samples from 33 patients with NPC and 30 non-NPC control subjects were recruited in this study. Using ClinProTools bioinformatics and statistical software, 4 prominent protein peaks with the mass to charge ratio at 1205.93 (m/z), 1304.13 (m/z), 1911.70 (m/z) and 228.77 (m/z) were selected as a model for NPC diagnosis. The former two proteins were down-regulated whereas the later two were up-regulated in cancer patients as compared to controls. This four-peak protein model was able to discriminate NPC from non-NPC subjects with sensitivities of 73% and specificities of 70%, respectively. The results of our study suggest that proteins represented by these 4 peaks could be new biomarkers for NPC diagnosis. Further investigation is currently underway to validate the potential of this serum protein pattern for the early detection of NPC in Thailand.

Poster Presentation 14

PURIFICATION AND CHARACTERIZATION OF THE NATURAL SUBSTRATES OF OS4BGLU12 RICE β-GLUCOSIDASE

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Rice Os4BGlu12 is a glycosyl hydrolase family 1 β-glucosidase. The full-coding cDNA coding for Os4glu12 was previously isolated from rice seedlings by RT-PCR and its recombinant protein was expressed as a soluble, active form in *Escherichai coli*. The Os4BGlu12 protein was purified by Co^{2+} IMAC column and used for identifying its natural substrates from rice tissue extracts. The natural substrates were purified by LH-20 column chromatography, TLC, and HPLC. Recombinant Os4BGlu12 protein expressed in *E. coli* could hydrolyze compounds extracted with methanol from 7-day old rice seedlings and rice plants at flowering stage. The substrates of Os4BGlu12 found in rice leaf and stem at flowering stage were named C1/sD1, Cl/sD2 and Cl/sD3. ^{1}H-NMR analysis indicated the presence of aromatic groups and anomeric protons of sugar molecules in the structures of three C1/sD compounds. The molecular weights of C1/sD1 glycoside, was determined by ESI-MS to be 492 amu, which matches many flavonoid glycosides, while Cl/sD2 and Cl/sD3 were both found to have the MW of 688 amu. This data suggests that Cl/sD2 and Cl/sD3 could be isomeric compounds. In addition, the enzyme could hydrolyze the oligosaccharide products of rice cell wall hydrolysis by a wounding induced rice endo-1,3-1,4-β-glucanase.

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Poster Presentation 15

ALTERATIONS IN CELLULAR PROTEOME AND SECRETOME UPON DIFFERENTIATION FROM MONOCYTE TO MACROPHAGE: INSIGHTS INTO BIOLOGICAL PROCESSES

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Monocyte and macrophage are mainly involved in immune response and inflammatory processes. Monocytes circulate in the bloodstream and migrate to various tissues where they can differentiate to macrophages. However, the molecular basis of biological processes involved in this cellular differentiation remains ambiguous. This study was to investigate alterations in cellular and secreted proteins after this differentiation phase. Macrophage was differentiated from U937 human monocytic cell line by treatment with 100 ng/ml phorbol myristate acetate (PMA) for 48 h. Cellular and secreted proteins extracted from PMA-treated cells (macrophages) were compared with those of untreated cells (monocytes) using 2-DE (n=5 gels/condition; stained with Deep Purple fluorescence dye). Quantitative intensity analysis revealed 81 and 67 protein spots whose levels were significantly altered in cellular proteome and secretome. These proteins were subsequently identified by Q-TOF MS and/or MS/MS analyses. The altered levels of cellular elongation factor-2 (EF-2) and secreted α-tubulin were confirmed by Western blot analysis. Global protein network analysis demonstrated that these altered proteins were involved in cell death, lipid metabolism, cell morphology, cellular movement, and protein folding. Our data may provide some insights into molecular mechanisms of biological processes upon differentiation from monocytes to macrophages.

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Poster Presentation 16

VALIDATION OF DOCKING METHODS TO STUDY THE INTERACTION OF TYROSINE KINASE OF EGFR AND ITS INHIBITORS

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Several docking methods are currently available, most of which are claimed to be the best docking algorithm among others. However, we believe that there is no docking method or scoring function that is suitable for every protein. For each docking application, one needs to validate all available protocols in order to select the best docking method and scoring function. In this study, we have evaluated the following docking program packages: MOE, GOLD (CCDC), GLIDE (SCHRÖDINGER), AUTODOCK versions 3 and 4 for binding interaction of tyrosine kinase EGFR-TK and its inhibitors as obtained from its available crystal structures. RMSD was calculated with both individual docking program and Discovery Studio Visualizer program for comparison. The efficiency of ligand docking algorithm and scoring function was judged by the ability to reproduce the inhibitor conformation resembling that observed in the co-crystal structures with low RMSD value. Subsequently, the best method after program validation for docking of the EGFR-TK structure can be used in virtual screening process.

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Poster Presentation 17

SERINE HYDROXYMETHYLTRANSFERASE FROM *PLASMODIUM VIVAX* IS DIFFERENT IN SUBSTRATE SPECIFICITY FROM ITS HOMOLOGUES

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Plasmodium vivax serine hydroxymethyltransferase (PvSHMT) catalyzes the conversion of L-serine and tetrahydrofolate (THF) to glycine and methylene-THF. The enzyme was cloned and expressed in *Escherichia coli*. The purified enzyme is a dimeric protein with a monomeric molecular mass of 49 kDa. PvSHMT has a maximum absorption peak at 422 nm with a molar absorption coefficient of 6,370 $M^{-1}cm^{-1}$. The K_d for binding of the enzyme and PLP was measured to be 0.14 $\pm$ 0.01 mM. An alternative assay for measuring the THF-dependent SHMT activity based on the coupled reaction with 5,10-methylenetetrahydrofolate reductase (MTHFR) from *E. coli* was developed. PvSHMT uses a ternary-complex mechanism with the k_{cat} value of 0.98 $\pm$ 0.06 s^{-1} and the K_m values of 0.18 $\pm$ 0.03 and 0.14 $\pm$ 0.02 mM for L-serine and THF, respectively. Although L-serine is a physiological substrate for SHMT in the THF-dependent reaction, PvSHMT can also use D-serine as a substrate for catalysis. This property is different from that of mammalian SHMTs, indicating the dissimilarity between PvSHMT and the mammalian SHMT that may be exploited for further development of specific inhibitors against PvSHMT. In addition, SHMT can also catalyze a retro-aldol cleavage reaction of many amino acids. Our results have shown that PvSHMT can catalyze the retro-aldol cleavage of L-threonine, *allo*-threonine and β-phenylserine. Kinetic parameters for these substrates were measured. Recently, we have used another type of assay employing 5,10-methylenetetrahydrofolate dehydrogenase (MTHFD) to measure the THF-dependent SHMT activity. This assay may be more advantageous than the MTHFR assay since it does not require a strict anaerobic condition.

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Poster Presentation 18

COMPARATIVE ENERGY ANALYSIS OF HUMAN EGFR AND ERBB2 TYROSINE KINASES IN COMPLEX WITH 4-ANILINOQUINAZOLINE

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Abnormalities in receptor tyrosine kinases of ErbB family (EGFR, ErbB2, ErbB3 and ErbB4) have been associated with several types of human cancer. Overexpressions and mutations on tyrosine kinase (TK) domain of both EGFR and ErbB2 are commonly found in breast and lung cancer with poor prognosis. Although the TK domain of ErbB2 shares ≈90% sequence similarity to that of EGFR, it has been found that 4-aniliniquinazoline (AQ4), an anti-cancer drug, could inhibit the activity of EGFR-TK more specifically than that of ErbB2-TK. This study aimed at exploring the detailed interaction between the two TKs and AQ4 in order to understand the mechanisms causing drug specific in EGFR-TK and ErbB2-TK. Structural model of ErbB2-TK was constructed based on sequence homology to EGFR-TK. The inhibitor was docked into the ATP-binding site of both EGFR- and ErbB2-TKs. Molecular dynamics (MD) simulation methods were performed to evaluate the binding free energy for these two complexes. Comparison of interaction energies between the amino acid residues located in the binding site of each complex and the inhibitor has revealed the key residues or pharmacophore regions that may be responsible for specificity of AQ4.

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Poster Presentation 19

DIFFERENTIAL PROTEIN EXPRESSION IN SPECIFIC CELL TYPE OF *PENAEUS MONODON* HEMOCYTE DURING VIRAL INFECTION.

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The study of immune mechanism in crustacean including Black Tiger shrimp (*Penaeus monodon*) has been a challenge for the global aquaculture industry. Many reports indicated that the hemocyte tissues are the first line of defense, playing important roles in immune defense reaction. In this present research, we have demonstrated that at least three different types of hemocytic cells, hyaline, semi-granular and granular cells were successfully characterized and isolated by Percoll® gradient centrifugation method. Flow cytometric analysis revealed that relative abundance of each hemocytic population was altered upon the course of virus infection. A significant decreasing of hyaline cells was found in contrast to an increasing of semi-granular cells, suggesting their different roles in shrimp defense mechanism. SDS-PAGE analysis of the proteins extracted from each type of hemocytic cell showed differential expression of protein associated with immune function, such as 14-3-3 like protein, alpha-2 macroglobulin and histones. Comparative two-dimensional gel electrophoresis of protein expression between hyaline and semi-granular cells revealed a number of differentially expressed proteins upon viral infection. Further analysis of 2D gels by fluorescence staining also suggested the differences in specific protein phosphorylation between infected and the controlled groups. This comparative characterization of differential and phosphorylated proteins could provide a clue to understand specific roles and functions of each hemocytic cell type in viral defensive mechanism of the shrimp system.

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Poster Presentation 20

EXPRESSION AND PURIFICATION OF RICE β-GALACTOSIDASE C-TERMINAL DOMAIN FOR STRUCTURAL ANALYSIS

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β-Galactosidases (βgals) (EC 3.2.1.23) catalyse hydrolysis of the glycosidic bond of terminal nonreducing β-D-galactosyl residues of oligosaccharides and β-D-galactopyranosides. In rice (*Oryza sativa* L.), 15 genes for β-galactosidases (OsBgals) have been identified, 12 of which contain a conserved putative galactosyl binding domain at their carboxy-terminus. To understand the function and structure of this domain, a cDNA encoding the C-terminal domain of OsBgal1 (Bgal1Cter) was amplified by RT-PCR, cloned into pENTR- D/TOPO, then subcloned into pET32a(+)/DEST, and expressed in high yield as an N-terminal thioredoxin fusion protein in *Escherichia coli* strain Origami B(DE3). The soluble thioredoxin/Bgal1Cter fusion protein was purified by immobilized metal (Co^{2+}) affinity chromatography (IMAC). The OsBgal1Cter was cleaved from the fusion protein using enterokinase or thrombin protease, which digested the OsBgal1Cter from the tag of about 90% and 100% of the fusion protein, respectively. Then, the free Bgal1Cter was purified by a 2nd IMAC step. To demonstrate the presence of a stable and intact domain and to remove the leader peptide, the free OsBgal1 Cter protein was digested with trypsin protease, which was later removed from the digested OsBgal1 Cter with a benzamidine column. The purity of the protein obtained was approximately 95%, which allowed screening for crystallization. To eliminate the need for this step, a shortened construct, which placed the BgalCter immediately after the thrombin site was also designed. The purified OsBgal1Cter was used for crystallization screening with the JY, Hampton 134 and Emerald Wizard I/II crystal screens.

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Poster Presentation 21

PROTEIN ENGINEERING IN β-GLUCOSIDASE FOR HYDROLYSIS OF SOY BEAN ISOFLAVONE GLUCOSIDES

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β-glucosidases are hydrolytic enzymes catalyzing the cleavage of β-O-glucosidic linkage between non-reducing glucose and aglycone groups with various structures. These substrates of β-glucosidases play important roles in plant genera. For example, cyanogenic glucoside linamarin or isoflavone glucosides are involved in defense mechanism and plant regulation, respectively. Soybean isoflavone glucosides have been wildly studied due to the biological properties of its aglycone part, including osestrogenic, antioxidant, and antitumoral activities. Previous studies have shown that β-glucosidases from *Dalbergia cochinchinensis* (dalcochinase), *D. nigrescens* and *Glycine max* (GmICHG) could hydrolyze soybean isoflavone glucosides with different efficiencies, even though they show 60-80 % amino acid sequence identity. To identify the residues that may be involved in hydrolysis of soybean isoflavone glucosides, four mutant enzymes of dalcochinase were generated, in which the residues of dalcochinase were changed to corresponding residues of GmICH (D400N, A454F, E455A and S459V). These mutants were expressed, purified, and their relative activities towards *p*NP-β-D-glucoside, dalcochinin glucoside (isoflavone glucoside, natural substrate of dalcochinase) and soybean isoflavone glucosides (genistin and daidzin) studied. Compared to the wild-type recombinant enzyme, D400N showed between 2- to 6-fold decreases in relative activities towards all substrates tested, while A454F showed slight increases. On the other hand, E455A had 4- to 5-fold higher relative activity towards all substrates. The relative activities of S459V increased 3- and 2-fold towards genistin and daidzin, respectively, but increased only slightly towards *p*NP-β-D-glucoside and dalcochinin glucoside. Therefore, it appeared that residue D400 was favorable for hydrolysis of soybean isoflavone glucosides, while E455 and S459 were not.

Poster Presentation 22

PROTEIN FRACTIONATION BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) FOR QUANTITATIVE PROTEOMICS OF RICE ROOT CULTURES DURING SALINITY STRESS

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A simple and efficient way to analyze the differential expression of complex protein mixtures in rice root cultures during salinity stress is developed. Two-month-old rice root cultures were treated with 513 mM NaCl for 0, 6, 9 12 and 24 h. Total proteins were extracted and separated by one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands were excised corresponding to protein markers, and the tryptic peptides from in-gel digestion of each gel plug were analyzed by label-free quantitation based on LC-MS technique. The LC-MS data were analyzed using DeCyder MS 2.0 followed by MASCOT software. Quantitative differences in the relative abundance of 12 proteins were found during salt stress. This strategy offers the ability to quantitatively analyze changes in protein abundance that correlate with salt stress response with enhanced dynamic range of analysis and improved identification of low-abundance proteins.

Poster Presentation 23

MUTATIONAL ANALYSIS OF JUNCTIONAL REGION OF *PLASMODIUM FALCIPARUM* DIHYDROFOLATE REDUCTASE-THYMIDYLATE SYNTHASE

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Plasmodium falciparum dihydrofolate reductase-thymidylate synthase (*pf*DHFR-TS) is an important target of antimalarial drugs such as pyrimethamine (Pyr) and cycloguanil (Cyc). Previous investigations on this bifunctional enzyme revealed that amino acids remote from active site residues could be important in regulating the enzyme activity and conferring drug resistance. Results from our laboratory and others further confirmed the importance of this junctional region (JR) which links between the *pf*DHFR and *pf*TS domains in conferring the *pf*TS activity. Using TS-deficient *E. coli*, we have been able to develop a complementation system which shows that both DHFR and TS domains interacted with each other and that the length of JR was important for the *pf*TS activity. A series of recombinant *pf*JR-TS with variation in the lengths of JR were constructed and were co-transformed into TS-deficient *E. coli* harboring the plasmid which expresses catalytically active *pf*DHFR. Complementation studies revealed that in order for the *pf*JR-TS construct to complement the growth of *E. coli*, the minimal length of JR needed to start from Tyr 278 and any *pf*JR-TS constructs with shorter JR would not be able to complement the growth of TS-deficient *E. coli*. A more sensitive method employing [^{3}H]-FdUMP also confirmed the above findings which highlight the possibility of exploring parasite new target through targeting the non-active site region of this important enzyme.

Poster Presentation 24

PROPERTIES OF ALMOND β-GLUCOSIDASE-FUNCTIONALIZED MAGNETIC NANOPARTICLES

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The use of nanomaterials provides many advantages due to their unique sizes and physical properties. Hybrid nanoscale materials are well established in various bioprocesses such as nucleic acid detachment, protein separation and immobilization of enzymes. Immobilization of enzymes has received great attention for industrial uses because of many advantages, including easy separation of immobilized enzyme from the reaction mixtures by use of an external magnetic field. This provides a convenient platform for recycling and recovery of expensive biocatalysts. Moreover, small nanoparticles, with greater specific surface area than that of microparticles, offer unique favorable properties in kinetics and diffusion in catalysis.
In our work, almond β-glucosidase, the most common enzyme with biotechnological applications in production of glucosides, was chosen for immobilization on iron-oxide superparamagnetic nanoparticles (SPIONs). Each milligram of SPIONs contained over 30 microgram of the enzyme. The specific activity of immobilized enzymes was found to be 1/100 that of the free enzyme. Recyclability of β-glucosidase-conjugated magnetic nanoparticles (MNPs) was demonstrated. No significant change was observed in the first 3-5 rounds and the enzyme activity was roughly diminished by 2.5% in each cycle. The storage stability of immobilized enzyme was higher than free enzyme. The pH profile and thermal stability of immobilized and free enzyme will be presented.

Poster Presentation 25

IMPAIRMENT OF BINDING OF ALIX TO LYSOBISPHOSPHATIDIC ACID ON CELL SURFACE DELAYS DENGUE VIRUS REPLICATION IN HUMAN ENDOTHELIAL CELLS

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The most severe form of dengue virus (DENV) infection is dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). The hallmark of DHF/DSS is an increased vascular permeability, indicating the effect of DENV infection on endothelial cells. A screening study on DENV-induced changes in proteome profiles in human endothelial cells (EA.hy926) by subcellular proteomic analysis showed up-regulation of Alix (apoptosis-linked gene-2-interacting protein X) in cytosolic fraction of DENV-infected cells comparing to mock-control cells. Interaction between late endosomal lipid lysobisphosphatidic acid (LBPA) and Alix has been associated with the fusion of viral envelope with endosomal membrane. Subsequent functional studies revealed that pretreatment of EA.hy926 cells with anti-LBPA antibody could prevent the binding of Alix to LBPA, and resulted in the reduction of viral protein synthesis and DENV replication. Our data indicate that the interaction between LBPA and Alix is required in the early phase of DENV replication, particularly when it arrives at the late endosome. Blocking this step may lead to a novel approach to control DENV replication *in vivo*.

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Poster Presentation 26

STRUCTURAL BIOLOGY USING SYNCHROTRON PROTEIN CRYSTALLOGRAPHY AT NSRRC

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Three-dimensional structures of biological macromolecules provide valuable information to help us understand their structure-function relationships and mechanisms of action. The high-throughput determination of a high quantity of protein structures is generally the indication of success in the challenging pursuit of structural genomics studies. Since the function of a gene product is tightly coupled to its three-dimensional structure, determining the structure or its folding pattern may provide important insight into its biochemical function, which, in turn, may help to place it in a particular cellular pathway. By using the power of synchrotron radiation to facilitate protein crystallographic structure determinations, structural biology can play an important role in the search for the functions of these novel genes.

The need for synchrotron radiation for biological research has increased dramatically over the past decade. The unique capabilities of synchrotron sources for generation of higher intensity X-ray beams at tunable wavelengths have resulted in a tremendous growth in numbers of three-dimensional structures determined by protein crystallography. Here, we report the current status and development of facilities for protein crystallography and examples of research results from these facilities at the NSRRC.

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Poster Presentation 27

A PRELIMINARY STUDY OF PROTEINS FROM TROPICAL LICHENS

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Lichens are co-existing symbiotic associations between fungi and algae. Seven crustose lichens specimens were selected as the representatives for this study. The proteins from thallus; a unique, castle-like structure, were extracted and separated by SDS-PAGE. Differences in the soluble protein fraction between the freshly isolated lichen were found. The protein bands of interest were excised, in-gel digested and the tryptic peptides were recovered for a subsequent analysis using LC-MS/MS.

Poster Presentation 28

DISCOVERY OF A GENUS-SPECIFIC ANTIGEN AND PROTEIN HETEROGENEITY IN INTRA-SPECIES OF *AEROMONAS* SPP.

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Aeromonas spp. is Gram-negative bacteria found widely in the environment. Some of them are pathogenic for animals and humans, causing gastroenteritis and serious infections of various internal organ systems. The classification of this bacterium is still arguable. This study then aimed to identify a genus- and/or species-specific antigen of this bacterium using Western blot analysis with immune sera. Groups of three mice were immunized each with whole cell lysates of *A. hydrophila*, *A. sobria*, *A. caviae*, *A. trota*, *A. jandaei*, *A. veronii*, or *A. media*. The immune sera were collected and tested against all seven *Aeromonas* species- and other non-related Gram-negative bacterial extracted antigens. Our results showed that all immunized sera reacted to a single antigen of molecular mass of 8.5 kDa of all 123 *Aeromonas* isolates of those seven species, but did not react with the antigen of other Gram-negative bacteria, indicating of *Aeromonas* genus-specific antigen. However, we did not find any species-specific antigen. As analyzed by using UPGMA-grouping system (SynGene software) and Jaccard's coefficient (Sj) $\geq$0.5, heterogeneity of the antigens from the bacteria within the same species were found. Twenty-six isolates each of *A. sobria*, *A. caviae*, *A. trota* were classified into 11, 11, and 13 groups, respectively; 27 isolates of *A. hydrophila* into 12 groups; 8 isolates of *A. jandaei* into 6 groups; 7 isolates of *A. veronii* into 3 groups; and 3 isolates of *A. media* into 3 different individuals. This is the first report on the success of *Aeromonas* genus-specific antigen discovery that would be further developed a diagnostic test.

Supported by Faculty of Tropical Medicine, Mahidol University and Mahidol University

Poster Presentation 29

EFFECTS OF CURCUMIN ON INVASIVE PHENOTYPE AND VASCULOGENIC MIMICRY OF HUMAN HEPATOCELLULAR CARCINOMA CELLS

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Vasculogenic mimicry is a characteristic of cancer cells in which cells form vascular channels without endothelial cell lining. Vasculogenic mimicry is associated with the capability of cancer cells to migrate and produce matrix metalloproteinases (MMPs). Curcumin, a component of turmeric (*Curcuma longa*) found in traditional herbs, has been reported to have anti-tumor properties. We investigated the effect of curcumin on vasculogenic mimicry *in vitro* with SK-Hep-1 human hepatoma cells. Curcumin inhibited the formation of capillary-like tubular networks of SK-Hep-1 cells on Matrigel (tube formation) in a dose-dependent manner. Cell migration assay (Transwell) and gelatin zymography revealed that curcumin decreased cell migration by 75 percent and reduce the production of MMP-9 in SK-Hep-1cells, respectively. These results demonstrated that curcumin does exert inhibitory effects on vasculogenic mimicry in human hepatoma cells.

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Poster Presentation 30

NON-PHOTOCHEMICAL ENERGY DISSIPATING PROCESSES OF UNICELLULAR GREEN ALGAE (*DUNALIELLA SALINA*) DURING CARBON SOURCE STARVATION

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Non-photochemical quenching (NPQ) is one of many mechanisms that photosynthetic organisms use to protect themselves from photo-oxidative stress. Apart from the previous studies which have been shown that there are many processes involved in NPQ induction, such as zeaxanthin accumulation from the xanthophylls cycle or increased in pH gradient across the thylakoid membrane, little is known in detail with respect to the factors that controls the rate or degree of NPQ. In this study, chlorophyll fluorescence measurement and pigment composition analysis were used to study the effect of carbon source starvation by both direct (changing media composition to bicarbonate free medium) and indirect (changing media composition to different salt concentration) methods on NPQ induction. The results showed that carbon starvation induced both the rate and degree of NPQ, along with zeaxanthin accumulation.

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Poster Presentation 31

HIGHLY STEREOSELECTIVE HYDROLYSIS OF CASSAVA LINAMARASE AND KINETIC RESOLUTION USING TWO-ENZYME SYSTEM

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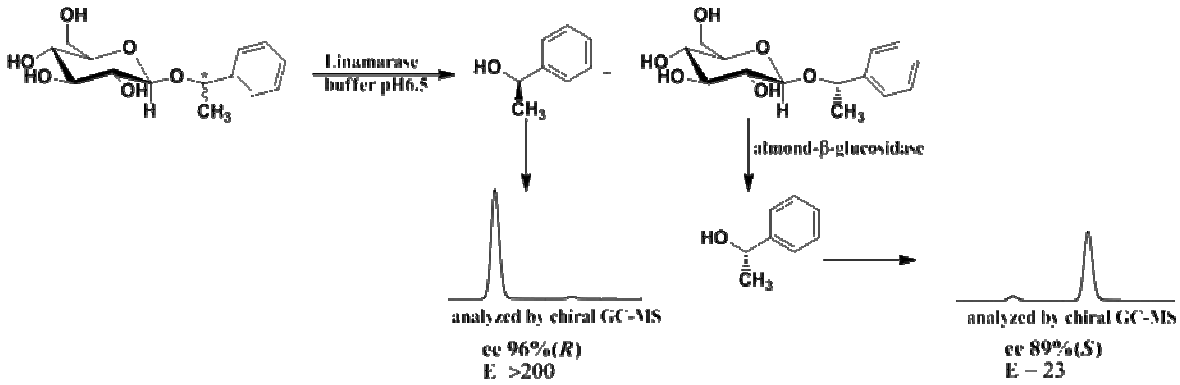
Cassava (*Manihot esculenta* Crantz) linamarase, a β-glucosidase, catalyzes the hydrolysis of the cyanogenic glucoside, linamarin, to yield acetone cyanohydrin. This enzyme plays an important role in the defense mechanism of the plant. Linamarase can be found in most tissues of the plant from the food storage root to leaves, with the highest amount found in the petioles. Besides the hydrolytic reaction, the enzyme can catalyze the transglucosylation reaction from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc), the glucosyl donor, to various alcohol substrates, as the glucosyl acceptor, to produce β-D-glucopyranosides with retention of configuration at the anomeric carbon. The unique property of the cassava enzyme is its reactivity towards secondary and tertiary alcohols. Therefore, we have investigated the stereoselectivity of β-glucosidases from cassava, almond and Thai rosewood to obtain enantiomerically pure (+)-(R)-1-phenylethanol by hydrolysis of (±)-1-phenylethyl-β-D-glucoside. Linamarase possesses the highest stereoselectivity with % enantiomeric excess (ee) up to 96%, E (relative rate) up to 200. The yield of (+)-1-phenylethanol was further optimized by varying the incubation temperature, time and enzyme amount. The (±)-1-phenylethyl-β-D-glucoside was prepared by Konique-Knorr synthesis as a model and the stereoselectivity was analyzed by chiral GC-MS with optically active 1-phenylethanol of known configuration as a standard.

Two enzymes, cassava linamarase and almond β-glucosidase, were employed in the kinetic resolution of (±)-1-phenylethanol. Cassava linamarase was able to resolve (±)-1-phenylethanol with high stereoselectivity. The (+)-(R)-1-phenylethanol hydrolysis product could be easily separated by extraction with ethyl acetate and then the unhydrolyzed glucoside was further hydrolyzed by almond-β-glucosidase. Using the two-enzyme system, the kinetic resolution of (±)-1-phenylethanol was successfully demonstrated to obtain enantiomerically pure (+)-(R)-1-phenylethanol and (-)-(S)-1-phenylethanol in 96% ee, and 89% ee, respectively.

Scheme 1. The two-enzyme system is used to resolve (±)-1-phenylethanol.



Poster Presentation 32

IDENTIFICATION OF PROTEINS EXPRESSED IN THAI HUMAN PLEURAL EFFUSIONS

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Pleural effusion, an accumulation of fluid in the pleural cavity due to trauma or disease, contains proteins originating from plasma filtrate through the capillary endothelium. This report presents data of the proteomic analysis of Thai human pleural effusion. In order to compare protein expression in each sample, the proteomic profiles of five pleural effusions were studied by two-dimensional gel electrophoresis. Protein spots with differential expression were identified by LC/MS/MS. The proteomic patterns revealed six unique spots whose protein expression have not been reported in plasma and may represent proteins specifically found in pleural fluid. These proteins are Ig kappa chain C region, inter alpha trypsin inhibitor heavy chain H4, S100 calcium binding protein A9, peroxiredoxin 2, ferritin light chain and serum amyloid A1. These proteins are potential biomarkers for lung cancer and will be further studied and verified by Western blot analysis.

Supported by the Chulabhorn Research Institute

Poster Presentation 33

PROTEOMIC ANALYSIS OF CASSAVA-LEAF PROTEINS DURING CASSAVA DEVELOPMENT, FROM PLANTING TO TUBERIZATION

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Tuberization in cassava (*Manihot esculenta* Crantz) occurs simultaneously with plant development, indicating the competition of photoassimilate partitioning between the shoot and the root organs. In potato, which is the most widely studied tuber crop, there is an ample evidence suggesting that metabolism and regulatory processes in leaf may have an impact on tuber formation. To search whether leaf proteins probably involved in regulating tuber production and/or development in cassava, comparative proteomic approach has been applied to monitor differentially expressed leaf proteins during the transition from fibrous to tuberous roots. Statistical analysis between the two groups with different plant ages using Student's T-test with 95% confidence level revealed that around 48-122 protein spots were significant changes with respect to their abundance levels ($p<0.05$) at least at one plant age. Of these, 39 differentially expressed protein spots were successfully identified by ion trap LC-MS/MS. The proteins span various functional categories from sugar metabolism (28%), defense (13%), cyanogenesis (8%), photosynthesis (8%), to miscellaneous (23%) and unknown (13%). This study provides a basis for further functional characterization of differentially expressed leaf proteins which can help understanding of the biochemical processes in cassava leaves that may be involved in storage root initiation and development processes.

Poster Presentation 34

ROLE OF DEG PROTEASE IN *CHLAMYDOMONAS REINHARTII* D1 PROTEIN TURN OVER

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Oxygenic photosynthetic organisms are unable to avoid the frequent-occurring photooxidative damage to the 32-kDa reaction center protein of photosystem II (PSII). Such damage, occurred under irradiance stress, leads to a phenomenon so called 'photoinhibition' which causes declines in plant productivity and eventually to death. Nature has evolved a repair mechanism to rectify such drastic effect of the excessive irradiance. A repair process operates to selectively replace the damaged D1 protein subunit within PSII with a newly synthesized copy. In Arabidopsis, Deg2 has been proposed to play an important role in the PSII repair though D1 protein degradation. However, in cyanobacteria, the mutant lacking all Deg proteases shows a slight sensitivity in high light. In this work, we aim to elucidate role of Deg protease in unicellular green alga, *Chlamydomonas reinhardtii*, by generating a mutant strain whose expression level of Deg proteases, especially Deg2, is down-regulated. Information from this study will provide a new evolutionary insight whether the functional role of Deg protease in D1 proteolysis is a new acquisition in higher plants or is unique to eukaryotic photosynthetic organisms.

Poster Presentation 35

SYNTHESIS OF ASYMMETRIC POLAR-HEAD CHOLESTEROL-BASED CATIONIC LIPIDS WITH POTENTIAL DNA DELIVERY

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Gene therapy represents an important advance in the alternative treatment of a variety of diseases of both genetic and acquired origin. In this process, the corrected exogeneous genes or portions of a gene are introduced into targeted cells to replace defective DNA sequences. In recent years much effort has been focused on the development of a variety of DNA carriers, and cationic liposomes have become the most common non-viral gene delivery system. Twenty four asymmetric divalent head group cholesterol-based cationic lipids were designed and synthesized by parallel solid phase synthesis technique. These asymmetric head groups composed of amino functionality together with trimethylamino, di(2-hydroxyethyl)amino or guanidinyl groups. Spacers between cationic heads and linker were both equal and unequal in length. These lipids were subjected to evaluate for DNA binding affinities by gel retardation assay and were screened for their transfection efficiency on HEK293 cells. Cationic lipids with equal chain length exhibited high transfection efficiency when polar part contained asymmetric polar heads. In contrast, lipids with unequal chain length exhibited high transfection efficiency when polar part contained symmetric heads. According to the optimal formulation, seven lipids exhibited higher transfection efficiency than commercially available transfection agents, Effectene^{TM}, DOTAP and DC-Chol, to deliver DNA encoding β-galactosidase as a reporter gene into PC3 human prostate adenocarcinoma cells. 3β-[*N*-(*N*′-guanidinyl)-2'-aminoethyl)-*N*-(2-aminoethyl)carbamoyl] cholesterol bearing amino and guanidinyl polar heads exhibited highest transfection efficiency with minimal toxicity. The morphology of active liposome was observed by transmission electron microscopy (TEM) and size of liposomes were around 200-700 nm.

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Poster Presentation 36

EVIDENCE OF COVALENT ADDUCT BETWEEN HUMAN SERUM ALBUMIN AND GAMBOGIC ACID BY FLUORESCENCE BIOORTHOGONAL CHEMISTRY

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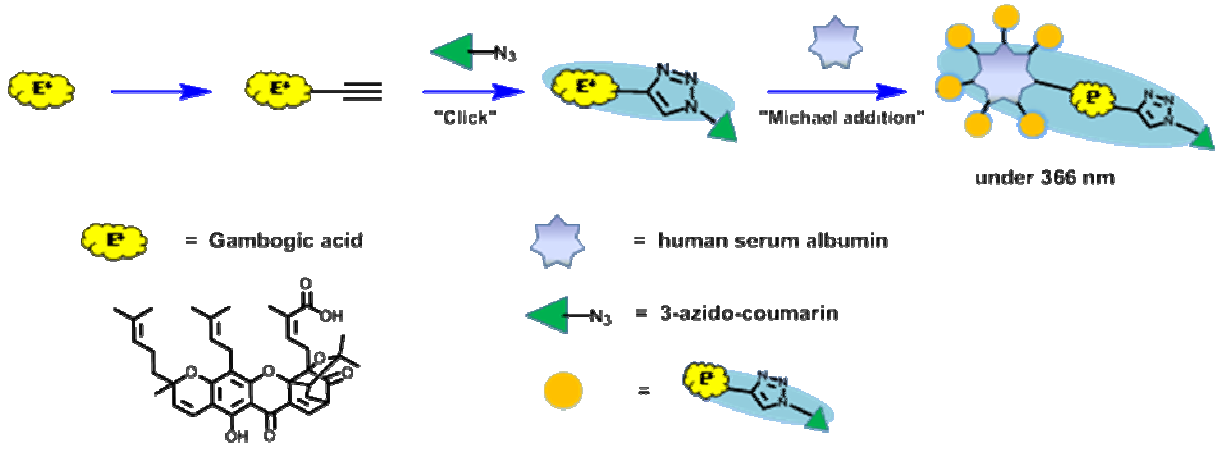
A bioorthogonal chemical reporter is a modern tool in chemical biology for tagging and visualizing of biomolecules. Gambogic acid (GA), the principal active component from the resin of *Garcinia hanburi*, is a polyprenylated xanthone with potent cytotoxicities against various cancer cell lines. The use of GA in this study is to modify to GA-alkyne. This alkyne was then reacted with a fluorogenic 3-azido-coumarin probe (Q-N_3) via Cu(I)-catalyzed azide-alkyne cycloaddition known as "click chemistry" to obtain a novel fluorescent derivative, gambogic-coumarin product (GAQ). GAQ was used as a reactive compound to monitor the interaction with human serum albumin (HSA). The 609-residue HSA of size 67.5 kDa has a free cysteine (cys-34) and lysine residues that can nucleophilically react with the α,β-unsaturated carbonyl moiety at C-10 of GAQ via the Michael addition. The HSA-fluorescent gambogic conjugate, a fluorescent biomolecule, was detected on gel electrophoresis and visualized with a general UV lamp. In addition, this reaction was confirmed by HSA titration with GA using spectrophotometric method. Upon reaction of GA with HSA, the maximum absorption wavelength was shifted from 360 nm to 330 nm, and the 330 nm absorbance increased with HSA addition. These results clearly demonstrated that cysteine or lysines in human serum albumin were reactive towards gambogic acid. The fluorescent property and easy-to-perform reaction of this bioorthogonal reaction provide an excellent tool that can be applied towards biomolecules tagging in living cells.



Poster Presentation 37

ROLE OF EARLY LIGHT INDUCIBLE PROTEIN (ELIP) IN *CHLAMYDOMONAS REINHARDTII*

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Elip is known as a protein whose expression is related to various environment stresses. It is a nuclear-encoded protein located in the chloroplast thylakoid membrane. It had been proposed that one of its functions is for photoprotection of plants under irradiance-stress condition as a chlorophyll binder or participating in energy dissipation. Yet, the exact role of Elip is still unclear. In this study, we have cloned and expressed *elip* cDNA from *Chlamydomonas reinhardtii* in *Escherichia coli* for production of Elip polyclonal antibodies for protein analysis. To elucidate the function of Elip in *C. reinhardtii*, we first cloned *elip* cDNA in pSL19 vector for overexpression in *C. reinhardtii*. We also constructed *elip* RNAi vector in pSL19 using inverted repeat RNAi method, aiming for post-transcriptional suppression of its expression. Currently, we have successfully transformed these vectors into *C. reinhardtii* strains cc400 (WT), cc503 (WT), cc3682 (*npq1* mutant) and and cc3683 (*npq2* mutant), and obtained the polyclonal antibodies. Verification of the transformants and effects of overexpression and suppression of *elip* are being investigated.

Poster Presentation 38

CLONING, EXPRESSION, AND CHARACTERIZATION OF TWO NON-HOMOLOGOUS N-ACETYL-GLUCOSAMINIDASES FROM VIBRIO HARVEYI

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N-acetylglucosaminidases (EC 3.2.1.52) are family-20 glycosyl hydrolases that hydrolyze terminal, non-reducing GlcNAc residues in chitobiose and higher chitooligosaccharides. We recently cloned the genes encoding for two N-acetyl glucosaminidases, namely *VhNAGase1* and *VhNAGase2*, from *Vibrio harveyi*. The *VhNAGase1* gene contains 2,335 bp, which encodes 779 amino acids with a putative MW of 88,979 Da, while the *VhNAGase2* gene contains 1,918 bp, and encodes 640 amino acids with a predicted MW of 73,114 Da. Although both enzymes are predicted to be non-secretory enzymes, the identity of their amino acid sequences was found to be very low (20%). VhNAGase1 was most active at pH 7.5, while VhNAGases2 was most active at pH 7.0. VhNAGase2 displayed higher hydrolyzing activity than VhNAGase1 by two fold. Moreover, VhNAGase2 showed broad substrate specificity towards various chitin substrates, whereas VhNAGase1 did not hydrolyze any substrate other than pNP-GlcNAc. In consistentent with the kinetic data, time course analysis by quantitative HPLC and thin layer chromatography revealed that VhNAGase2 hydrolyzed chitotetraose and chitotriose much more efficiently than chitobioase, chitiohexaose and colloidal chitin, indicating that the substrate binding cleft of the enzyme may contain four binding subsites, including subsites -1, +1, +2, and +3.

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Poster Presentation 39

EXPLORING *PLASMODIUM FALCIPARUM* SHIKIMATE PATHWAY FOR NEW DRUG TARGETS

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The shikimate pathway is the major route of de novo synthesis of aromatic compounds which are precursors for several compounds in other pathways, such as folate pathway, ubiquinone, aromatic amino acids, etc. Shikimate kinase (SK, EC 2.7.1.71), the fifth enzyme in the shikimate pathway, catalyzes the phosphate transfer of ATP to shikimate to generate shikimate 3-phosphate and ADP. The enzyme, found in bacteria, yeast, protozoa, plant, except for mammal, also catalyzes the committed step for the biosynthesis of chorismate. In *Plasmodium falciparum*, SK has been reported to be a potential drug target. The enzyme exists as a bifunctional protein with 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) with SK located at the C-terminus of the protein. We report here a cloning of the 1,620 bp gene encoding *Pf*SK from strain K1 into an expression vector containing 10X His-tag at the N-terminus of the protein. Unfortunately, all attempts to express the recombinant enzyme in *E. coli* host using various expression conditions failed to express the protein at the expected molecular size. Experiments are still undergoing to examine whether the EPSPS domain is critical for the SK expression. In such case, the expression may need to be carried out in yeast or baculovirus as the insert size might be too big for the *E. coli* system.

Poster Presentation 40

MOLECULAR MECHANISM OF ETOPOSIDE RESISTANCE IN A549 LUNG CANCER CELLS WITH ACQUIRED DRUG RESISTANCE

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Drug resistance is a major obstacle to effective cancer chemotherapy. To understand the mechanism of drug resistance, an etoposide-resistant lung cancer cell line A549RT-eto was established by continuous exposure of the A549 cell line to increasing concentrations of etoposide, leading to approximately 28-fold greater resistance to etoposide compared to the parental A549 cell line. Moreover, A549RT-eto resistant cells showed cross-resistance to doxorubicin but not to taxol and cisplatin. The expression levels of drug resistance-related genes were determined using real-time PCR and showed that only *mdr1* transcript levels were drastically increased in A549RT-eto cells compared to the parental cells. Since the *mdr1* gene encodes for P-glycoprotein (P-gp), a membrane protein involved in drug efflux pumps, the expression and transport activity of P-gp were studied. Western blot analysis showed increased P-gp expression in the resistant cells compared to A549 cells. The levels of calcein transported in the drug resistant cells determined by flow cytometry were also enhanced, correlating with the increased P-gp expression found in the Western blot. These studies strongly suggest that expression of *mdr1* induced by etoposide plays a major role in the development of drug resistance in the A549RT-eto cell line.

Poster Presentation 41

EFFECTS OF ACTIVE SITE RESIDUES ASPARTATE 313 AND TYROSINE 435 IN *VIBRIO HARVEYI* CHITINASE A ON CHITIN HYDROLYSIS

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Chitinase A (EC.3.2.1.14) is the major enzyme from *Vibro harveyi* that catalyzes the hydrolysis of chitin from a marine biosphere. We previously demonstrated, by the x-ray structures of chitinase A in complex with chitooligosaccharides, that Asp313 may involve in the stabilization of the oxazolanium intermediate, while Tyr435 may aid the terminal of the sugar chain movement within the substrate binding cleft. Point mutations of the residues Asp313 and Tyr435 were introduced by site-directed mutagenesis to generate four mutants including D313A, D313N, Y435A and Y435W. The hydrolyzing activites against colloidal chitin, chitosan and crystalline chitin were completely abolished in the mutated of Asp313. On the other hand, the specific hydrolysis activity of the mutant Y435A on these substrates were higher than the wild-type's activity by 1.4-fold, 1.1-fold and 1.1-fold, respectively, whereas the mutant Y435W gave less hydrolytic activities compared with wild-type's activity (0.9-fold, 0.8-fold and 0.7-fold, respectively). Both D313A and D313N revealed a complete lack of the hydrolyzing activity towards colloidal chitin. On the other hand, the mutant Y435A displayed significantly higher activity than the wild-type and mutant Y435W displayed a slight decrease in the activity. The binding activities towards crystalline chitin, colloidal chitin and chitosan were compared. All the enzymes exhibited highest binding activity towards colloidal chitin, followed by chitosan and then crystalline chitin. Mutants D313A and D313N showed lower binding activity than the wild-type, whereasY435A and Y435W displayed higher activity. The results suggested that the cleavage of chitin chain by chitinase A may be influenced by the steric effect of Tyr435. In addition, Asp313 seems to be crucial for the catalytic process of the enzyme.

Supported by the Suranaree University of Technology and the Thailand Research Fund

Poster Presentation 42

PURIFICATION AND CHARACTERIZATION OF LACCASE FROM *LENTINUS POLYCHROUS* LÉV

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Laccases (EC 1.10.3.2) are enzymes that catalyze the oxidation of phenolic compounds and aromatic amines using molecular oxygen as electron acceptor. They have potential uses in many areas such as pulp and textile dye bleaching, as well as effluent detoxification. In this study, laccase from *Lentinus polychrous* Lév. was partially purified using 40–85% saturated ammonium sulfate fractionation and column chromatography techniques including Concanavalin A, DEAE-cellulose and superdex 200 HR. Enzyme properties, substrate specificity, and effects of salts and inhibitors were characterized. The partially purified laccase sample contained three laccase isozymes with molecular masses of 65.6, 52.6 and 44.8 kDa. The optimum pH and optimum temperature were pH 4.5 and 50°C respectively, when 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was used as a substrate. Laccase activity was completely inhibited by 0.1 mM dithiothreitol, thioglycolic acid, sodium azide, cysteine or 10 mM *p*-cumaric acid and was inhibited more than 50% in the presence of 10.0 mM $MnCl_2$ or $MgCl_2$. However, with ABTS as a substrate, the laccase activity was slightly stimulated by $CuSO_4$ or $CdSO_4$. The enzyme had a greater affinity for ABTS (K_m of 19.19 μM) than 2,6-dimethoxy-phenol (DMP) (K_m of 185 μM) and guaiacol (K_m of 472 μM).

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Poster Presentation 43

BACTERIAL MEMBRANE DISRUPTION BY *BAUHINIA PURPUREA* EXTRACT

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In this study, we reported the antibacterial activity of *Bauhinia purpurea* seed extract. The crude extract showed strong antibacterial activity against six Gram-positive and six Gram-negative bacteria. Moreover, the bacterial inhibition activity has remained even after heat treatment at 121°C for 30 min. Sodium chloride up to 1.0 M had no effect on *Pseudomonas aeruginosa* and *Staphylococcus aureus* growth-inhibiting activity, while calcium chloride at 10 mM or higher concentration abolished this activity. Scanning electron microscopy showed disruption of the bacterial membranes after treatment with the crude extract.

Supported by The Thailand Research Fund and The Commission on Higher Education and Center of Excellence for Innovation in Chemistry, Faculty of Science, Mahasarakham University

Poster Presentation 44

TWO DIMENSIONAL VIRUS OVERLAY PROTEIN BINDING ASSAY FOR IDENTIFICATION OF JAPANESE ENCEPHALITIS VIRUS BINDING PROTEIN ON MICROGLIAL CELLS

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Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is a major cause of viral encephalitis in Asia. Even though the principle target cells for JEV in the central nervous system are neurons, the microglia is activated in response to JEV infection. Generally, for viral infection, viral attachment to the host cell is the first step of the viral entry. This study aimed to identify the JEV binding protein(s) on the surface of mouse microglial cells (BV-2). Using virus overlay protein binding assay (VOPBA) followed by liquid chromatography-mass spectrometry (LC/MS/MS), we identified JEV binding protein bands of 43 kDa laminin receptor precursor protein and 60 kDa chaperonin. Two-dimensional gel electrophoresis, to separate the membrane proteins before VOPBA, further confirmed the 43 kDa laminin receptor precursor protein as a JEV binding protein. This newly identified JEV binding protein is a potential candidate for JEV receptor protein on microglial cells.

Supported by The National Research Council of Thailand and Laboratory of Biochemistry, Chulabhorn Research Institute, Thailand

Poster Presentation 45

CHARACTERIZATION OF THE TWO COMPONENT NS2B(H)-NS3 PROTEASE OF JAPANESE ENCEPHALITIS VIRUS.

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Japanese encephalitis virus (JEV), a member of the Flaviviridae family, is a mosquito-borne neurotropic flavivirus which causes severe diseases of the central nerve system. Currently, several vaccines are employed clinically. However, their use is restricted due to high costs and occasional adverse effects. The development of small molecule drugs against viral target enzymes such as the NS2B-NS3 protease is therefore urgently needed. In this study, we have initiated a work on structure-activity relationships of the JEV NS2B-NS3 two-component protease. The sequence of NS2B-NS3 from JEV was obtained by generation of a synthetic gene. The protease complex NS2B(H)-NS3pro was obtained by SOE-PCR. Constructs were cloned and overexpressed in *E. coli*, purified by metal affinity chromatography and protein samples were subjected to analysis by SDS-PAGE, Western blotting and detected by fluorogenic substrates GRR-amc and RTKR-amc. The NS2B-NS3 protease from JEV was cloned, expressed and purified. Initial experiments revealed predominant expression in high amounts as soluble protein at 18°C. Western blot analysis identified 3 major immunoreactive products at molecular weights of approx. 36, 21 and 10 kDa. Purification by metal ion affinity chromatography yielded major product bands of 36 kDa and 21 KDa. Preliminary activity assays with the GRR-amc and RTKR-amc have, for the first time, revealed kinetic data such as K_m and k_{cat} for this enzyme.

Poster Presentation 46

DIFFERENTIAL EXPRESSION OF FASCIN IN CHOLANGIOCARCINOMA AND HEPATOCELLULAR CARCINOMA CELL LINES

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Cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) occur with relatively high incidence in Thailand. Proteomic and sub-proteomic analyses of Thai cholangiocarcinoma (HuCCA-1) and Thai hepatocellular carcinoma (HCC-S102) cell lines have identified significant involvement of cytoskeletal proteins. Of these proteins, fascin, an actin-bundling protein which induces membrane protrusions and increases cell motility in various transformed cells, showed higher expression in HuCCA-1 as compared with HCC-S102. In this study, subcellular fractionation of HuCCA-1 and HCC-S102 cell lines were performed and compared with two other hepatocellular carcinoma cell lines (HepG2 and Alexander) using 1-D and 2-D immunodetection. Fascin was detected in both membrane and cytosolic fractions of HuCCA-1, HCC-S102, HepG2 and Alexander cell lines, according to 1-D Western blots. Consistently, the expression of fascin was higher in the cytosolic fraction than in the membrane fraction. The highest expression of fascin was detected in HuCCA-1, followed by HepG2 and HCC-S102 cell lines. Fascin in Alexander cell line was almost undetectable. Moreover, two-dimensional immunoblots revealed the presence of at least three spots of fascin with the same molecular weight but differing in pH. Our results confirmed the presence of fascin with post-translational modifications in HuCCA-1 and HCC-S102 cell lines. The differential expression of fascin in these cell lines may suggest different characteristic or behavior in each cell line, which require further investigations.

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Poster Presentation 47

CRYSTALLIZATION AND PRELIMINARY X-RAY STRUCTURE DETERMINATION OF TRYPANOSOMAL CHIMERIC DIHYDROFOLATE REDUCTASE-THYMIDYLATE SYNTHASE

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Dihydrofolate reductase (DHFR) has been validated as a drug target for the treatment of African sleeping sickness caused by *Trypanosoma brucei*. The enzyme is expressed as a bifunctional protein with thymidylate synthase (TS) located at the C-terminus. Due to expression and crystallization problems of TbDHFR-TS, chimeric protein of *T. brucei* DHFR and *T. cruzi* TS (TbDHFR-TcTS) was created without the junction region. The protein was expressed in *Escherichia coli* BL21 (DE3) and purified with MTX-sepharose affinity chromatography. Most of the purified sample was in its 59-kDa full-length form, with a very faint band of the truncated protein, which was removed by Sephacryl S200 chromatography. The protein was crystallized in ammonium sulfate solutions using the microbatch technique. X-ray diffraction data were collected to 3.1 Å resolution using an in-house X-ray generator. TbDHFR-TcTS structure was determined with the molecular replacement using partially refine structure of TbDHFR and TcTS as a search template. The trigonal $P3_2$ crystal contained two bifunctional homodimers per asymmetric unit. The 24 N-terminal residues of DHFR made similar contacts with the TS domain as seen in other structures of Kinetoplastids DHFR-TS, although detailed description of the interactions was limited by the low resolution data. Without ligand, the DHFR active site differs from the structure of substrate-bound TbDHFR ternary complex. The data provides important information for drug development against TbDHFR.

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Poster Presentation 48

A NOVEL HOMOZYGOUS MUTATION IN THE PROMOTER REGION OF THE *RMRP* GENE ASSOCIATED WITH SEVERE IMMUNE DEFICIENCY AND MILD SKELETAL MANIFESTATIONS IN CARTILAGE-HAIR HYPOPLASIA

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Cartilage-hair hypoplasia (CHH) is a rare autosomal-recessive pleiotropic disorder caused by mutations in the *RMRP* gene. Several mutations have been identified and are located in the promoter region or transcribed sequence. We report on a Thai girl who first presented at 8 months of age with chronic diarrhea caused by recurrent infections and cytomegaloviral enterocolitis, recurrent infections, and severe failure to thrive without apparently disproportionate dwarfism. The immunologic profiles were consistent with combined immune deficiency with decreased CD4, progressively declined CD8, decreased CD19, and neutropenia. The diagnosis of CHH was made after the severe wasting was corrected, and disproportionate growth became noticeable. The patient had other characteristic features of CHH including sparse hair and metaphyseal chondrodysplasia. The patient died from enteroviral meningoencephalitis at 23 months of age. Mutation analysis identified a novel homozygous mutation, g.-19_-25 dupACTACTC, in the promoter region of the *RMRP* gene. The patient is the first CHH case with a homozygous *RMRP* promoter mutation. This disproves the previous hypothesis that a homozygote of the *RMRP* promoter mutations is not compatible with life. Severe immune deficiency and mild chondrodysplasia in this patient imply that mutations in the *RMRP* promoter region affect proliferative bone marrow functions more than cartilage growth. Identification of the mutation enabled us to provide a prenatal diagnosis in the subsequent pregnancy. Because of marked phenotypic variability, CHH should be considered as a differential diagnosis in patients with primary T-cell or combined immune defects, even without apparently disproportionate short stature.

Poster Presentation 49

THE EFFECT OF pH ON THE MECHANISM OF FLAVOPROTEIN PYRAONSE 2-OXIDASE

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Pyranose 2-oxidase (P2O) from basidiomycete *Trametes multicolor* mainly catalyzes oxidation of D-glucose at the C2 position, yielding 2-keto-D-glucose and hydrogen peroxide as products. This enzyme is a flavoprotein containing a covalently bound FAD (flavin adenine dinucleotide) through histidyl linkage of histidine residue to the C8 methyl position of FAD. Stopped-flow experiment in both reductive-half-reaction and oxidative half reaction was done in pH 7 at 4C°. In the reductive half-reaction showed that the oxidized enzyme rapidly bound to D-glucose forming an enzyme substrate complex ($E\text{-}Fl_{ox}:G^*$) with the K_d 45 mM following the isomerization to another fully active form of the complex ($E\text{-}Fl_{ox}:G$) with the rate of 160 s^{-1} and the reverse rate of 10 s^{-1}. The isotopic substrate using 2-*d*-D-glucose showed an inverse isotope effect of 0.6 on the k_{obs} of this phase implying that the C2-H bond of D-glucose is more rigid in the $E\text{-}Fl_{ox}:G$ complex compared to in the free form. A large normal primary isotope effect (k_H/k_D= 8.84) was detected in the flavin reduction step. The reduction step is independent on pH in the range of pH 5.5 to 10. In the oxidative half-reaction, the reduced enzyme reacted with oxygen to form C4a-hydroperoxy flavin intermediate with a forward rate of 5.8 x 10^4 $M^{-1}s^{-1}$ and a reverse rate of 2 s^{-1} and decayed with the rate of 18 s^{-1}. This is the first time that C4a-hydroperoxy flavin intermediate can be detected in oxidase class of flavoprotein. The decay rate of C4a-hydroperoxy flavin is decrease when pH is increase and the pK_a was calculated to be 7.6. The pKa that obtain is may be the Histidine 548 which is in the active site of enzyme. The deprotonated form of histidine may from hydrogen bond to the N5 proton of the flavin and stabilize theC4a-hydroperoxy flavin. Steady-state kinetics at pH 7.0 shows a pattern of parallel lines which is implied a ping-pong mechanism. Kinetics of formation and decay of C-4a-hydroperoxy-FAD is the same in absence and presence of 2-keto-D-glucose, implying that the sugar does not bind to P2O during the oxidative half-reaction. This suggests that the kinetic mechanism of P2O is likely to be the Ping Pong type where the sugar product leaves prior to the oxygen reaction. The movement of the active site loop when oxygen is present is proposed to facilitate the release of the sugar product. Correlation between data from pre-steady state and steady-state kinetics has shown that the overall turnover of the reaction is limited by the steps of flavin reduction and decay of C4a-hydroperoxy-FAD.

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Poster Presentation 50

DEFICITS IN THE ACTIVATION OF THE STRESS INDUCED UPR PATHWAY IN ERYTHROBLASTS FROM β-THALASSAEMIA/HbE PATIENTS

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Cellular stress has been observed to play a role in pathology of many diseases including neurodegenerative diseases, haemophilia and diabetes. Cells respond to stress by activating several pathways such the unfolded protein response (UPR), autophagy and apoptosis. While several studies have reported that thalassemic erythroblasts eventually undergo apoptosis, no other stress responses have been studied in these cells. This project aims to investigate UPR induction in thalassemic compared to normal erythroblasts by isolating $CD34^+$ haemopoietic progenitor cells from mononuclear cells of peripheral blood using MACS^{TM} isolation kit system and subsequently grown in normal medium before incubated in growth factors-deprived medium in day 10 of culture. The results show that both UPR–responsive mRNA and proteins analyzed by real time RT-PCR and western blot decreased (fold reduction) significantly ($p<0.05$) during time-course starvation in thalassemic erythroblasts as compared to the normally induced (fold induction) in normal. Moreover, dissociation of PERK and ATF6 from GRP78 examined by confocal microscopy clearly show that only in normal but not in thalassemic erythroblasts responsded to starvation. These results reveal the inability of thalassemic erythroblasts to modulate stress upon starvation *in vtro* which may underline the significantly accelerated apoptosis in thalassemic erythroblasts *in vivo* during erythropoiesis.

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Poster Presentation 51

PROTEOMICS ANALYSIS OF ERYTHROID PRECURSOR CELLS IN β-THALASSEMIA/HbE PATIENTS

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β-thalassemia/HbE disease, one of the commonest forms of β-thalassemia in Thailand, is characterized by the co-inheritance of HbE with β globin gene mutation. Previous studies have shown a number of genetic modifier genes or SNPs associated with the disease severity. However, the relatively low correlation between the abundance of a genomic or mRNA and that of the encoded protein makes it important to characterize the protein profile directly of thalassemic cells in addition to the transcriptome. The protein profiles were compared among 4 mild β-thalassemia/ HbE patients and 4 normal healthy volunteers. The $CD34^+$ stem cells were selected from peripheral blood mononuclear cells by positive immunomagnetic selection. Erythroid lineage specific differentiation was induced in the $CD34^+$ progenitor cells by erythropoietin (EPO), interleukin 3 (IL3), and stem cell factor (SCF). Cytosolic proteins were extracted from D7 cultured precursor cells and subjected to two-dimensional gel electrophoresis. Three hundred and eighty one protein spots were detected in both groups of samples and 13 spots were strikingly different. The identification and characterization of the protein profiling will be of great value for the understanding of the pathogenesis of β-thalassemia/HbE that will lead to the better design of novel protein-targeted therapy for the patients.

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Poster Presentation 52

PRODUCTION OF TELOMERASE BY TRANSIENT EXPRESSION OF GENE-CONTAINING PLASMIDS FOR TELOMERASE INHIBITION ASSAY

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Telomerase is a new target for anticancer therapy since it is expressed in 85-90% of cancer cells but not in normal somatic cells. To find telomerase inhibitors, a good screening assay is essential. The conventional telomerase assay uses whole cell extract from cancer cells as the source of telomerase. However, the content of telomerase in cancer cells is rather small; therefore, it is sensible to increase the production of telomerase by transient expression. Human telomerase is a ribonucleoprotein whose catalytic function depends minimally on two components: the hTERT (human telomerase reverse transcriptase) protein and telomerase RNA (hTR). We produced telomerase using the transient expression of hTERT-containing plasmid and hTR-containing plasmid in HEK293T cells. Each plasmid (hTERT and hTR) was first amplified by transforming into *E. coli* stain DH5α. The purified hTERT-containing plasmid and the hTR-containing plasmid (ratio 1:5) were co-transfected into HEK293T cells by lipofectamine 2000. The protein level of hTERT was then analyzed by Western Blot analysis, and the activity of telomerase was analyzed by fluorescent telomeric repeat amplification protocol assay (TRAP assay). We found that the activity of telomerase increased no less than 32 fold in the extract from the transfected cells. Finally, we used the extract from these transfected cells to demonstrate the telomerase inhibitory effect of some G-quaduplex ligand-based telomerase inhibitors.

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PROCEEDINGS

Proceedings 1

STRUCTURE DETERMINATION OF *PLASMODIUM FALCIPARUM* PLASMEPSIN-II MUTANT CONTAINING PLASMEPSIN-I ACTIVE-SITE RESIDUES

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ABSTRACT

Plasmepsins are aspartic protease enzymes found in *Plasmodium* parasites, which cause malaria disease. *Plasmodium falciparum* plasmepsins I and II (PM-I and PM-II) are involved in hemoglobin degradation in the parasite's food vacuole. Although PM-I and PM-II are 73% identical, they strikingly differ in terms of substrate specificity and molecular properties. PM-II is highly soluble but PM-I aggregates badly upon concentration. This prevents successful crystallization and X-ray structure determination of PM-I. Recently, an attempt has been made to generate a crystallizable plasmepsin mutant with PM-I active-site structure incorporated into the PM-II structural core for further rational design studies. Based on protein homology, nine different amino acids located in the active site of PM-II have been mutated to the corresponding amino acids of PM-I. Interestingly, the resulting PM-II M9 mutant still retained most of the PM-II native specificity despite having the PM-I active-site residues.

In this study, we aimed to determine the three-dimensional structure of this PM-II M9 mutant in order to understand the structural effect of those mutations and to provide the structural clues on PM-I active site specificity. The M9 mutant with and without pepstatin A inhibitor were successfully crystallized. Crystals of unliganded and pepstatin-complexed M9 diffracted X-rays to 2.70 and 3.10 Å resolutions, respectively. The crystal of ligand-free M9 belongs to the orthorhombic space group $P2_12_12$ with 49.8% solvent content and the crystal of pepstatin-M9 complex belongs to the monoclinic space group $P2_1$ with 49.2% solvent content. Currently, the unliganded M9 structure is refined to an R-factor of 26% (R-free 28%). Superposition of PM-II M9 and PM-II WT (PDB ID: 3F9Q) structures shows different conformations of the flap region. The structure is being refined further and details of the differences are being investigated.

Keywords: Plasmepsins, *Plasmodium falciparum*, crystallization, structure determination

INTRODUCTION

Malaria is a serious disease in tropical countries and is caused by protozoan parasite in the genus *Plasmodium*. Each year, malaria afflicts an estimate of 500 million people and kills nearly 2 millions, mostly children [1]. Infection is passed to humans through a female anopheles mosquito and is due to four species of the parasite, namely *P. falciparum, P. vivax, P. ovale, and P. malariae* [2]. These species can deliver the disease in various forms and *P. falciparum* is the most widespread and perilous of the four species of *Plasmodium.* During its development in host

erythrocytes, the parasite engulfs and degrades up to 80% of host hemoglobin [3, 4]. Scientists have studied the parasite functions to understand crucial biological pathways of the parasite for development of anti-malarial drugs through rational drug design. Despite the scientists' endeavor, neither vaccine nor new effective drugs are available to fight against the drug-resistant parasites.

Plasmepsin I (PM-I) is the initial enzyme that cleaves the native hemoglobin at Phe-33–Leu-34 linkage of the α-globin chain, prior to its further degradation by a set of proteases [5]. Consequently, it could become a candidate target for anti-malarial chemotherapy [6, 7] through the design of specific inhibitors based on its three-dimensional structure. However, the 3-D structure of PM-I is not available. The recombinant PM-I protein is expressed in *Escherichia coli* as inclusion bodies which proves to be more difficult to refold comparing to its homologue plasmepsin II (PM-II), in spite of the high identity (~73%) between their amino-acid sequences. Furthermore, the low solubility of PM-I leads to poor PM-I production which hinders its crystallization study, while the higher solubility of PM-II has led to deposition of several of its 3-D structures in the Protein Data Bank. Since the structure and chemical interactions around the active site of PM-I is the crucial information for the rational design of its specific inhibitors, we have aimed to obtain this active-site structure in the crystallizable protein core of PM-II.

MATERIALS AND METHODS

The PM-II M9 protein was designed, expressed and purified as described [8]. Crystallization experiments were set up at 22^{o}C using the micro-batch crystallization method. X-ray diffraction data from mature PM-II M9 crystals were collected on an in-house X-ray source, Rigaku/MSC RU-H3R rotating-anode X-ray generator (Cu *Kα*; λ = 1.5418 Å) operating at 50 kV and 100 mA located at the Center for Excellence in Protein Structure and Function (CPSF), Faculty of Science, Mahidol University, Thailand. Complete X-ray diffraction data were recorded on an R-AXIS IV^{++} image plate system (Rigaku/MSC). X-ray diffraction data from a crystal of mature PM-II M9–pepstatin complex were collected on mar-*dtb* CCD detector (MarResearch) using Bruker rotating-anode X-ray generator (Cu *Kα;* λ =1.5418 Å) operating at 45 kV and 60 mA located at the Synchrotron Light Research Institute (SLRI), Nakhon Ratchasima, Thailand. All data were processed and scaled with the *CrystalClear* program version 1.3 [9]. The initial phases were determined by the molecular replacement method in the *CCP4* version 6.2 program suites [10]. The structure of wild-typed PM-II in complex with pepstatin A (PDB ID: 1SME) was used as a search template (98% identity by NCBI BLAST). The program *O* [11] was used for iterative model rebuilding. Programs from the Uppsala software factory and *CNS* version 1.2.1 were used for map manipulation and model refinement [12].

RESULTS AND DISCUSSIONS

The protein PM-II M9 was successfully purified by strong anion-exchange chromatography on Q-sepharose in denatured and native conditions with the final recovery yield of 22%. The enzyme sample was of >90% purity based on the Coomassie-Brilliant Blue-stained SDS-PAGE analysis (Fig. 1). Crystals of mature PM-II M9 were obtained from 1 µl of activated protein solution (~15 mg/ml) mixed with 1 µl of precipitant solution (100 mM bis-Tris, pH 6.5, 18–20% PEG 6000 and 80–120 mM $CaCl_2$). Small rod-shaped crystals appeared in one day then proceeded to their maximum sizes of approximately 30 x 40 x 200 µm in 2 weeks (Fig. 2a). PM-II

M9 was also co-crystallized with pepstatin A, a general tight binding inhibitor of aspartic proteinases, in similar conditions as the free form. Thinner, but longer rod-shaped crystals appeared in one day, then proceeded to their maximum sizes of approximately 30 x 40 x 680 µm in 3 weeks (Fig. 2b).

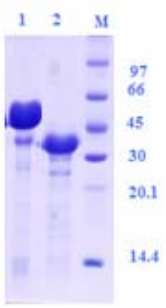

Fig. 1 Protein purity on SDS-PAGE.
Lane 1: pro-form PM-II M9
Lane 2: mature-form PM-II M9
Lane M: protein markers

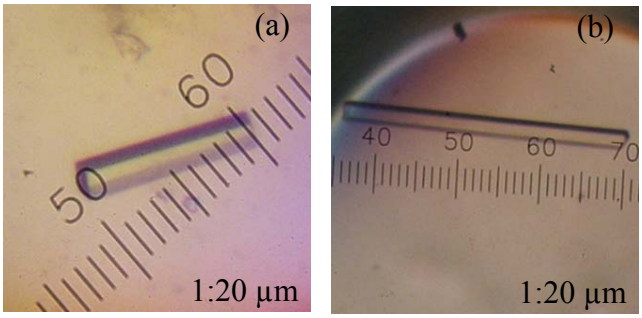

Fig. 2 Crystals of mature PM-II M9 in both (a) free form and (b) co-crystallized complex with Pepstatin A.

Cryoprotectant solution was not required and the crystals were quickly dipped in liquid nitrogen as fast as possible to prevent ice-ring formation for X-ray data collection. In this regard, the best PM-II M9 crystals could diffract X-rays to 2.70 Å resolutions, and belonged to the primitive orthorhombic space group *P*21212. Currently, the best crystals from pepstatin A co-crystallization could diffract X-rays to 3.10 Å resolution, and belong to primitive monoclinic space group *P*21. Crystallographic parameters and statistics of data collection from different crystals are summarized in Table 1.

Table 1 Summary of X-ray data collection statistics of mature PM-II M9 crystal and mature PM-II M9 co-crystallized with Pepstatin A. Values in parentheses are data obtained in the highest resolution shell.

Compounds	**Mature PM-II M9**	**Mature PM-II M9 co-crystallized with Pepstatin A**
Space group	Orthorhombic $P2_12_12$	Monoclinic $P2_1$
Unit cell lengths (Å)	a = 81.8, b = 111.3, c = 80.3	a = 91.4 b = 80.5 c = 107.8
Unit cell angles (degree)	$\alpha = \beta = \gamma = 90$	$\alpha = 90$, $\beta = 115$, $\gamma = 90$
Cell volume (Å^3)	731,078	793,160
Resolution limits	40.9–2.70 (2.79–2.70)	44.3–3.10 (3.21–3.10)
Number of reflections	Observed = 150,093 Unique = 39,111	Observed = 105,775 Unique = 15,962
Completeness (%)	99.1 (99.1)	99.6 (99.1)
I/σ (I)	5.3 (1.7)	6.2 (2.4)
R_{merge} (%)	11.3 (40.4)	13.2 (40.9)
Solvent content (%)	49.8	49.2
No. molecules/asym. unit	2	4

The initial phases of both unliganded and pepstatin-complexed forms were determined with the molecular replacement technique with the native structure as a search template. The obtained molecular replacement solutions resulted in 43.7% correlation and 47.6% R-factor for the free PM-II M9 crystal form and 41.3% correlation and 52.7% R-factor for the pepstatin-complexed form. A structural model

of the unliganded form is being rebuilt and refined in iteration with current R-factor of 26% and R-free of 28%.

The overall structures of both PM-II WT and PM-II M9 are similar, as expected since there are only nine different amino-acid residues (Fig. 3a). Specifically focusing at each mutation point around the active site, all the mutated residues are in the conformations consistent with their clear σA-weighted *2Fo–Fc* electron density. Superposition of PM-II WT and PM-II M9 structures produces the overall RMSD values of 0.98 and 1.31 Å for monomers A and B of PM-II M9, respectively. The high residue-based RMSD values came from residues 76–81 located at the "flap region" of the active site, and residues around the active-site entrance (Table 2), making the active site of PM-II M9 slightly wider than that of PM-II WT. Interestingly, the "flap regions" of both monomers of PM-II M9, which are in different conformations, appear more opened than that of PM-II WT (Fig. 3). These differences may be contributed by either intrinsic conformational flexibility of the mutated amino acids, or the differences in intermolecular packing of the different crystal forms. The basis of these differences will be investigated in more details with a better refined structure.

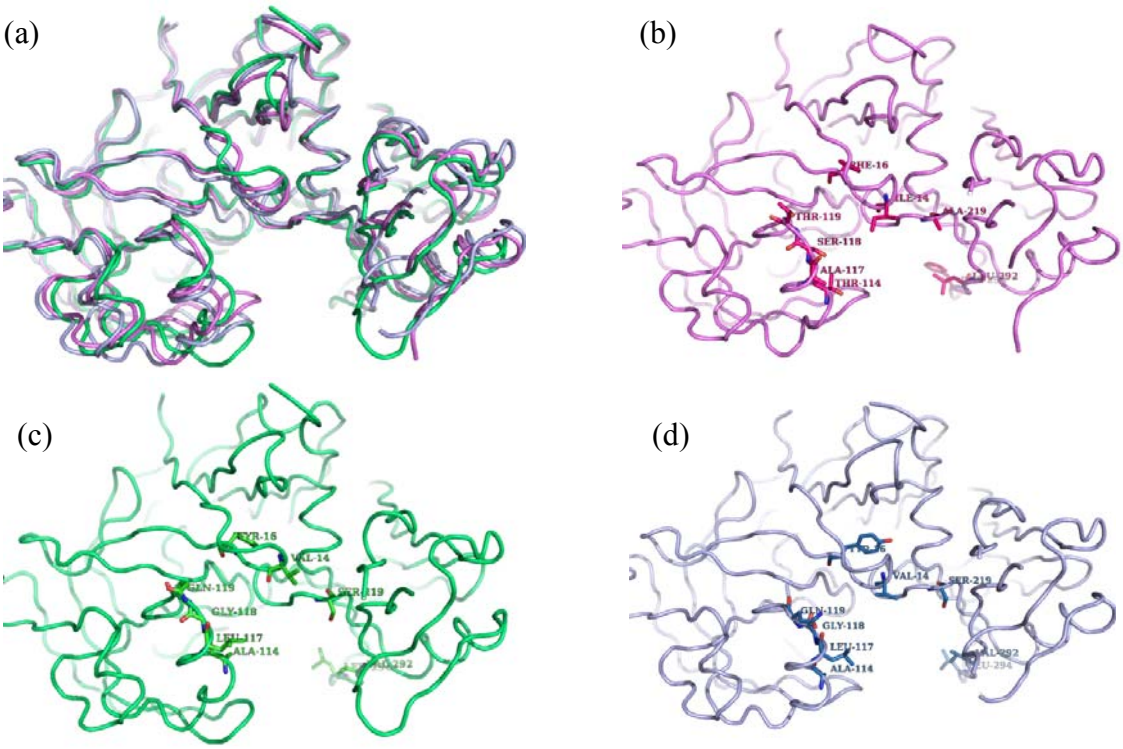

Fig. 3 Comparison of the crystal structures of PM-II WT and PM-II M9. (a) Superposition of the three crystal structures, which are shown individually in panels b–d. PM-II WT (PDB ID: 3F9Q) is drawn in magenta (b), while mol A (c) and mol B (d) of PM-II M9 are drawn in green and light blue, respectively. The nine different amino acids around the active site are labeled.

Table 2 List of twenty amino-acid pairs with the highest root mean square deviation (RMSD) between C_α atoms of PM-II M9 and PM-II WT (PDB ID: 3F9Q) structures.

3F9Q *vs* PM-II M9 monomer A	RMSD (Å)	3F9Q *vs* PM-II M9 monomer B	RMSD (Å)
Overall structure (315 C_α atoms)	0.98	Overall structure (313 C_α atoms)	1.31
S 79 | S 79	5.65	S 132 | S 132	7.42
G 80 | G 80	4.88	L 131 | L 131	6.92
V 78 | V 78	4.43	T 108 | T 108	5.66
Y 77 | Y 77	4.20	P 240 | P 240	4.68
D 279 | D 279	4.14	D 162 | D 162	4.03
N 76 | N 76	3.89	N 109 | N 109	3.77
V 280 | V 280	3.69	I 133 | I 133	3.68
M 75 | M 75	3.66	H 161 | H 161	3.38
E 278 | E 278	3.25	K 163 | K 163	3.35
T 81 | T 81	3.23	D 107 | D 107	3.31
V 296 | V 296	3.03	V 239 | V 239	3.25
N 233 | N 233	2.98	V 78 | V 78	3.06
G 281 | G 281	2.94	G 134 | G 134	3.05
P 240 | P 240	2.89	G 281 | G 281	2.96
P 297 | P 297	2.88	S 118 . G 118	2.91
P 295 | P 295	2.87	L 284 | L284	2.88
P 282 | P 282	2.77	P 282 | P 282	2.86
I 277 | I 277	2.69	V 280 | V 280	2.86
L 206 | L 206	2.65	L 287 | L 287	2.81
D 235 | D 235	2.59	M 286 | M 286	2.74

CONCLUSIONS

The crystals of mature PM-II M9 in free-form and a form co-crystallized with pepstatin A diffracted X-rays to 2.70 Å and 3.10 Å resolutions, respectively. X-ray data processing indicated that the crystals belonged to the orthorhombic space group $P2_12_12$ and monoclinic space group $P21$, respectively. At 26% R-factor and 28% R-free, the monomeric structures of PM-II M9 exhibit some conformational differences with PM-II WT.

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Proceedings 2

STABILIZATION OF MOLECULAR CONTACTS IN PLASMEPSIN II CRYSTALS THROUGH ENGINEERED DISULFIDE BRIDGES

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ABSTRACT

Plasmodium falciparum plasmepsin II (*Pf*-PMII) is an aspartic protease of the malarial parasite that plays a key role in the degradation of hemoglobin in its food vacuole. Since inhibition of aspartic proteases leads to starvation of the parasite, *Pf*-PMII has been considered as an important target for the development of new antimalarial drug. In this research, we are interested in reducing the conformational change in *Pf*-PMII crystal during flash cooling by incorporation of disulfide bridges at the protein-protein interface. A low-resolution structure of *Pf*-PMII crystal has been obtained and the geometries of amino-acid side chains at the molecular interfaces were screened for the design of such disulfide bridges. *Pf*-PMII mutants were constructed, expressed, purified, and crystallized for protein structure. Three mutant enzymes, with one or two amino-acid mutations (E108pC, H161C, E108pC and H161C), spontaneously cross-linked to form oligomers faster than the wild-type *Pf*-PMII. β-mercaptoethanol was used to reduce any pre-formed disulfide bonds in order to stabilize the monomeric enzyme form. This reduced form was found to be capable of auto-activation, suggesting it forms a correct, functional protein fold. Crystallization of the plasmepsin II mutants is underway with the aim to investigate the effects of these newly incorporated disulfide bridges.

Keywords: Disulfide crosslinking, plasmepsin, crystallization.

INTRODUCTION

Cryocrystallographic study of macromolecules has grown rapidly in recent years [1]. Single-crystal X-ray diffraction at temperatures near those of liquid nitrogen is now routinely conducted at X-ray laboratories and synchrotron beamlines. Cryocrystallography greatly reduces radiation damage to the crystals, increases diffraction resolution, and simplifies sample handling in the experiment [2]. However, increased conformational change is commonly observed in rapidly cooled crystals.

We aim to stabilize the crystal lattice to reduce the conformational change in the *Plasmodium falciparum* proplasmepsin II (*Pf*-PPMII) crystal during flash cooling by incorporation of disulfide bridges at the protein-protein interface. For a known crystal lattice, we can identify amino-acid residues on the surface that are involved in crystal packing and that have proper geometries for possible disulfide linkage. Then, we would be able to mutate the residues to Cys and try to crystallize the mutant protein in the same crystal form under thiol-reducing condition. At this stage, the mutant protein would theoretically be cross-linked over time throughout the whole crystal. Then, we would flash-cool the crystal and collect X-ray diffraction data for comparison with that of the native protein crystal.

MATERIALS AND METHODS

Construction of mutant proteins

The general criteria for the design of such disulfide bridges were based on the analysis of interatomic distances between C_α or C_β atoms located at the molecular surfaces from the known lattice, possibly at low resolution. Three PPMII mutants; a, E108pC; b, H161C; and c, E108pC and H161C, were constructed by site-directed mutagenesis. Oligonucleotide primers were designed in pairs for Polymerase Chain Reaction (PCR) mutation using the *Pfu* enzyme. The PCR products were treated with *DpnI* to digest the parental, non-mutated plasmid DNA template, and the DNA was used to transform competent *Escherichia coli* XL1-Blue. The transformed cells were selected with ampicillin-containing LB agar and the plasmid DNAs were extracted. The gene coding for the Pf-PMII was sequenced to ensure incorporation of the correct mutations. The mutated plasmid DNA was used to transform *E. coli* BL21(DE3) pLysS for protein expression.

Purification

The wild-type (WT) and mutant (MT) *Pf*-PPMIIs were overexpressed as inclusion bodies which were sequentially washed with washing buffer solutions [3]. The dialysates were applied to a Q-Sepharose column in denaturing condition. The column was eluted with a linear NaCl gradient (0–1 M in urea-containing buffer), and the fractions containing the 43-kDa protein band expected to be proplasmepsins as analyzed by SDS-PAGE were pooled and stored at 4°C. The PPMII from WT and MT were refolded by rapid dilution, and the refolded proteins were applied to Q-Sepharose HP column in non-denaturing condition. The protein fractions from the Q-Sepharose HP column were pooled based on protein purity and activity according to auto-activation analysis as observed on SDS-PAGE. The eluted protein was concentrated to about 16 mg/ml by ultrafiltration using a 10-kDa cut-off concentrator.

Activation of pro–plasmepsins

For an auto-activation assay, the mature forms of plasmepsins were generated by addition of one-tenth volume of 1 M sodium acetate, pH 4.5, and incubated for 8 h at room temperature. Autocatalytic processing of each zymogen (43 kDa) to its respective mature forms (37 kDa) was monitored by SDS-PAGE.

Crystallization

All crystallization experiments were carried out at 22°C using the hanging-drop vapor diffusion method. X-ray diffraction data were collected on a 165-mm Mar CCD detector system using an in-house rotating-anode X-ray generator (Cu K_a; λ = 1.542 Å) at the Synchrotron Light Research Institute, Nakorn Ratchasima, Thailand. The data were processed and scaled with the *CrystalClear* Program suite [4].

RESULTS AND DISCUSSION

Three proplasmepsin II mutants were successfully constructed, expressed and purified. The purified *Pf*-PPMII wild-type and mutant enzymes were evaluated by *in vitro* auto-activation assay and analyzed on 12.5% SDS-PAGE and 8.5% native PAGE. (Fig. 1) In contrast to the wild-type pro-PMII, the mutant enzymes could be easily cross-linked to form oligomers as seen on the non-reducing SDS-PAGE and native PAGE. β-mercaptoethanol (BME) was used to keep the thiol groups in reduced state in order to stabilize the monomeric form prior to crystallization. Nevertheless,

this form was found to be capable of auto-activation, suggesting the protein forms a correct, functional folding as shown in Fig. 2.

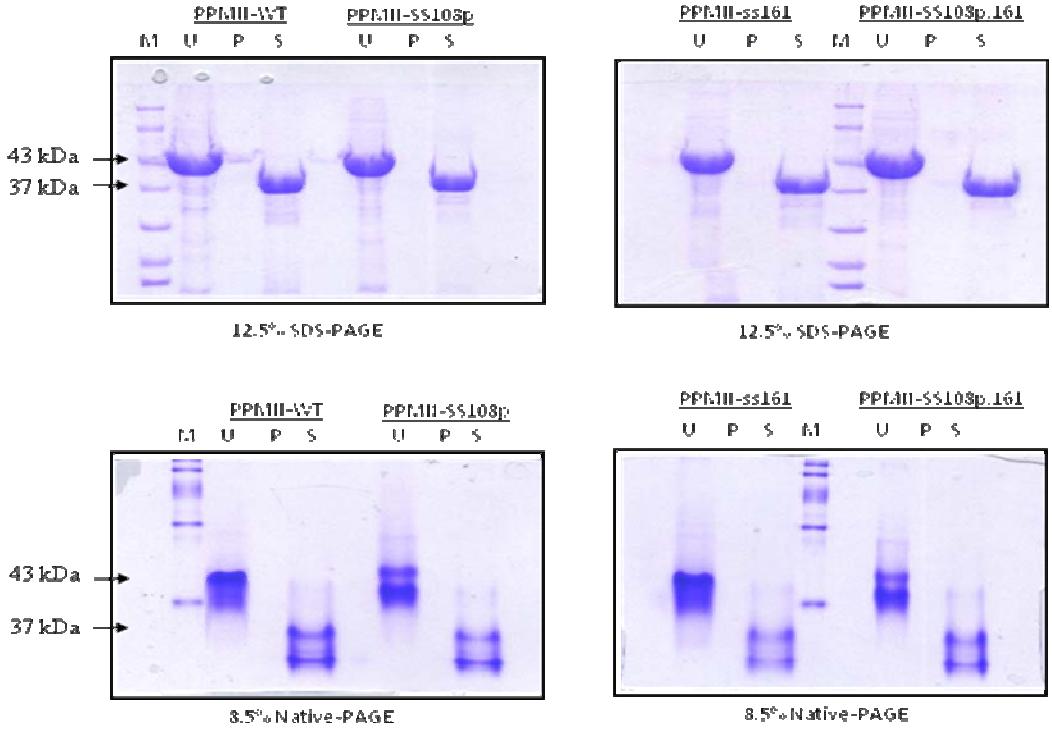

Fig. 1 Auto-activation assay of wild-type and munt *Pf*-PPMII proteins, 1) E108pC, 2) H161C and 3) E108pC and H161C as detected on 12.5% SDS-PAGE (upper panel) and on native-PAGE (lower panel). Conversion of the unactivated proteins (U, 43 kDa) to soluble, activated forms (S, ~37 kDa) was performed with little precipitates. (P) indicated that all four proteins have correct protein folding. Lanes M were protein molecular-weight markers.

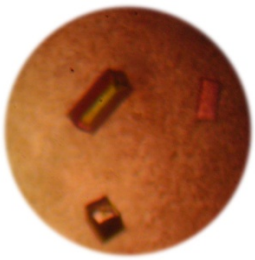

Fig. 2 H161C mutant crystal of *Pf*-PPMII

Mutant crystals of the H161C *Pf*-PPMII were obtained from a hanging-drop vapor-diffusion crystallization, in which 1 µl of the protein solution was mixed with 1 µl of the precipitant solution (23 % (V/V) PEG 4000, 100 mM Tris-HCl pH 7.5, 85 mM Na, K tartrate, and 20 mM BME) as shown in Fig. 2. Crystals appeared within 5 days and reached the maximum size in about 14 days. The data of the H161C *Pf*-PPMII mutant were processed and scaled with the CrystalClear/d*TREK program suit. The statistics of the data collected are summarized in Table 1.

Table 1 Summary of the statistics of the data collected.

Space group	$P4_12_12$
Unit cell dimensions	a=83.29 Å, b=83.29 Å, c=116.73 Å $\alpha=\beta=\gamma$=90.00°
Resolution range	41.65 - 3.45 (3.57 - 3.45)
Total number of reflections	78,202
Number of unique reflections	5,751
Average redundancy	13.60 (13.26)
% completeness	99.2 (98.8)
R_{merge} †	0.094 (0.433)
Reduced Chi^2	1.86 (1.78)
Output <I/sigI>	25.1 (7.6)

† $Rmerge = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i \langle (hkl)\rangle$, where I_i is the intensity for the i^{th} measurement of an equivalent reflection with indices hkl.

Using the CCP4 program package [5], the initial phases were solved with the molecular replacement (MR) method. Initial $2F_o–F_c$ electron-density map was calculated with the CNS program [6] and visualized in program O [7]. Continuous electron density was observed in the $2F_o–F_c$ map between side chains of H161C *Pf*-PPMII MT of two symmetry mates, suggesting possible formation of an intermolecular disulfide bond (Fig. 3).

Our preliminary result shows that a single mutant H161C *Pf*-PPMII MT could form a dimer through a disulfide mutation at the molecular surfaces. Further characterization is needed with improved crystallization and X-ray data collection to higher resolution. Data from the expected mutant with multiple disulfide cross-linking are being pursued as these would result in further cross-linking of molecules in the lattice.

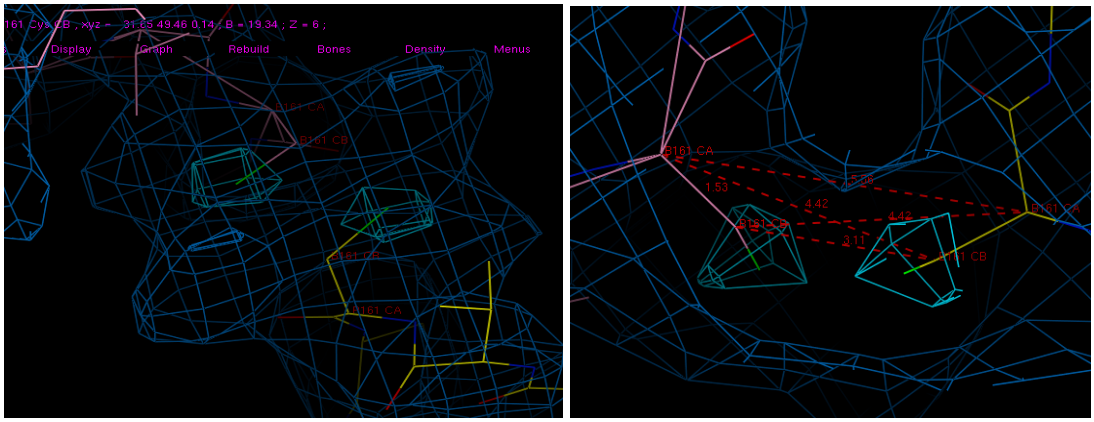

Fig. 3 Continuous $2F_o–F_c$ electron density around the side chains of H161C *Pf*-PPMII MT of two symmetry mates, suggesting a possible intermolecular disulfide bond.

Although this engineered disulfide cross-linking strategy requires the knowledge of known lattice and protein structure, it is expected to help improve crystallization as well as X-ray diffraction data quality. Moreover, it may help restricting protein orientation and thus facilitate crystal formation, especially for routine crystallization applications such as in rational drug design where structures of various complexes of the same target protein are required. In addition, the technique may be useful for the case of crystallization of highly homologous proteins where similar protein folding and molecular packing are expected to be probable.

ACKNOWLEDGEMENTS

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Proceedings 3

ESTABLISHMENT AND IDENTIFICATION OF ACQUIRED DOXORUBICIN RESISTANCE IN HUMAN BREAST CANCER CELLS

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ABSTRACT

Doxorubicin is considered as one of the first line chemotherapies in breast cancer. Unfortunately, resistance to this agent continues to be a major problem in the treatment of breast cancer. Understanding the mechanism(s) underlying this resistance would be useful for treatment and prognosis of cancer patients. In this study, we established a doxorubicin-resistant MDA-MB-231/DOXO subline from parental MDA-MB-231 human breast cancer cells. MDA-MB-231/DOXO was 100-fold more resistant to doxorubicin than its parental cells. It showed cross-resistance to taxol, vinblastine, etoposide and colchicine, but not to curcumin, cisplatin and 5-fluorouracil (5-FU) compared to the parent cells. Currently, comparisons of subcellular proteins (cytosolic proteins, membrane proteins, nuclear proteins and cytoskeletal matrix proteins) in both cell lines are in progress. The identified proteins may help in predicting drug resistant mechanisms and provide targets for the development of therapeutic agents in breast cancer cells.

Keywords: doxorubicin, resistance, human breast cancer cells, MDA-MB-231, subcellular proteins

INTRODUCTION

Cancer is considered to be one of the leading causes of death worldwide second to heart disease [1]. Nowadays, breast cancer in Thailand was estimated to have the highest incidence rate among cancer cases and also has dramatically increased every year [2]. After several years of treatment, more than half of breast cancer cases become resistant to chemotherapy and the mechanisms are not well understood [3-5]. Doxorubicin or hydroxyl daunorubicin is an anthracycline antibiotic derived from bacterial called *Streptomyces*. The anthracyclines are considered to be one of the most effective agents against wide range of cancers, including breast cancer, lung cancer (small cell and non small cell lung cancer), leukemia (lymphocytic and myeloblastic leukemia), bladder cancer, and gastric cancer. Doxorubicin is an inhibitor of topoisomerase II or topoisomerase α that is involved in the process of DNA and RNA synthesis [6, 7]. Several mechanisms have been reported by which normal cancer cells to grow and become resistant to the treatment [8-10]. The major mechanisms of doxorubicin resistance have been reported to be the over-expression of multiple drug resistance (MDR1) and the multidrug resistance protein (MRP) family [7, 9, 11].

In order to investigate the mechanisms of doxorubicin resistance, we established doxorubicin resistant cells from MDA-MB-231, a human breast cancer cell line, by stepwise increasing the concentrations of doxorubicin until resistant cell line showed more than 100-fold increased resistance to doxorubicin compared with parental cells and also showed cross-resistance to other anticancer drugs. Both the parental cell line and its sub-line were fractionated into cytosolic, membrane, nuclear and cytoskeletoal components, then sub-fractions were separated by SDS-PAGE gel electrophoresis.

MATERIALS AND METHODS

Cell line and cell culture

The human breast cancer, MDA-MB-231 cell line, was maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO^{TM}). Completed media contained 100 U penicillin streptomycin (GIBCO^{TM}), amphotericin B (GIBCO^{TM}), and 10 % fetal bovine serum (FBS) (GIBCO^{TM}). The cell line was maintained at 37°C in 5 % CO_2.

Establishment of doxorubicin resistant cell

The doxorubicin resistant cell (MDA-MB-231/DOXO) was established from the parental MDA-MB-231 cell by stepwise increasing of the doxorubicin concentrations every week until 50 % of inhibitory concentration (IC_{50}) of doxorubicin for MDA-MB-231/DOXO was 100-fold higher than that of its parental cells. The MDA-MB-231 and MDA-MB-231/DOXO cells were treated with various concentrations of vinblastine, etoposide, colchicine, cisplatin, curcumin and 5-FU to evaluate cross resistance using an MTT assay.

Subcellular protein extraction

The MDA-MB-231 and MDA-MB-231/DOXO cells were scraped from the plates in 1X PBSA buffer and pelleted by centrifugation at 2,500 rpm, 4°C for 15 minutes. The cells were fractionated into cytosolic, membrane, nuclear and cytoskeletal components with a subcellular proteome extraction kit (EMD Biosciences) according to the manufacturer's protocol. Proteins were precipitated in 10% trichloroacetic acid overnight, washed twice in 25% acetone and re-suspended in lysis buffer (thiourea, urea, sodium dodecyl sulfate and 3-(4-heptyl)phenyl-3-hydroxypropyl)dimethylammoniopropanesulfonate (C_7BzO)). The concentration of protein was quantified by the Bradford assay (Bio-Rad).

One dimension gel electrophoresis

Twenty micrograms of cytosolic protein, 25 μg of membrane protein, 50 μg of nucleic protein and 15 μg of cytoskeletal matrix protein were loaded onto SDS-polyacrylamide gel electrophoresis (14%). Electrophoresis was performed at constant 11 mA per gel for 4 - 4.5 h.

RESULTS AND DISCUSSION

The sensitivity for doxorubicin of parental cells was 0.04 μM, whereas that of doxorubicin resistant cells which had been exposed stepwise by doxorubicin for 8 months was 4.05 μM. The cytotoxicity assay demonstrated that this resistant cell line was about 101-fold more resistance than the parental cells (Table 1). Both the resistant cells and its parental cells were compared with respect to cross resistance to

other anticancer drugs. Table 1 shows that the MDA-MB-231/DOXO subline also exhibited approximately 60-fold more resistance to taxol, 137 fold cross resistance to vinblastine, 45-fold more resistance to etoposide and 25-fold more resistance to colchicines as compared to the parental cells. In contrast, as presented in Table 1, MDA-MB-231/DOXO subline showed no cross resistance to curcumin, 5-FU and cisplatin.

Table 1: IC_{50} values of MDA-MB-231 and MDA-MB-231/DOXO cells

Drug	IC_{50} (µM)		Fold difference
	MDA-MB-231	MDA-MB-231/DOXO	
Doxorubicin	0.04	4.05	101.25
Etoposide	0.3398	15.29	44.99
Curcumin	65	70	1.07
Cisplatin	16.66	16.66	1
Colchicine	0.025	0.626	25.04
5-FU	615.02	1,537.55	2.5
Taxol	0.00586	0.35	59.95
Vinblastine	0.0018	0.247	137.2

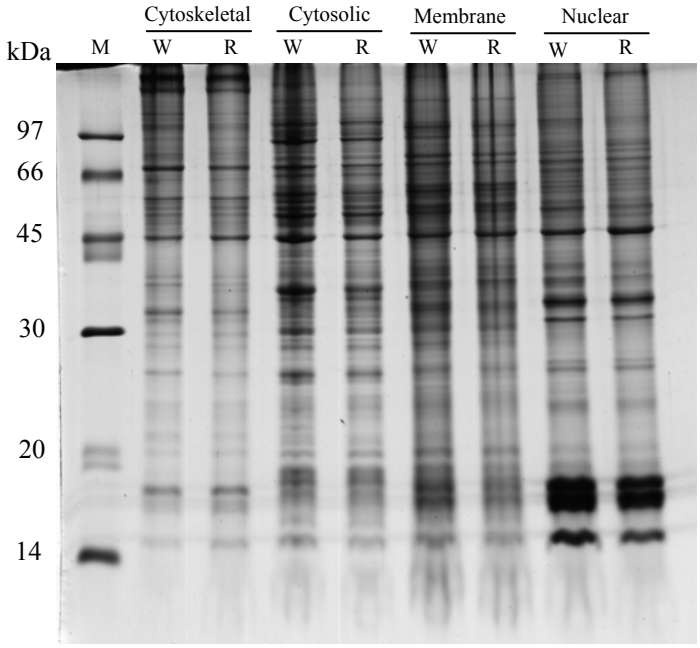

Fig. 1 SDS-polyacrylamide gel electrophoresis of four protein fractions of MDA-MB-231 and MDA-MB-231/DOXO cells. Each protein fraction was fractionated with a subcellular proteome extraction kit and loaded onto SDS-PAGE. M; protein standard markers. W; parental cell line (MDA-MB-231) and R; doxorubicin resistant cell line (MDA-MB-231/DOXO).

Both the MDA-MB-231 cell line and its sub-line were fractionated into subcellular fractions, precipitated in 10% trichloroacetic acid, washed in 25% acetone and re-suspended in lysis buffer. Twenty micrograms of cytosolic protein, 25 µg of membrane protein, 50 µg of nucleic protein and 15 µg of cytoskeletal matrix protein. The proteins were loaded onto SDS polyacrylamide gel electrophoresis and the resulting gel was stained with Coomassie blue R250 (Fig. 1). Some of differential expressed proteins are marked by arrows. Two dimension gel electrophoresis of each

fraction and ddifferential protein expression will be performed to better understand te drug resistant mechanisms. The studies would provide targets for the development of therapeutic agents in breast cancer cells

CONCLUSIONS

In this study, we established doxorubicin resistant cell line and found that it shows cross resistance to taxol, vinblastine, etoposide and colchicine, but not to curcumin, cisplatin and 5-FU compared to the parent cells. Moreover, SDS-PAGE showed differential protein expressions between the parental cell line and its sub-line. Currently, 2D gel electrophoresis and protein identification are in progress.

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Proceedings 4

PROTEOMIC ANALYSIS OF PLASMA PROTEIN EXPRESSION AND OXIDATIVE MODIFICATION IN β-THALASSEMIA/Hb-E PATIENTS

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ABSTRACT

Thalassemia is a group of genetic disorders resulting from different mutations in the globin gene clusters leading to an imbalance in globin synthesis. Unpaired globin chains are not stable and are susceptible to oxidation. Patients with β-thalassemia/Hb-E are prone to increase oxidative stress as indicated by increased lipid peroxidation and protein oxidation. As oxidative stress can be easily quantifiable by detecting carbonylation, we investigated the carbonyl formation of proteins using redox proteomics of thalassemic patients. Specific protein carbonyl levels of prothrombin, fibrinogen, α1-β-glycoprotein, and α1-antitrypsin were significantly increased in β-thalassemia/Hb-E patients compared with normal subjects whereas haptoglobin, hemopexin and apolipoprotein A-I were not oxidized. Thus, these proteins could be potential targets as biomarkers under different pathological and physiological conditions.

Keywords: free radicals, oxidative stress, protein carbonyl, redox proteomics

INTRODUCTION

β-thalassemia/Hb-E, the most common β-thalassemia disease found in Southeast Asia [1], is a group of genetic disorders resulting from defects in the rate of β-globin chain synthesis. In the absence or inadequate of β-globin chain production, the amount of α-globin chain is out of balance. It is present in excess and precipitates within developing erythroid progenitors in bone marrow as well as in peripheral red cells. These excess α-chains are unstable. Following oxidation, the unstable subunits generate free oxygen radical species, such as superoxide and potent hydroxyl radicals [2]. These free radical can induce oxidative damage to cell macromolecules including DNA, proteins, and lipids by a number of different mechanisms such as formation of DNA adducts, protein carbonylation, and lipid peroxidation [3].

Oxidative damage of proteins is one of the modifications leading to a severe failure of biological functions and to cell death [4, 5]. However, oxidative stress is also an inevitable part of normal metabolism in an aerobic environment in which reactive oxygen species (ROS) are generated. ROS may oxidize amino acid side chains into ketone or aldehyde derivatives [6]. Histidine, arginine, and lysine are the most susceptible amino acids for the ROS-mediated carbonyl formation [7]. Measurement of these carbonyl groups is thought to be a good estimation for the extent of oxidative damage of proteins associated with various conditions of oxidative

stress, aging, physiological disorders, and diseases [8, 9]. In order to investigate the oxidative modifications to proteins in β-thalassemia/Hb-E disease, we used a parallel proteomic approach to identify protein expression and protein carbonylation of plasma proteins in these patients.

MATERIALS AND METHODS

Subject

Fifteen β-thalassemia/Hb-E patients (five were splenectomized), were enrolled after giving their informed consent. Their hemoglobin levels were between 6-9 g/dl, and ages ranged from 14-40 years. The controls were healthy normal subjects with hemoglobin A and A_2. The ages range was between 22-39 years.

Blood fraction preparation

Blood samples were collected in EDTA-coated tubes from normal subjects and thalassemia/HbE patients. Blood samples were centrifuged at 800 x g at 4 °C for 10 minutes to separate plasma then stored at -80 °C.

Samples preparation

Thirty-five microliters of plasma samples were depleted of two major proteins (albumin and IgG) using the ProteoExtract™ Albumin/IgG removel kit (EMD Biosciences, Inc., an Affiliate of Merck KGaA, Darmstadt, Germany). Proteins were then precipitated by addition of ice-cold 100% TCA to obtain a final concentration of 10% TCA for 30 min and then resuspended with lysis buffer. Protein concentrations were determined using Bradford method (Bio-Rad Laboratories, CA) [10]. Two hundred micrograms of protein were used. Seventy-five microliters of rehydration buffer were then added.

Preparative 2-D electrophoresis

The non-linear pH 3-10, IPG gel strips (GE Healthcare, Buckinghamshire, UK) were used for applying the sample. The first dimension (IEF) was performed at 55,000 Vh, using a Ettan IPGphor II Focusing system (GE Healthcare). Each IPG gel strip was equilibrated at room temperature in SDS equilibration buffer. Proteins were separated according to their MW on 12.5% SDS-polyacrylamide gels (100×105×1.5 mm). After electrophoresis, proteins were visualized by Coomassie brilliant blue R-250 staining (SERVA Electrophoresis GmbH, Heidelberg, Germany).

Western Blotting

Two hundred micrograms of protein were incubated with 20 mM 2,4-dinitrophenylhydrazine at room temperature (25 °C) for 20 min, and the electrophoresis was carried out as described above. The proteins from the 2-D electrophoresis gels were transferred to PVDF membrane (Immobilon-P; Millipore, Billerica, MA, USA) using a Transblot-Blot® SD semidry transfer cell (Bio-Rad) at 30 V for 15 h. The 2,4-dinitrophenylhydrazone (DNP) adducts of the carbonyls of the proteins were detected on the PVDF membrane using a primary goat antibody (Bethyl Labolatories, Montgomery, TX) specific for DNP-protein adduct (1:2000) followed by a secondary rabbit anti-goat IgG (Invitrogen, Carlsbad, CA, USA) antibody, and color was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (SigmaFast tablets, Sigma-Aldrich, St. Loius, MO, USA).

Gel scanning and image analysis

The gels were scanned using ImageScaner II (GE Healthcare). The ImageMaster 2D Platinum software 6.0 (GE Healthcare) were then used for computer analysis.

Liquid chromatography-mass spectrometry (LC/MS/MS)

Different protein spots were selected and subjected into in-gel digestion with trypsin and then the extracts were subjected to LC/MS/MS. The database search was performed with proteinLynx screening SWISS-PROT and NCBI. For proteins that were difficult to find, the Mascot search tool available on the Matrix Science site screening NCBInr was used.

Data analysis

Statistical analysis was performed using one sample *t*-test.

RESULTS AND DISCUSSION

The specific carbonyl levels were obtained by dividing the carbonyl level of a protein spot on the PVDF membrane by the protein level of its corresponding protein spot on the gel. Such number gives the carbonyl level per unit of protein. We used a parallel approach to quantify the protein levels by coomassie brilliant blue R-250 staining (Fig. 1) and the extent of DNP-bound proteins by immunohistochemistry (Fig. 2). In comparison with normal subjects, thalassemic plasma proteins were significantly oxidized as shown in Table 1. Western blot immunoassay showed that fibrinogen, among other plasma proteins, was highly susceptible to attack by oxidants. In contrast to haptoglobin, hemopexin and apolipoprotein A-I were not oxidized in both normal subjects and β-thalassemia/Hb-E patients.

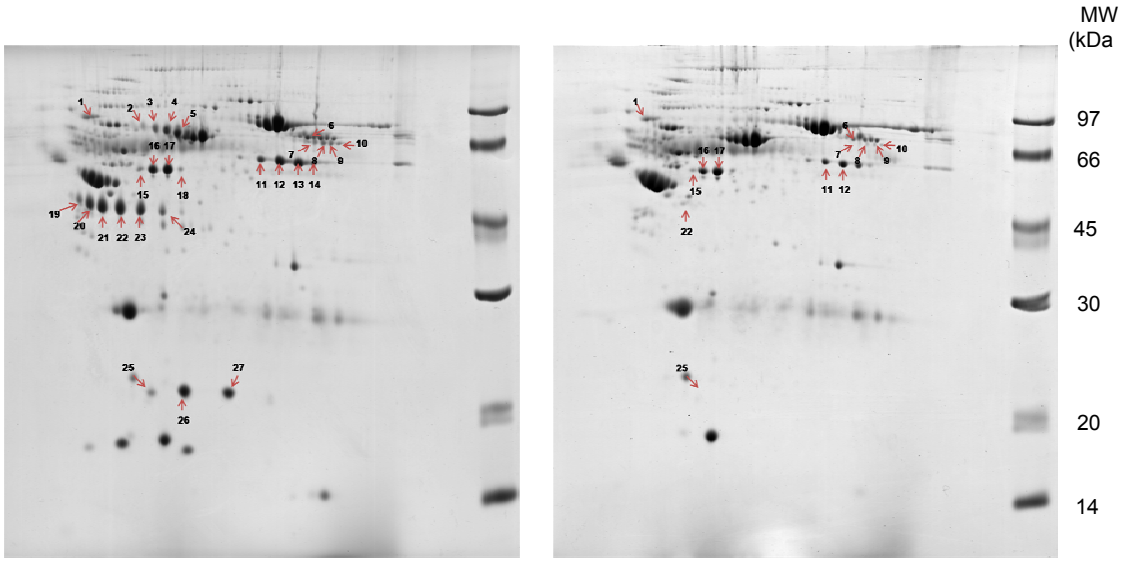
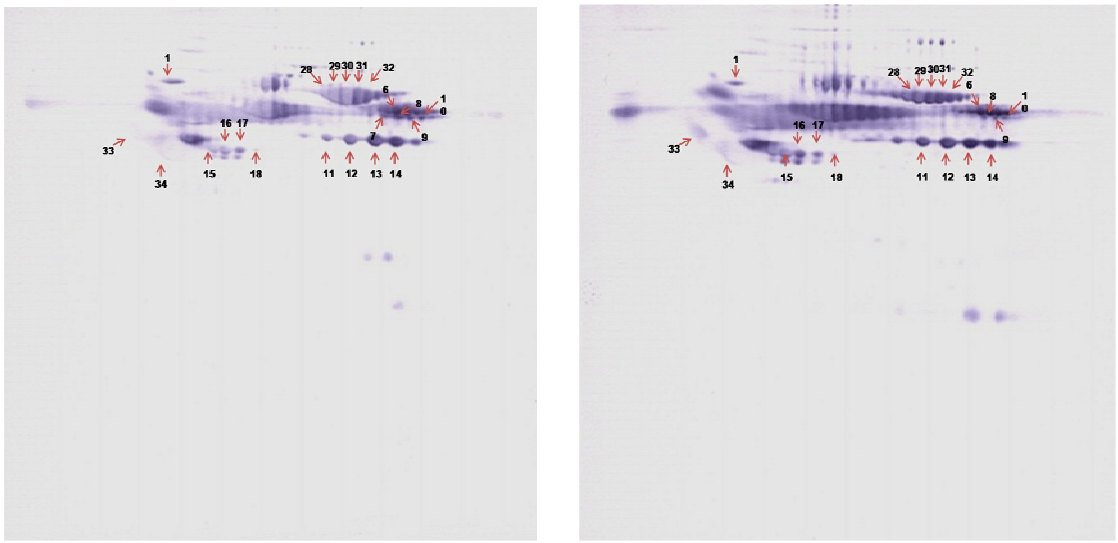


Fig. 1 Two-dimensional electrophoresis images of plasma proteome from normal subjects and β-thalassemia/Hb-E patients.



Normal subjects **β-thalassemia/Hb E**

Fig. 2 Carbonyl immunoblot of plasma proteome from normal subjects and β-thalassemia/Hb-E patients. Arrows indicate the differential oxidized protein expression shown in Table 1.

Table 1. Protein carbonyl levels in non-splenectomized (NS) and splenectomized (SP) β-thalassemia/Hb-E patients as compare to normal subjects. Values are shown as fold increase. *$p < 0.001$

Spot no.	Protein names	Peptide Match	pI/MW	Average Fold of Change	
				NS-Baseline	SP-Baseline
1	Prothrombin	11	5.05/80.15	2.03±0.09*	1.97±0.05*
6-10	Fibrinogen α-chain	4	6.75/65.92	3.61±0.43*	3.31±0.18*
11-14	Fibrinogen β-chain	3	6.27/55.70	4.12±0.24*	2.72±0.28*
15-18	Fibrinogen γ-chain	13	5.34/49.57	2.85±0.16*	1.92±0.13*
28-32	Transferrin	10	5.21/42.23	2.19±0.14*	1.76±0.21*
33	Alpha1-Antichymotrypsin	9	4.97/54.95	+	+
34	Alpha1-Antitrypsin	10	5.49/54.24	2.88±37*	1.84±0.14*

+ absent in normal subjects but found in β-thalassemia/Hb-E patients

CONCLUSION

Fibrinogen is a high abundant plasma protein and the major plasma coagulation factor. It consists of two sets of three disulphide-bridged chains (Aα, Bβ, and γ) of 610, 461, and 411 amino acid residues, respectively [11–14]. Our finding showed that this protein was highly susceptible to oxidative stress. Oxidant-induced carbonyl formation in fibrinogen derives largely from amino acid oxidation and not from oxidation of carbohydrate groups [15]. Therefore, oxidized forms of fibrinogen circulating in blood could be interesting in monitoring oxidative stress.

ACKNOWLEDGEMENTS

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CHANGES IN PROTEIN PROFILE OF *BRASSICA ALBOGLABRA* LEAVES AT DIFFERENT TIME OF DEVELOPMENT

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ABSTRACT

Plant proteomics has been extensively studied in recent years since the establishment of a complete genome sequence of the reference model plant, *Arabidopsis thaliana*. Plant proteomics enables the research of plant growth and development and also genes and pathways crucial for stress response and tolerance. The recent proteomic analysis on plant developmental processes has been carried out mostly in crops such as rice, soybean and wheat but only few in vegetables. Therefore, *Brassica alboglabra* (Chinese kale) or also known as khana in Thailand which is a commonly consumed in Southeast Asian country was studied. Chinese kale is extensively grown in Thailand to fulfill the market demand as well as it is easy to grow due to the tolerance to heat and humidity. Farmers normally harvest *Brassica alboglabra* starting from the fifth week of growth and sell it to the market. In this study, the changes in protein at two different time points during plant growth were investigated. Two methods were used to compare protein extracts from *Brassica alboglabra* leaves. Proteins were extracted most efficiently by grinding in liquid nitrogen and precipitating with TCA/acetone. Subsequently, the proteins extracted from leaves at second and fifth weeks were subjected to two-dimensional gel electrophoresis. Using ImageMaster 2D Platinum 6.0 software to analyze the 2D-PAGE, 47 and 209 spots were identified in the second and fifth week, respectively. Differential protein expression was carried out in the fifth week and 5 protein spots were found to increase by at least 2-folds. These include ATP synthase beta chain and Rubisco large subunit, while 173 spots were present in the fifth week. These proteins include Rubisco activase, catalase, Rieske FeS protein, N-glyceraldehyde 2-phosphotransferase, and 30 S ribosomal protein S5 family protein. The functions of the fifth-week proteins were mainly involved in photosynthesis and photorespiration, defense, and energy. Full identification of proteins in the proteome of *Brassica alboglabra* could serve as a reference for studying the effects of stress on the protein expression levels.

Keywords: proteomics, *Brassica alboglabra,* different time-point

INTRODUCTION

B. alboglabra is a green leafy vegetable which is commonly consumed in Thailand and other Southeast Asia countries due to high nutritive values and availability throughout the year. It is known as a dicotyledon plant with broad and

thick waxy leaves and it tastes slightly bitter and best served as stir-fried in Chinese-style dishes. The vegetable, like other higher plants, possesses smany unique pathways which include photosynthesis that provides major food for other organisms [1]. To understand the plants metabolism, proteomic approach has been used as a tool to facilitate the identification of changes in protein expression and serves as the central bridge to integrate plant physiology, genomics, and metabolomics. Plant proteomics has been intensely studies since the availability of the full 2-DE mapping of the reference plant, *Arabidopsis thaliana*. Since then, progress has been made towards identifying and cataloguing proteins from various plants which include rice, soybean, barley and maize correspond to their developmental processes [2]. Protein preparation is a crucial step in proteomics. In order to obtain a reproducible protein patterns from gel to gel for comparison, the protein needs to be well–resolved, and proteolysis which amy result in artifacts which would interfere the gel interpretation, should be avoided [3]. In this study, two methods of protein extraction from *B. alboglabra* leaves were compared. Protein from leaves were identified and categorized into 7 functional groups and 1 group of unknown proteins. Protein identification of *B. alboglabra* has not yet been established, and this study would provide a baseline for the assessment of plant stress and tolerance as well as changes during plant processes and development.

MATERIALS AND METHODS

Plant samples

The eeds for the *B. alboglabra* were grown in a pot of 8 cm in diameter filled with soil. The plants were taken in the morning of the second and the fifth weeks and the leaves were excised for protein analysis.

Protein extraction and urea homogenization

The leaves (0.1 g) were homogenized in 1 mL of extraction buffer containing 8M urea, 2% CHAPS and 0.28% DTT. The samples were frozen and thawed for 3 times, followed by centrifugation at 10,000 g for 15 minutes at 4°C. The supernatant was collected for protein determination using Bradford assay method, with IgG2 as standard at 595 nm.

TCA/ acetone precipitation

The leaves (0.1 g) were ground in liquid nitrogen and resuspended in 1 mL solution of 10% TCA and 0.07% DTT in cold acetone. The samples were incubated at 20°C for 1 h, followed by centrifugation at 14,000 rpm for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 1 ml of 0.07% DTT in cold acetone, incubated and centrifuged for 10 min twice at 14,000 rpm. The pellet was vacuum-dried for 30 minutes and resuspended in 500 μL of rehydration buffer consisted of 8 M urea, 2% CHAPS, 40 mM DTT, 0.2% ampholytes 3–10, and 2 mM tributylphosphine (TBP). The samples were then vigorously vortex for 5 min and incubated at 4 °C overnight. The next day, the samples were centrifuged at 14,000 rpm for 10 min. The supernatant was collected for protein determination using Bradford assay as described above.

Two-dimensional electrophoresis

The two-dimensional electrophoresis was performed using Immobiline™ DryStrip pH 3-10 Non-Linear, 7 cm (GE Healthcare). The sample (125 μL),

containing 150 µg of protein and rehydration buffer, was loaded and kept at room temperature overnight. The first IEF was conducted at 200 Vhrs, and 55 µA/strip. Prior to the second dimension SDS-PAGE gels, the strips were soaked with equilibration buffer 1 (0.5 M Tris-HCl pH 6.8, urea, glycerol, SDS, and DTT) with gentle agitation at room temperature for 10 minutes followed by soaking in equilibration buffer 2 (0.5 M Tris-HCl pH 6.8, urea, glycerol, SDS, and Iodoacetamide). The IPG strips were embedded within the molten agarose directly placed on the top of a 1 mm 12.5 % SDS-PAGE gel. The SDS-PAGE was performed at 200 V and 44 mA. The gels were stained with Commassie Blue, and destained with 40% methanol and 5% acetic acid for 2 hours and 10% of methanol and 5% of acetic acid for 1 h. The gels were scanned by an Amersham Biosciences image scanner. The gel Images were analysed with the Image Master 2D Platinum 6.0 from GE Healthcare.

Liquid Chromatography-mass spectrometry (LC/MS/MS)

The protein spots were excised and destained with 0.1M NH_4HCO_3/50% acetonitrile, reduced the disulphide bonds with 0.1M NH_4HCO_3/10mM DTT/1mM EDTA, alkylated with 100mM iodoacetamide/0.1M NH_4HCO_3 and digested with trypsin. The LC/MS/MS analyses were carried out using a capillary LC system (Waters) coupled to a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray ion-source working in the nanoelectrospray mode. Glu-fibrinopeptide was used to calibrate the instrument in MS/MS mode. The tryptic peptides were concentrated and desalted on a 75 µm ID X 150 mm C18 PepMap column (LC Packings, Amsterdam, Netherlands). Eluents A and B were 0.1 % formic acid in 97% water, 3% acetonitrile and 0.1% formic acid in 97% acetonitrile respectively. Six microliters of sample were injected into the nanoLC system, and separation was performed using the following gradient: 0 min 7% B, 35 min 50% B, 45 min 80% B, 49 min 80% B, 50 min 7% B, 60 min 7% B. The database search was performed with ProteinLynx screening SWISS-PROT and NCBI. The Mascot search toll available on the Matrix Science site screening NCIBnr was used for proteins which were not available in previous database.

RESULTS AND DISCUSSIONS

The seeds of *B. alboglabra* were obtained from a local seed company, with the information of 80% germination and 98% purity provided. The seeds were grown in the soil supplied with organic and chemical fertilizers with no other treatments except for water and sunlight. The plants were harvested in the morning to minimize the possibility of protein changes.

We characterized the protein extracts of the plant by comparing 2 methods, namely the TCA/ acetone precipitation [4] and urea homogenization [5]. By using 20 µg of protein from the fifth-week leaves, the TCA/acetone method showed a better protein separation as revealed by 1-dimension SDS/PAGE compared to urea homogenization (Fig. 1). This is probably because the TCA precipitation efficiently inhibits protease activity in the plant tissue and therefore gives highly reproducible gels with good spots resolution in large pH and Mr ranges [3]. Therefore, the TCA/acetone method was selected for the 2-dimensional electrophoresis of *B. alboglabra* leaves.

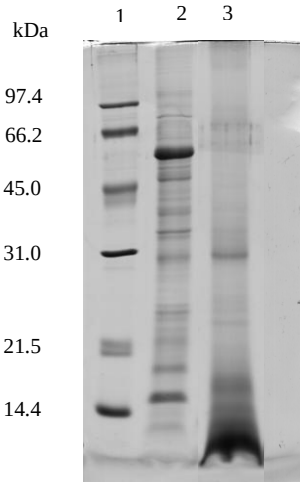

Fig. 1 Optimization of conditions for extracting proteins from the fifth week of *Brassica alboglabra* leaves. One-dimensional electrophoresis was performed using leaves grounded in liquid nitrogen and TCA/acetone precipitated (Lane 2) or homogenized in 8 M urea (Lane 3). Lane 1 represents the molecular weight standard.

There were significant growth changes of plants by approximately about 50% in the fifth-week compared to the second-week as shown in the small photographs. Using Image Master Software analyses, there were a total of 209 and 47 spots observed on the 2-DE gel in the fifth and the second weeks, respectively. Of the 209 spots in the fifth-week leaves, we identified 99 spots by LC/MS/MS (Fig. 2).

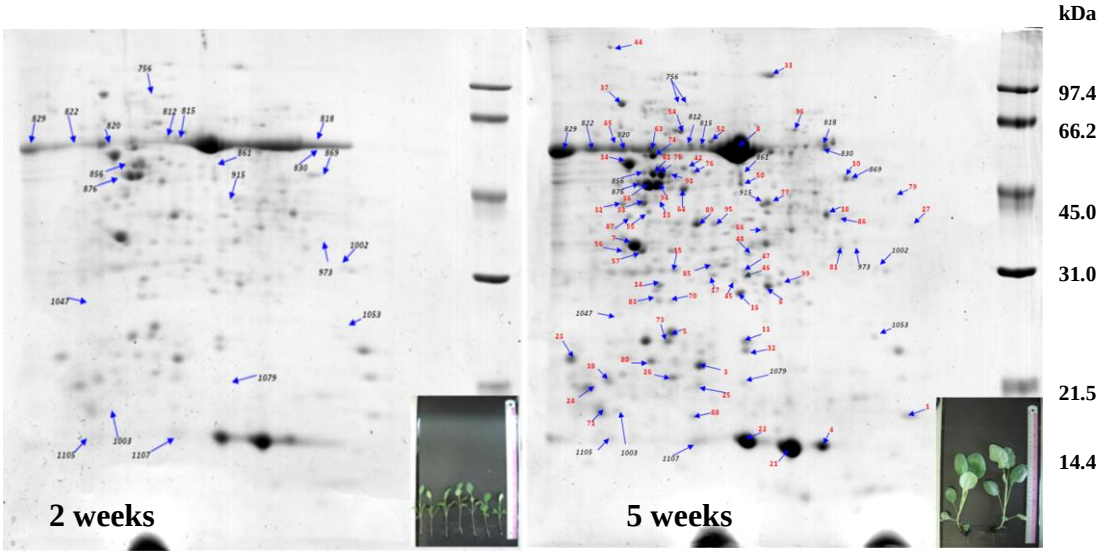

Fig. 2 Two dimensional gel maps of the second and the fifth week of *B. alboglabra* leaves indicating the growth of the whole plants corresponding to the 2-D PAGE. Numbers in italics indicate protein spots which were significantly increased in the fifth week leaves compared to those in the second-week leaves. The gel with the fifth-week leaves indicates all 99 protein spots identified by LC/MS/MS.

From the 2-DE maps, there were differential protein expressions observed in the fifth-week leaves compared to those in the second-week leaves (Fig. 2). Five spots were found significantly increased by at least 2 folds, with 3 of them identified as Rubisco large subunit chain (Spot no 822 and 829) involved in photosynthesis and photorespiration and ATP synthase beta (Spot no 815) which functions in ATP synthesis to provide energy for biosynthesis and promotes plant growth [1]. While, of the 173 spots appeared in the fifth weeks were increased about 10^4 folds, and only 18 spots were identified. The 3 spots (Spot no 812, 820 and 1003) are Rubisco large

spots were identified. The 3 spots (Spot no 812, 820 and 1003) are Rubisco large subunit, 2 spots (Spot no 1105 and 1107) were Rubisco small subunit and 2 spots (Spot no 856 and 876) were Rubisco activase (Table 1). The possible roles of Rubisco activase are in the activation of Rubisco [6]. The 30S ribosomal protein S5 family protein (Spot no 973) functions in protein synthesis and tends to be expressed in photosynthetically active cells[7]. Therefore, all these proteins were present due the process of photosynthesis and photorespiration in the leaves [1, 6].

Table 1. List of spots identified by LC/MS/MS with significant increase in protein expression in the fifth week compared to the second week of *B. alboglabra* leaves. Significant changes referred to ≥2-fold changes of percent volume using Image Master Software analysis.

Spot ID	Protein description	pI/MW	Fold change
815	**ATP synthase beta chain, mitochondrial, putative [Arabidopsis thaliana]**	6.06/59.82	2.41
822	**Ribulose bisphosphate carboxylase large chain precursor RuBisCO large subunit**	5.84/52.92	5.38
829	**Ribulose bisphosphate carboxylase large chain precursor RuBisCO large subunit**	6.18/24.80	2.12
756	**Transketolase chloroplast**	5.36/72.95	10^4
812	**Ribulose bisphosphate carboxylase large chain precursor**	5.84/52.92	10^4
818	**SHM1 (Serine hydroxymethyltransferase 1)**	8.13/57.36	10^4
820	**Ribulose bisphosphate carboxylase large chain precursor**	6.57/47.11	10^4
830	**catalase [Brassica juncea]**	6.35/54.96	10^4
856	**RuBisCO activase**	5.50/42.72	10^4
861	**N glyceraldehyde 2 phosphotransferase like Arabidopsis thaliana**	4.95/31.71	10^4
869	**aminomethyltransferase, putative [Arabidopsis thaliana]**	8.55/44.42	10^4
876	**RuBisCO activase**	7.79/45.68	10^4
915	**fructose-bisphosphate aldolase [Pandanus amaryllifolius]**	6.48/38.30	10^4
973	**30S ribosomal protein S5 family protein [Arabidopsis thaliana]**	9.38/32.63	10^4
1002	**Predicted protein Phsycomitrella patens**	6.11/17.08	10^4
1003	**Full=Ribulose bisphosphate carboxylase large chain; Short=RuBisCO large subunit**	5.88/52923	10^4
1047	**putative poly ADP ribose glycohydrolase Oryza sativa japonica cultivar group**	5.91/26.53	10^4
1053	**RBF (Ribosome recycling factor, chloroplast precursor)[Arabidopsis thaliana]**	9.46/30.40	10^4
1079	**Rieske FeS protein [Arabidopsis thaliana]**	8.80/24.33	10^4
1105	**F101910 Arabidopsis thaliana**	5.60/14.69	10^4
1107	**Ribulose bisphosphate carboxylase small chain F1, chloroplastic**	8.23/20.17	10^4

Ninety-nine spots were identified in the fifth-week of *B. alboglabra* leaves and were categorized according to the functional groups (Fig. 3). The vast majority of proteins were found to involve in photorespiration and photosynthesis which accounted for 41% followed by proteins involved in stress response (17%) and glycolysis and gluconeogenesis (16%), protein synthesis (8%), C-compound and carbohydrate metabolism (6%), amino acid metabolism (4%) and cellular communication (2%). The proteins identified were mainly Rubisco large subunit, possibly in accordance to the fact that Rubisco is the most abundant protein in the plants and large amount Rubisco are required to support photosynthesis and up to quarter of the total protein in leaves is invested in Rubisco [6].

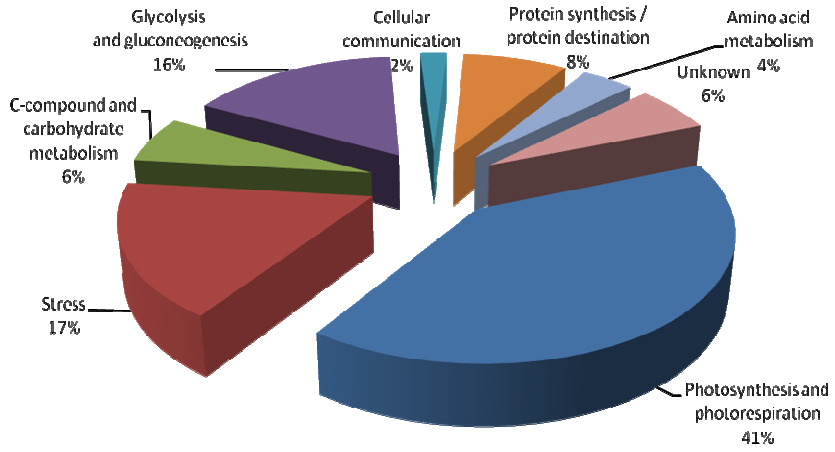

Fig. 3 Functional distribution of 99 identified proteins in the fifth week of *B. alboglabra* leaves.

CONCLUSION

Two methods, namely TCA/acetone precipitation and homogenized in urea, were used to compare proteins in two different time points. The proteins from *B. alboglabra* leaves were successfully extracted with TCA/acetone method and were subjected to 2-dimensional electrophoresis. By using Image Master Software analyses, changes in differential protein expression by at least 2 folds were observed in the second and the fifth week of *B. alboglabra* leaves which include ATP synthase beta chain and Rubisco large subunit. These proteins play important roles in photosynthesis and photorespiration and provide energy to promote plant growth. The protein spots were identified with LC/MS/MS and were grouped into 7 functional groups and 1 unknown group. The proteins involving photosynthesis and photorespiration were accounted for 41% of the total proteins identified.

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CRYSTALLIZATION AND PRELIMINARY X-RAY ANALYSIS OF *PLASMODIUM FALCIPARUM* PROPLASMEPSIN-II

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ABSTRACT

Plasmepsin-II (PM-II) is one of the aspartic proteases present in the food vacuole of the malarial parasite *Plasmodium falciparum*. This enzyme belongs to a subfamily of the plasmepsins which play important roles in host hemoglobin degradation. Structures of both pro- and active forms of PM-II have been determined, providing important structural information for inhibitor design against the plasmepsins. Yet, the structural information of an important active-site loop is missing from the available proplasmepsin-II (PPM-II) structure, likely due to its high flexibility. In this study, we attempted to crystallize proplasmepsin-II in various different conditions, aiming to obtain its structure in a new crystal form with well defined structure of the active-site loop. X-ray diffraction data from a crystal obtained from the best conditions were collected on a Rigaku rotating-anode generator. It is anticipated that the representative structure of the active site may provide better understanding in binding interactions as well as activation mechanism advantageous to the development of new antimalarial drugs targeting plasmepsins.

INTRODUCTION

Malaria is one of the most common infectious diseases in human. It is caused by *Plasmodium* parasite, threatening half of the world's population. There were estimated 247 million malaria cases among 3.3 billion people at risk in 2006, causing nearly a million deaths, mostly of children under 5 years [1]. Malaria was endemic in 109 countries around the world in 2008 (World Health Organization, [1]). Infection in human is due to any of the four *Plasmodium* species, among which *P. falciparum* (Pf) is the most virulent as it can lead to fatal cerebral malaria, while *P. vivax* (Pv), *P. ovale* (Po) and *P. malariae* (Pm) cause more benign types of malaria. During the course of its infection, multiple plasmepsins have been identified in the digestive food vacuole (DV) of *P. falciparum* [2]. Four of these enzyme homologs appear to function in this organelle: plasmepsin-I (PM-I), plasmepsin-II (PM-II), plasmepsin-IV (PM-IV), and histidine aspartic protease (HAP). The digestion of hemoglobin probably occurs by a semi-ordered process involving the sequential action of different proteases [3]. PM-I and PM-II are the best characterized enzymes in the family and both are capable of cleaving native hemoglobin between phenylalanine and leucine residues located at positions 33 and 34 on the alpha-globin chain [4]. These residues are located in a conserved domain known as the hinge region, which is believed to be crucial in stabilizing the overall structure of hemoglobin. In a previous study [5], a large shift between the N-terminal sub-domain and the C-terminal sub-domain of PPM-II in comparison with the mature PM-II structure was reported to open the active-site cleft, preventing the formation of a functional aspartic proteinase active site. In the structure of PPM-II, the tip of the active-site flap (Asn 76–Thr 81) was not

defined, possibly due to the empty and severely distorted active site, or to the lack of intermolecular contacts. This lack of information yields an incomplete picture of the active site of the enzyme for rational design study. Therefore, we would like to crystallize the PPM-II in a different crystal form whose "active-site flap" is visible.

MATERIALS AND METHODS

Expression and Purification

The recombinant PPM-II construct used in this experiment contains the last 48 amino acids of the pro-segment at the N-terminus and the whole mature sequence at the C-terminus. This enzyme was expressed using the T7 dependent pET3a vector and was transformed into *Escherichia coli* expression cell line BL21(DE3) pLysS. The PPM-II enzyme was purified as described by Siripurkpong, P. [6] with some modifications. For the first Q-Sepharose anion-exchange chromatography, PPM-II was eluted with a gradient of 0–0.4 M NaCl in 20 mM Tris pH 8.0 buffer containing 6M urea. The resulting PPM-II was refolded by a rapid mixing with ten volumes of the refolding buffer (20 mM Tris-HCl pH 8.5, 10% glycerol, and 1 mM EDTA). The correctly folded enzyme was loaded into the second Q-Sepharose column and the proteins were eluted with a gradient of 0–0.3 M NaCl in 20 mM Tris pH 8.5 buffer. The quality of the refolded PPM-II sample was checked through its auto-activation activity. In a small scale purification, the mature forms of PPM-II were generated by addition of 10% volumes of 1 M sodium acetate, pH 4.5 to the sample and then incubated for 24 hours at room temperature. The reaction was stopped with 15% volumes of 2 M Tris-HCl pH 8.5. The activated samples were centrifuged at 13,000 rpm for 10 min and the resulting pellet was separated from the supernatant. Autocatalytic processing of PPM-II was monitored by observing the conversion of the zymogen (43 kDa) to mature form (37 kDa) in 12.5% SDS-PAGE. Fractions with complete processing were pooled and dialyzed against the buffer containing 50 mM NaCl.

Crystallization

PPM-II was concentrated to ~17 mg/ml and was used for crystallization trials using 60 screening conditions with the microbath technique [7], of which the conditions contained various salts (ammonium acetate, calcium acetate, KCl, $CaCl_2$, Li_2SO_4, $MgCl_2$, NaCl, sodium formate, Na,K-tartrate and zinc acetate, buffers (Acetate pH 4.6, citrate pH 5.6, imidazole pH 6.5, bis-Tris pH 6.58, HEPES pH 7.5 and Tris pH8.5), and PEG 4000. The PPM-II protein was crystallized using 1 µl of protein solution and 1 µl of precipitant solution. For each positive hit from the screen, a typical optimization was set up in two dimensions around the original condition to vary salt and PEG concentrations in order to reproduce the crystals as well as to obtain single crystals of high quality.

Data collection

Single crystals of PPM-II obtained from the optimization were exposed to the X-rays and checked for X-ray diffraction. The crystals were flash-cooled in a cold nitrogen gas stream prior to data collection. A crystal from conditions containing 200–300 mM ammonium acetate 20–25% PEG 4000, 0.1 M HEPES pH 7.5 diffracted X-rays to 2.75 Å resolution. X-ray diffraction data of this crystal was collected on an R-AXIS IV^{++} image plate system (Rigaku/MSC) using 10 min exposure time for each 1° oscillation to cover a total of 100° rotation. The X-ray radiation was generated by a

Rigaku RU-H3R rotating-anode X-ray generator (λ=1.5418 Å). All data were processed and scaled with the CrystalClear/d*TREK program suite [8].

Determination of initial phases

The initial phases of this new crystal form of PPM-II were solved by molecular replacement using PPM-II structure from another crystal form (unpublished data) as a search model in the *CCP4* version 6.2 program suites [9]. The rotation and translation functions gave clear solutions in the $P2_12_12_1$ space group for two molecules per asymmetric unit with a correlation coefficient of 53.5 % and an R-factor of 42.9 % in the resolution range of 200–2.75 Å. Electron density maps calculated using the CNS program [10] are being used to guide adjustment of the structural model into the electron density using the O program [11].

RESULTS

Purification and auto-activation test

Following the purification of PPM-II, the enzyme was concentrated to about 17 mg/ml for subsequent crystallization experiments. Correct protein folding was verified through auto-activation ability at an acidic pH. SDS-PAGE analysis of the auto-processing samples suggested that the purified PPM-II was of good purity and quality (Fig. 1). The change of molecular size from ~43 kDa to ~37 kDa corresponding to the conversion of PPM-II to its mature counterpart was an indication of native protein folding with proper activity. In addition, it had high protein solubility both before and after the auto-activation assay.

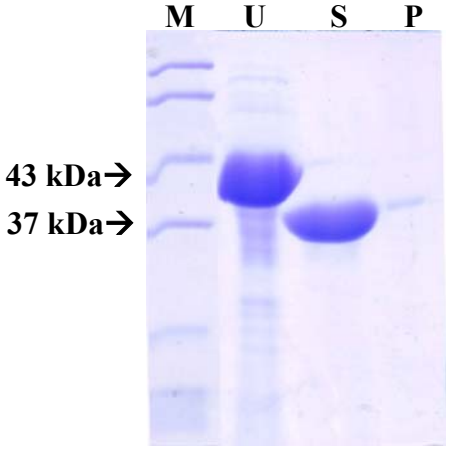

Fig. 1 SDS-PAGE analysis of PPM-II samples undergoing auto-activation test: M, molecular-weight markers; U, un-activated PPM-II; S and P, soluble and pellet fractions of activated PPM-II, respectively.

Crystallization

Following the crystallization trials using our 60 screening conditions, crystals were observed in various conditions, which were further optimized until well defined crystals were obtained (Table 1, Fig. 2). Some of these crystals were exposed to the X-ray beam in order to test for X-ray diffraction.

Table 1 Positive crystallization conditions for PPM-II obtained with the microbatch crystallization method [7]**.**

200 mM [Salts]	**0.1 M [Buffer]**
$NH_4O_2CCH_3$	Bis-Tris pH 6.6
$NH_4O_2CCH_3$	Hepes pH 7.5
NaO_2CH	Bis-Tris pH 6.6
Na, K tartrate	Bis-Tris pH 6.5
KCl	Hepes pH 7.5

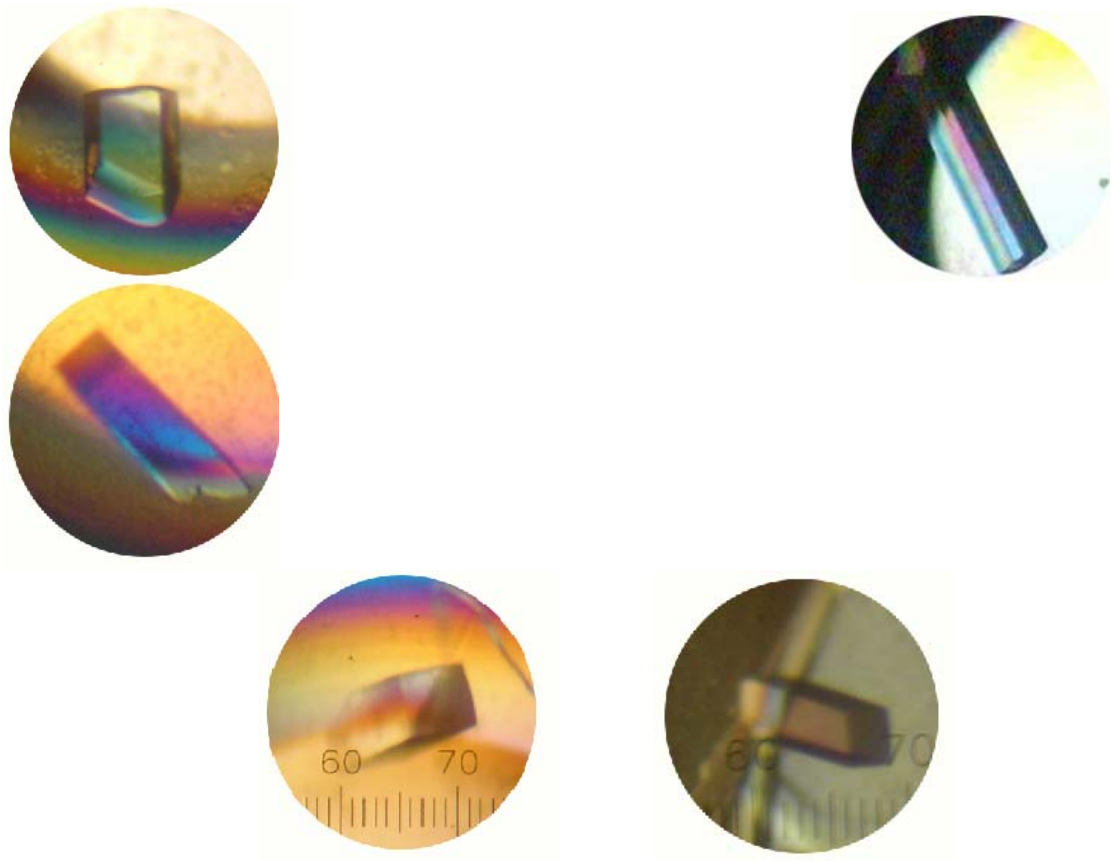

Fig. 2 Well defined PPM-II crystals obtained from optimized conditions using the microbatch crystallization method**.**

X-ray diffraction

Good-looking crystals from the optimization were flash-cooled for X-ray diffraction test. Among several of these, a crystal obtained from $NH_4O_2CCH_3$-containing condition diffracted X-rays beyond 2.8 Å. X-ray diffraction data were collected and processed with the statistics shown in Table 2.

Table 2 Data-collection statistics for X-ray data collection of PPM-II crystal.

	PPM-II
Wavelength (Å)	1.5418
Resolution limit (Å)	37.28–2.75
Completeness (%)	96.4
R_{merge}	0.10
$\langle I/\sigma(I) \rangle$	3.9
Mosaicity (°)	0.76
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 83.36 b = 83.36 c = 115.5 α =90° β = 90° γ = 90°
Total No. of reflections	21,558
Total No. of reflections used	20,723
V_M ($Å^3$ Da^{-1})	802,908
Solvent content (%)	47.3
No. of molecules per ASU	2

Structure determination and refinement

For the $P2_12_12_1$ crystal form, the molecular replacement search gave a correlation coefficient of 53.5% and R-factor of 42.9% for two molecules per asymmetric unit. The solution produced no overlapping of molecules with good agreement on most parts to the sigma-weighted $2F_o–F_c$ electron density map. The structural model of PPM-II was adjusted and rebuilt according to its electron density obtained from the X-ray diffraction data set. Iterative model building and refinement are being carried out to obtain the best representative model for the electron density of this crystal form.

CONCLUSIONS

New crystallization conditions that produced PPM-II crystals in various morphologies were identified. A crystal from a new set of conditions diffracted X-rays to 2.75 Å resolution. X-ray data processing indicated that the crystal belonged to the orthorhombic space group $P2_12_12_1$. Currently, iterative rebuilding and refinement of this model is in progress.

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Proceedings 7

AMPLIFICATION OF TROPOMYOSIN FROM FRESHWATER PRAWN (*MACROBRACHIUM ROSENBERGII*)

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ABSTRACT

Food allergies are a major health problem and are caused by an adverse immune response to food allergens. Such allergens are usually proteins that react with IgE antibodies to induce an allergic reaction. Among food allergens, shellfish are the frequent cause of adverse food reaction. Shellfish which include crustaceans such as crab, shrimp and crayfish, and mollusca such as squid, octopus, oyster and mussel, are common ingredients in many dishes. One of the major allergens in crustaceans was identified to be a heat stable muscle protein, tropomyosin. Tropomyosin is a cross-reacting allergen among invertebrates and is assumed to be the major allergen common among decapod crustaceans. However, the sequence of the tropomyosin from the freshwater prawn, *Macrobrachium rosenbergii* is not yet available. The present work reports a successful PCR amplification of the tropomyosin cDNA fragment of 900 bp of the sequencing is underway. Our planned studies will include cloning of a full-length gene of tropomyosin from *M. rosenbergii* and characterization of the protein for understanding their evolution.

Keywords: tropomyosin, allergen, freshwater prawn, *Macrobrachium rosenbergii*

INTRODUCTION

Food allergy is an immunological response to a food protein. This is a reaction which involves an IgE mediated type I hypersensitive reaction. When a protein or immunologically active protein fragment crosses the mucosal membrane surfaces, and is processed by macrophages and lymphocytes, this can lead to stimulation of the production of protein-specific IgE antibodies. The induced antibodies have the ability to bind to the surfaces of mast cells and basophils. Subsequent interactions or exposure to allergen can cross-link two or more cell-membrane-bound IgE antibodies, leading to release of preformed mediators as well as synthesis and release of newly formed mediators [1]. Food-induced allergic reactions are responsible for a variety of symptoms involving the skin, gastrointestinal tract, and respiratory tract and might be a cause of life-threatening anaphylaxis. It is estimated that about 3-4% of adults and about 6% of young children are affected by food allergies and the prevalence is still rising [2-3]. Two invertebrate groups, crustacean (shrimp, crabs, lobster, and crawfish) and mollusca (snails, mussels, oysters, scallops, clams, squid and octopus), are generally termed as “shellfish”. They are common constituents in the diet of many populations. Shellfish is one of the most common food allergens.

Tropomyosin is a heat stable major allergen in shellfish. It is a myofibrillar protein composed of two identical subunits with molecular mass of 35-38 kDa [4-5].

The protein molecule has highly conserved amino acid sequence. Tropomyosin is regarded as a pan-allergen that is implicated in IgE cross-reactivity among various invertebrates including crustaceans (decapod), mollusks, mites and cockroaches [6-8]. Tropomyosin cDNA sequences from black tiger prawn (*Penaeus monodon*), kuruma prawn (*Penaeus japonicus*), pink shrimp (*Pandalus eous*), king crab (*Paralithodes camtschaticus*), snow crab (*Chionoecetes opilio*), and horsehair crab (*Erimacrus isenbekii*) were elucidated by a cDNA cloning technique [9]. However, the sequence of tropomyosin from the freshwater prawn, *Macrobrachium rosenbergii* is not yet available. We report here the successfull PCR amplification of a tropomyosin cDNA fragment from *M. rosenbergii.*

MATERIALS AND METHODS

Freshwater prawn sample

M. rosenbergii (freshwater prawn) were purchased from Banglane, Nakhon-Pathom province. The muscle was dissected out and immediately frozen in liquid nitrogen and then stored at -80°C for molecular analysis.

Total RNA extraction

Total RNA was prepared from the muscle using Total RNA Extraction mini kit for Tissue (RBC) and was used as a template for the amplification of the tropomyosin fragment.

Primers design

Primers were designed based on the conserved amino acid and nucleotide sequences of the tropomyosin from other shrimp species using Clustal W computer program (Fig. 1 and Fig. 2, respectively).

Synthesis and amplification of cDNA fragment

Reverse transcription (RT) and Polymerase Chain Reaction (PCR) were performed using the Titan One Tube RT-PCR System (Roche). Amplifications were carried out by using the pairs of specific primers. The PCR conditions were as follows: reverse transcription at 50^{o}C for 30 min, initial PCR activation step at 94^{o}C for 2 min, followed by 35 cycles of 94^{o}C for 30 sec, 50^{o}C for 30 sec and 68^{o}C for 45 sec, and then a final extension step was performed at 68^{o}C for 7 min. Each PCR reaction (50 μl) was conducted in a Mastercycler (GENE AMP PCR SYSTEM 2400, perkin elmer), in a reaction mixture using 1 μg of total RNA, 200 μM of each dNTP, RT-PCR buffer (Tris-HCl, DMSO, 1.5 mM $MgCl_2$ pH 7.5), 10 mM forward (5'-CAGGCGATGAAGCTGGAGAAG-3') and reverse (5'-TTAGTAGCCAGACAGTTCGCTGA-3') primers, and 1 μl of Titan One-Tube RT-PCR enzyme mix (including AMV reverse transcriptase and Expand High Fidelity enzyme). The PCR products was kept at -20^{o}C, or immediately used for electrophoresis.

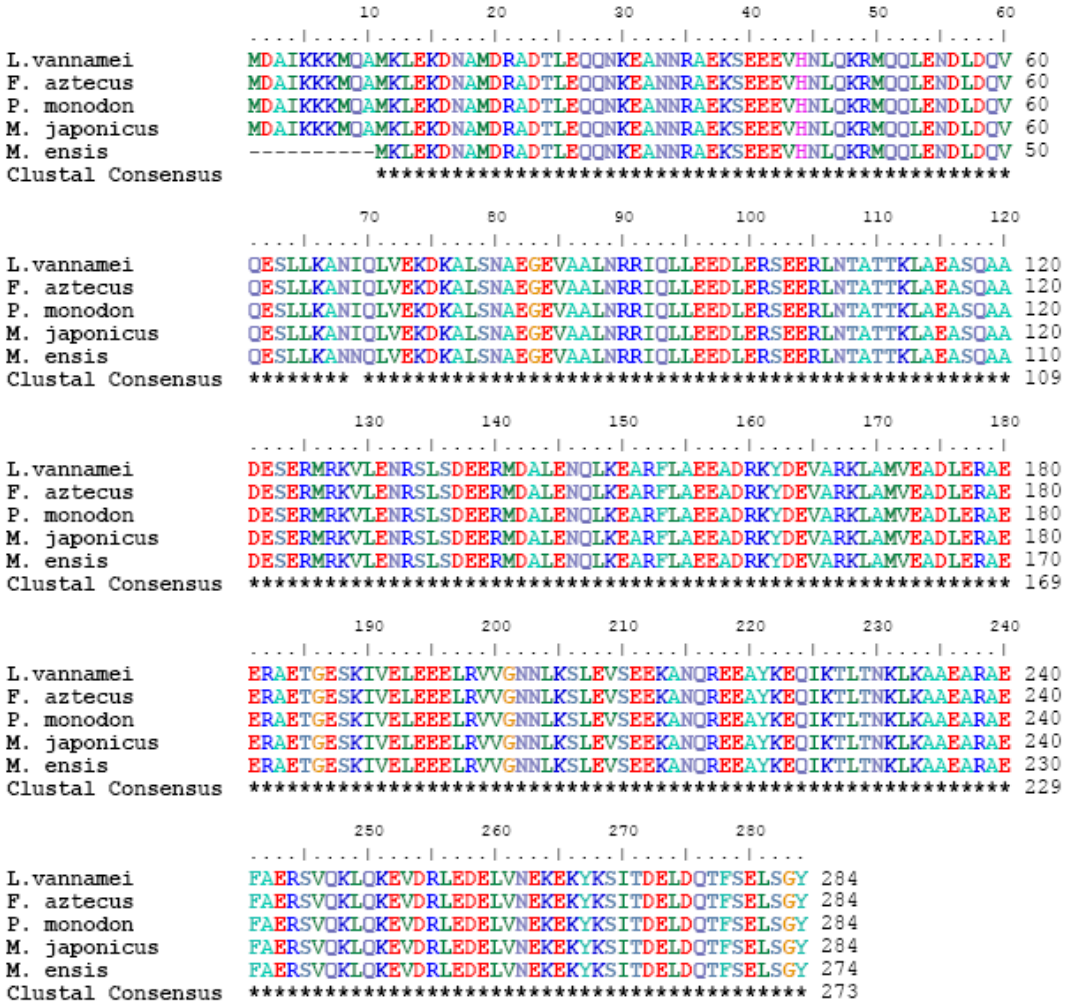

Fig. 1 Amino acid sequence alignment of shrimp tropomyosins. The shrimp names and their corresponding GenBank accession numbers are as following: *Litopenaeus vannamei* (white shrimp), ACB38288; *Farfantepenaeus aztecus* (brown shrimp), AAZ76743; *Penaeus monodon* (Black tiger shrimp), BAF47262; *Marsupenaeus japonicus* (Kuruma shrimp), BAF47263; *Metapenaeus ensis* (Greasyback shrimp or sand shrimp), Q25456. The numbers above the amino acid sequences indicate the position in the alignment of the amino acid residue. The (-) indicates a gap introduced into the amino acid sequence to allow for the maximum degree of identity in the alignment. Identical amino acid residue positions are indicated by asterisks.

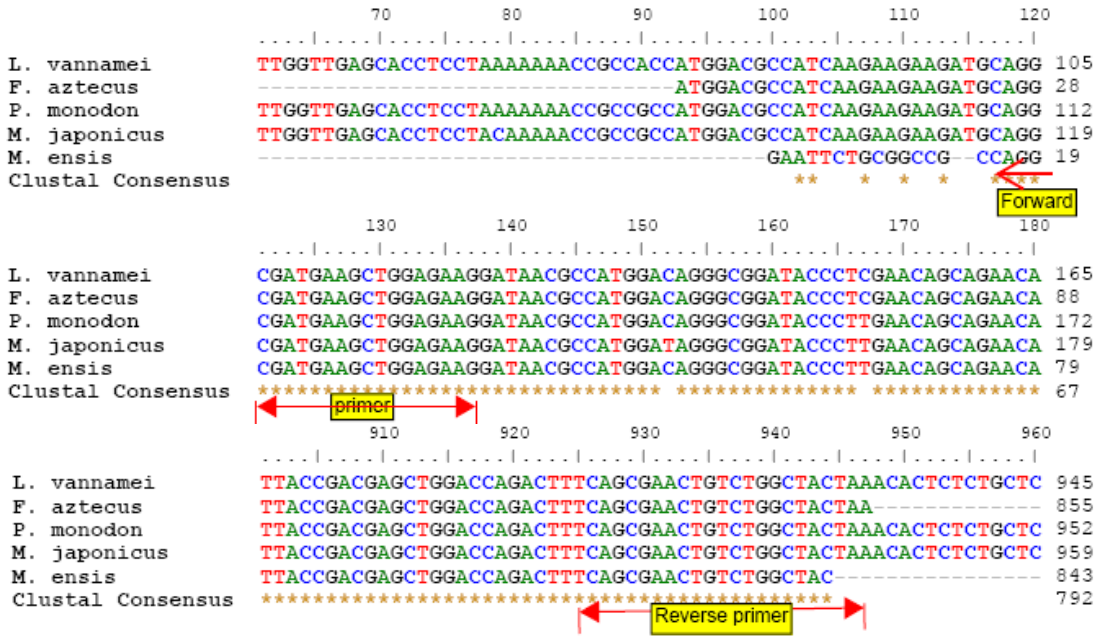

Fig. 2 Nucleotide sequence alignment of shrimp tropomyosins. The shrimp names and their corresponding GenBank accession numbers are as following: *Litopenaeus vannamei* (white shrimp), EU410072; *Farfantepenaeus aztecus* (brown shrimp), DQ151457; *Penaeus monodon* (Black tiger shrimp), AB270629; *Marsupenaeus japonicus* (Kuruma shrimp), AB270630; *Metapenaeus ensis* (Greasyback shrimp or sand shrimp), U08008. The numbers above the nucleotide sequences indicate the position in the alignment of the nucleotide residue. The (-) indicates a gap introduced into the nucleotide sequence to allow for the maximum degree of identity in the alignment. Identical nucleotide residue positions are indicated by asterisks.

RESULTS

The DNA fragment of tropomyosin gene of about 900 bp in length was amplified by PCR from freshwater prawn (Fig. 3). This fragment will be cloned and sequenced for investigate the open-reading frame (ORF) of tropomyosin from freshwater prawn.

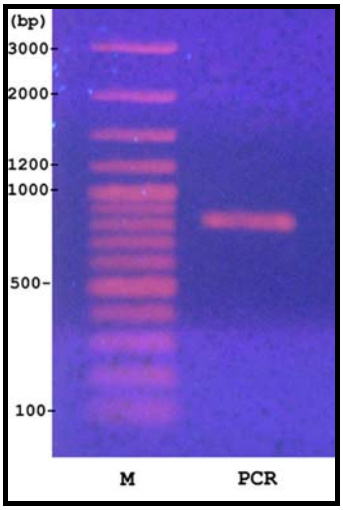

Fig. 3 PCR amplification of tropomyosin gene from freshwater prawn. M, DNA markers; lane 1, PCR amplification products.

CONCLUSION AND DISCUSSION

The present work reports the successful PCR amplification of a tropomyosin cDNA fragment of 900 bp from *M. rosenbergii* and the DNA sequence of the amplified product will be verified soon. The Open-reading frame (ORF) of tropomyosin from *M. rosenbergii* was obtained by RT-PCR. 5' and 3' rapid amplification of cDNA ends (RACE) will be investigated in the future for further understanding the evolution of the protein.

ACKNOWLEDGEMENTS

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Proceedings 8

EXPRESSION AND PHOSPHATASE ACTIVITY OF A NOVEL PROTEIN PHOSPHATASE TYPE2C AP2C IN *ARABIDOPSIS*

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ABSTRACT

In order to survive, plants need to develop a proper signaling for responding to specific stimuli. Protein phosphorylation is the principal strategy that plants use to cope with environmental stress. In *Arabidopsis*, protein serine/threonine phosphatases type 2C (PP2Cs) play important roles in stress responses. Here, an *Arabidopsis* PP2C, named AP2C was analyzed. The expression of gene coding for AP2C was performed by inducing with biotic and abiotic stress factors. The phosphatase activity of AP2C was found to be Mg^{2+} dependent manner and was abolished by EDTA. Glycine to aspartic acid substitution at the position 178 of AP2C, where metal coordination in the catalytic part of the protein is, was found to reduce approximately 70% of MPK6 phosphatase activity of the wild type.

Keywords: PP2C, AP2C, Arabidopsis, protein expression, phosphatase activity

INTRODUCTION

AP2C is a putative *Arabidopsis* protein phosphatase type 2C (PP2C) identified *in silico* by phosphatase catalytic domain of the protein [1]. It is encoded by a nuclear gene AT1G07160 and comprises 380 amino acids with molecular weight of ~40.7 kDa (www.arabidopsis.org). The protein structure of AP2C contains predicted chloroplast transit peptide (cTP) of 67 amino acids and a mitogen-activated protein (MAP) kinase interaction motif (KIM) in the N-terminus, and 11 catalytic subdomains with metal-binding site (Mn^{2+} or Mg^{2+}) in the C-terminus [2, 3]. The protein is grouped in cluster B of *Arabidopsis* PP2C according to amino acid sequence homology to MP2C, the alfalfa PP2C (*Medicago saliva*).

A number of evidence suggested that the AP2C protein might be involved in stress response signaling in *Arabidopsis*. First, its close homolog MP2C plays an important role as the regulator of signal transduction in response to environmental stresses. The MP2C has been identified as a negative regulator of stress-induced MAP kinase pathways in yeast and plants [4, 5]. Second, the AP2C protein has been shown to be highly upregulated in catalase-deficient plants CAT2HP1 exposed to high light stress [6]. Microarray data has shown that the AP2C protein transcript is induced by several biotic and abiotic stresses (AtGenExpress). Thus the AP2C protein is interesting as a possible component of stress response signaling cascade. However, its function has not been identified. Characterization of the function, expression and enzymatic activity of AP2C could possibly help regulate stress response in any specific way.

MATERIALS AND METHODS

Histochemical GUS staining and microscopy

Histochemical GUS reporter assay was performed according to Jefferson et al. [7]. Plant tissues were incubated for 12-18 h at 37°C in staining solution containing 100 mM sodium phosphate (pH 7.0), 0.5 mM $K_4[Fe(CN)_6]$, 0.5 mM $K_3[Fe(CN)_6]$, 0.01% v/v tween 20, and 0.5 mM X-Glc. The tissue was observed under Zeiss Axioplan2 microscope with Nikon Coolpix4500 camera.

RNA extraction and transcription

Total RNA from plant leaves was isolated using Tri®Reagent (Sigma, T9424). Approximately 25 mg of leaves were frozen in liquid nitrogen and then ground with a cold pestle and a mortar. cDNA was produced from total RNA. RT-PCR was performed using the following primers: *AP2C* fwd: 5'-AGGAGATCCCAAACAGGCAATA-3', *AP2C* rev: 5'-TATCCACATAACCGCCCGAGCTC-3', *ACT3* fwd: 5'-ATGGTTAAGGCTGGTT TTGC-3', and *ACT3* rev: 5'-AGCACAATACCGGTAGTACG-3'.

Creation of a loss-of-function mutation of AP2C

The cDNA of *AP2C* was mutated using the QuikChange Site II - Directed Mutagenesis kit (Stratagene) to introduce the single amino acid exchange glycine to aspartic acid at position 178. PCR using PfuTurbo DNA polymerase (Stratagene) was performed with 18 cycles of denaturation at 95°C 30 second, annealing at 55°C for 1 min, polymerization at 68°C for 5 min and 68°C for 10 min. Following cycling, the product was treated at 37°C for 1 h with DpnI endonuclease. The nicked PCR product was then transformed into XL1-Blue Supercompetent *E.coli* cells (Stratagene) according to the instruction. The mutated constructs were verified by DNA sequencing and cloned into pGEX-4T-1 vector.

Expression and purification of the recombinant AP2C Protein

Recombinant wild type and lof-AP2C were expressed as glutathione *S*-transferase (GST) fusion proteins. The transformed *E. coli* DH5α cell containing the cAP2C was grown at 37°C in 200 ml of Luria Bertani medium containing 50 μg/ml ampicillin until the absorbance at 600 nm reached 0.8. Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was incubated at 28°C for an additional 2 h, and then the bacterial cells were harvested by centrifugation. The cells were sonicated at 50/50 cycles for 10 sec 3 times. The GST-fusion proteins were purified by GST-sepharose bead (Amersham Biosciences) affinity chromatography. The protein concentrations were determined with the Bio-Rad detection system using BSA as a standard, and the purity of the protein fractions was determined by Coomassie Brilliant Blue staining after 10% SDS-PAGE.

Phosphatase assay

Phosphatase activity was measured using [^{32}P]-labeled GST-MPK6-K92M (MPK6-lof) as a substrate and incubated with 0.1 μg/reaction of each PP2C at 30°C for 30 min in triplicates [5]. The GST-MPK6 K92M was labeled by GST-MKK2 T220E, T226E (MKK-gof) [8].

RESULTS

The expression information of AP2C *in silico* was retrieved from AtGenExpress database. The data demonstrated that the expression of AP2C could be induced by biotic and abiotic stress factors including pathogens and oxidative stresses such as *Psudomonas syringae*, flg22 elicitor, high salt, heat, UV-B and methyl viologen (Fig. 1).

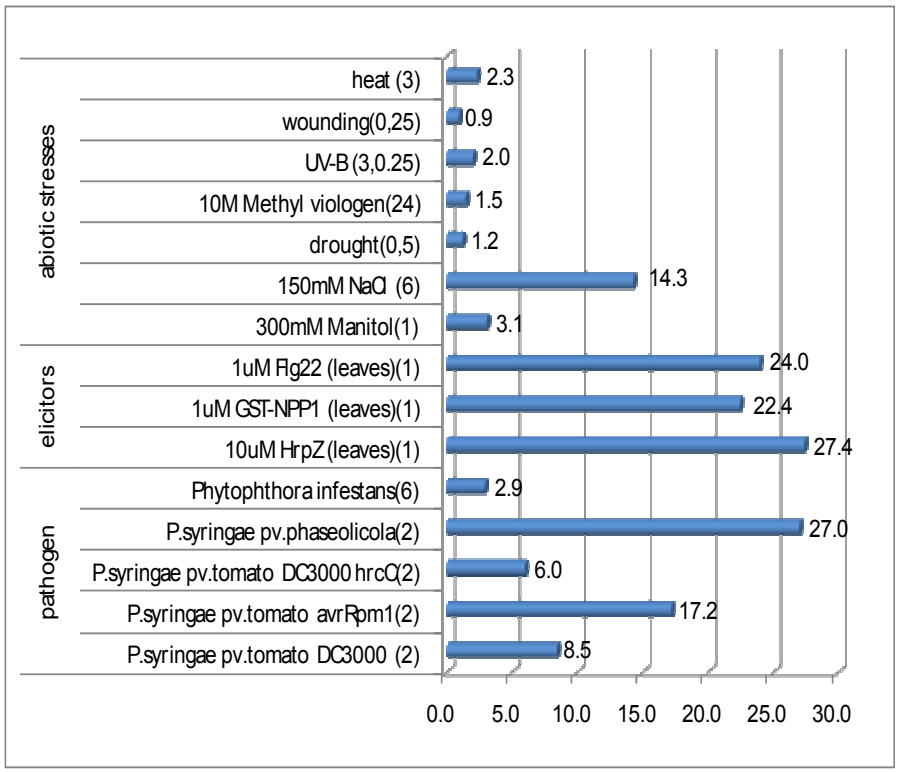

Fig. 1 Expression of AP2C under biotic and abiotic stresses *in silico*. The data were derived from AtGenExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp) developed by Christian K. Widmer. Only high fold changes of expression of AP2C are reported.

AP2C::GUS reporter analysis

To identify the inducers and to investigate the expression conditions of AP2C, promoter activation was studied using transgenic plants with promoter::GUS reporter gene activity in the background of WT plants *(AP2C::GUS/WT)* and CAT2HP1 plants *(AP2C::GUS/CAT2HP1)*. Whereas no GUS staining could be detected in the untreated AP2C::GUS/WT seedlings (Fig. 2A), it could be detected at the hydratode point of leaves and at root of seedlings after treatment with cellulase (Fig. 2B). On the other hand, the GUS staining was observed in AP2C::GUS/CAT2HP1 plants even without treatment (Fig. 2C). The GUS staining was stronger in *AP2C::GUS*/CAT2HP1 plants than in *AP2C::GUS*/WT plants after induction by cellulase. The induction was observed at the hydratode point of leaves, in stomata, in roots and around wounded area (Fig. 2D). Taken together, AP2C expression was found to be induced upon stresses. GUS staining could be detected where ROS was accumulated.

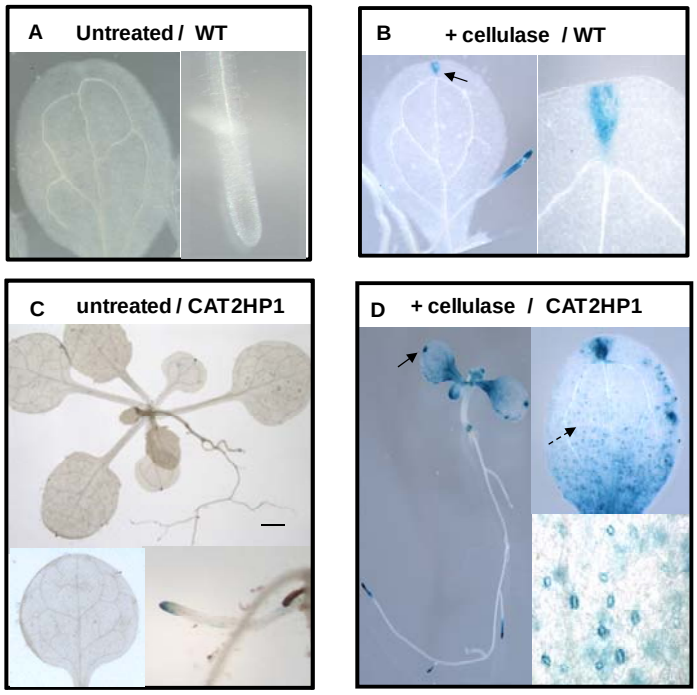

Fig. 2 Comparison of GUS activity between *AP2C*::GUS expression in wild type (AP2C::GUS/WT) and catalase-deficient plants (*AP2C*:GUS/ CAT2HP1). (A) untreated WT plant (B) cellulase-treated WT plant (C) untreated CAT2HP1 plant (D) cellulase-treated CAT2HP1 plant.

AP2C transcript was induced upon stresses.

Two-month old plants and five-day old seedlings were subjected to different stresses including 100 nM flg22, UVC irradiation for 3 h, wounding for 3 h, 0.1 mg/ml cellulase for 30 min or 100μg/ml catechin for 3 h. RNA was extracted from the frozen leaves and RT-PCR was performed. While no *AP2C* transcript could be detected in untreated plants, the *AP2C* transcript was strongly induced by flg22, cellulase, wounding, catechin and UVC respectively (Fig. 3).

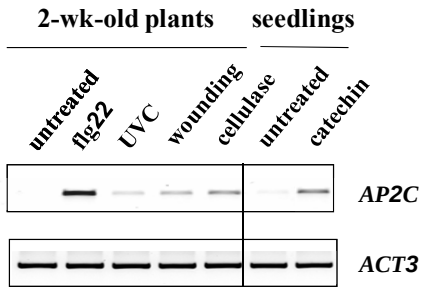

Fig. 3 *AP2C* transcript was induced upon stresses**.** Plants were stressed by: adding 100nM flg22 for 3 hr, spraying with 0.1 mg/ml cellulase for 30 min, UVC irradiation for 3 h, adding catechin for 3 h and wounding for 3 h before freezing leaves for RNA extraction. RT-PCR was performed from isolated total mRNA. ACT3 –control.

Analysis of phosphatase activity of AP2C

Plasmid pGEX-AP2C-wt and pGEX-AP2C-lof were used in this study. The loss-of-function mutation was created according to the G180D mutation in *ABI1* gene in the conserved motif involved in metal binding and catalysis [3, 9]. The result showed that the MPK6 phosphatase activity of GST-AP2C-lof was 70% reduced as compared to that of the GST-AP2C-wt protein activity (Fig. 4).

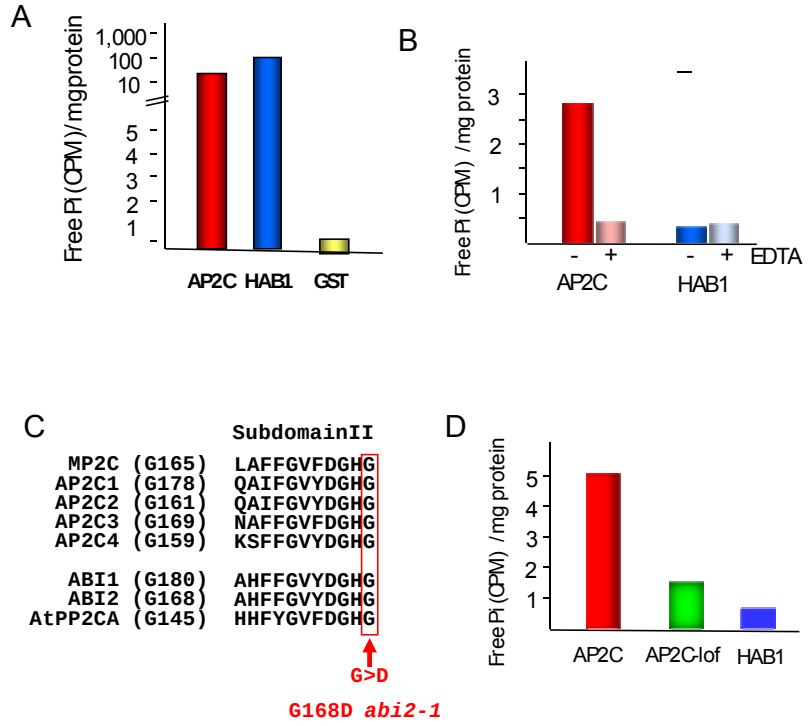

Fig. 4 Recombinant AP2C phosphatase activity. (A) AP2C and HAB1 proteins had phosphatase activity toward casein substate. (B) phosphatase activity of AP2C on MPK6 substrate was abolished by adding 10mM EDTA. (C) loss-of-function mutation of AP2C was created by substitution of glycine 178 to aspartic acid. D) MPK6 phosphatase activity of AP2C-lof reduced approximately 70% of the wide-type activity.

DISCUSSION

AP2C is a putative *Arabidopsis* protein Ser/Thr phosphatase type 2C (PP2C) group B. Since AP2C has amino acid sequence similar to MP2C, we hypothesised that the functions of AP2C might be related to stress response. MP2C expression is transiently induced by wounding after wound-induced activation of SAMK (stress-activated MAPK pathway that is activated by cold, drought, touch and wounding) in plants [4].

AP2C is an inducible protein. Its expression is highly specific and very low under normal growth condition according to microarray analysis (AtGenExpress) and promoter *AP2C*::*GUS* reporter analysis. However, the expression of AP2C is induced under both biotic and abiotic stress conditions such as flg22, cellulase, pathogens, UVC, salinity, wounding and paraquat. Moreover, the location of AP2C expression is at the site of ROS accumulation, for example at the hydratode point of leaves or around wounded area. Higher expression of AP2C is found in situation with higher ROS production, for example in catalase deficient plants, CAT2HP1. AP2C is 52-fold up-regulated in CAT2HP1 plants upon exposure to high light stress [6].

Phosphatase activity of AP2C is specific to MPK6 substrate. Its activity depends on Mg^{2+} and is sensitive to EDTA. Glycine at residue 178 is essential for catalytic activity of the enzyme, and its substitution to aspartic acid reduced 70% of the wild-type phosphatase activity. Glycine 178 of the protein was reported as the metal coordinating residues of the catalytic part of phosphatase activity.

ACKNOWLEDGEMENTS

The CAT2HP1, CAT2HP2 and empty vector control PTHW seeds were kindly provided by Dr.Frank Van Breusegem, Belgium, for use in this study.

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Proceedings 9

A NEWLY THERMOSTABLE LECTIN FROM *Kaempferia parviflora Wall. Ex Baker*

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ABSTRACT

The products from *Kaempferia parviflora* are herbal and are commercially popular at the present time. These products are available in several forms such as capsule and powder or beverage. Previous studies have focused on the organic compound that has the bioactivity. This study consisted of purification and characterization of lectin from *K. parviflora*. The lectin from *K. parviflora* lectin was isolated from rhizome using a combination of ammonium sulfate fraction, ConA Sepharose affinity and Sephacryls S-100 gel filtration chromatography. The molecular weight determined by SDS-PAGE was approximately 41.7 kDa. The lectin was still active at temparature up to 75 to 80°C.

Keywords: lectin, ConA sepharose, Kaempferia parviflora

INTRODUCTION

Lectins are carbohydrate-binding proteins that specifically recognize diverse sugar structures and mediate a variety of biological processes such as cell-cell and host-pathogen interactions, serum-glycoprotein turnover, and innate immune responses [1]. The lectins are widely distributed in the nature and can be found in microorganisms [2], in pluricellular animals [3]. However, plants are the main sources for purification of lectins [4].

Lectins could be valuable in biotechnological field, medical research, biology, pharmacology, and biochemistry. Plant lectins are found in many different species and in distinct organs and tissues. It is assumed that they play fundamental biological roles in defense mechanisms such as anti-insect [5, 6], mitogenic [7], and antifungal [8].

Kaempferia parviflora Wall. Ex Baker is one of the plants in the Zingiberaceae family, locally known in Thai as Kra-Chai-Dam. The rhizomes of this plant have been used for the treatment of allergy [9], and gastrointestinal disorders [10]. However, there is no report on lectin from *K. parviflora*. In this study, we purified and characterized lectin from rhizomes of *K. parviflora* and investigated its possible role as antifungal activity.

MATERIALS AND METHODS

Plant materials

Rhizomes of *K. parviflora* were purchased from the local market (Bangkok, Thailand). A voucher specimen (BK51772) is deposited at the Bangkok Herbarium (BK) of The Plant Variety Protection Division, Department of Agriculture, Thailand.

Isolation of lectin from *K. parviflora*

Fresh rhizomes (1 kg) were washed, and homogenized in a blender with 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (1:5 kg/L). The homogenate was centrifuged and $(NH4)_2SO_4$ was added to the supernatant to 80% saturation. The suspension was centrifuged, and the precipitate was resuspended in 20 mM Tris-HCl buffer, pH 7.4. The sample was then dialyzed against the same buffer and applied to ConA Sepharose fast-flow column (1.6x20 cm) installed on an AKTA prime instrument (GE Healthcare, Sweden) equilibrated with buffer A (20 mM Tris-HCl buffer, pH 7.4 containing 0.15 mM NaCl). Bound proteins were eluted with buffer B (buffer A containing 2.0 M methyl α-D-glucopyranoside). The fraction of bound proteins were collected and dialyzed against distilled water. The bound proteins were applied to a Sephacryl S-100 column (1.0x60 cm) equilibrated with 20 mM Tris-HCl buffer, (pH 7.4) containing 0.1 M NaCl, at a flow rate of 0.5 ml/min. The protein fractions were then lyophilized for further characterization by gel electrophoresis.

Determination of Protein Concentration

Protein concentrations were determined by Bradford method [11]. During the column chromatographic separations the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

Hemagglutinating activity test

Rabbit erythrocyte was obtained from National Laboratory Animal Centre Mahidol University. Fractions were tested for hemagglutinating activity by the standard method. Briefly, serial two-fold dilutions of the proteins in microtiter U-plates (50 µl) were mixed with 50 µl of a 2% (v/v) suspension of rabbit erythrocytes in 20 mM Tris-HCl buffer, pH 7.4 containing 0.15 mM NaCl, at room temperature. Precipitation of erythrocytes was assessed visually after 1 h when the blank was full sedimented.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using standard methods on the Bio-Rad Mini-Protean II system. The electrophoresis a discontinuous system with 15% separating gel (pH 8.8) and 15% stacking gel (pH 6.8) of size 7x10x0.1 cm, was performed at 20 mA per gel. The gels were stained with Brilliant Blue R concentrate for 30 min and were destained in 50% (v/v) methanol, 5% (v/v) acetic acid for 30 min or until protein bands appeared.

Effect of temparature on hemagglutinating activity and thermostability

The effect of temperature on hemagglutinating activity was determined by incubating lectin samples (pH 7) at various temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95°C) and at room temperature for 30 min, followed by an immediate chill at 4°C for 15 min. At least four replicates were done for each. Thereafter, the lectin solution was incubated at various temperatures (40, 50, 60, 70,

80 and 90°C) for 10, 20, 30, 60, 90, 120 and 150 min. The hemagglutinating activity was subsequently tested.

RESULTS AND DISCUSSIONS

The extract of *K. parviflora* rhizomes was obtained by $(NH4)_2SO_4$ precipitation. The crude protein was subjected to purification using affinity chromatography with ConA Sepharose as stationary phase. A retained protein with hemagglutination activity was eluted with 20 mM Tris-HCl buffer, pH 7.4 containing 0.15 mM NaCl with 2.0 M methyl α-D-glucopyranoside as a single protein peak. Then, gel filtration chromatography with Sephacryl S-100 column was select for a further purification. The result showed one major protein fraction. In addition, the gel filtration step gave the lectin with 40% yield and 22.5 fold purification as compared to the crude extraction step (Table 1). The fraction from Sephacryl S-100 gave a single peak with estimated molecular weight of 41.7 kDa (Fig. 1).

Table 1. Purification of the lectin from rhizomes of *K. parviflora*.

Purification step	Total protein (mg)	Total activity (HU)	Specific activity (HU/mg)	Yield (%)	Purification (fold)
Crude extract	1,958	25,600	13.07	100	1
80% $(NH_4)_2SO_4$ precipitation	1,758	24,000	13.65	94	1.04
Con A Sepharose	19.5	19,200	984.60	75	18.8
Sephacryl S-100	8.7	10,240	1,174.00	40	22.5

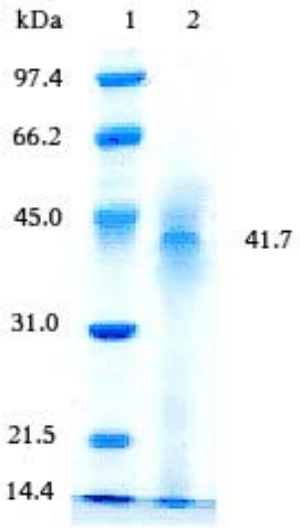

Fig. 1 SDS-PAGE analysis of *K.parviflora* lectin adsorbed on Sephacryl S-100. Lane 1, protein markers with include; phosphorylase b (97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trpsin inhibitor (21.5 kDa), and α-macroglobulin (14.4 kDa). Lane 2, purified lectin.

For the study on temperature stability, (thermostability) at 70°C, hemagglutinating activity of lectin was found to decrease to 40% of its activity at room temperature, suggesting conformational changes of lectin. At 85°C, the activity decreased to 60% compared to the activity at room temperature (Fig. 2A). This

phenomenon is quite similar to *Phaseolus vulgaris* lectin [12] which was found to be extremely thermostable at temperature around 82°C, and to *Astragalus mongholicus* lectin [13] which was still active at 65°C. Also, lectin from *Canavalia cathartica* has been reported to be active at 60°C, but its property was lost when heated at 70°C [14]. For the effect of results on hemagglutinating activity and thermostability, the results were shown in Fig. 2B. The hemagglutinating activities at 70, 80, and 90°C were found to increase with the incubation time between 30-60 min. The incubating temperature at 60°C had different pattern compared to other temperatures. The activity was rapidly decreased until fully inactive at 90 min. This result might imply that the conformation of lectin structure at 60°C might not be suitable for hemagglutination. Further studies are needed to better understand the reaction.

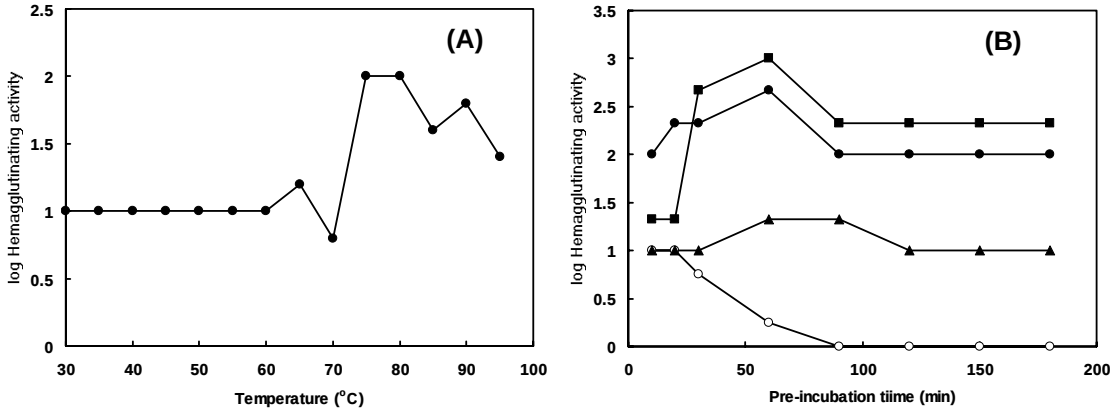

Fig. **2.** (A) Effect of temperature on the agglutinating activity of *K.parviflora* lectin and towards rabbit erythrocyte suspension each point on the line represents the average of duplicate results. Full activity (100%) corresponded to a titer of 2^4 at room temperature. (B) Thermostability of purified lectin towards rabbit erythrocyte suspension. The following temparature were tested using 20 mM Tris-HCl, pH 7.2: (○) 60°C; (▲) 70°C; (●) 80°C; and (■) 90°C. Full activity (100%) corresponded to a titer of 2^3.

CONCLUSION

Lectin was isolated from the rhizomes of *K. parviflora* by ConA Sepharose affinity chromatography and Sephacryl S-100 gelfiltration chromatography. The molecular weight of the purified lectin was approximately 41.7 kDa. The effect of temperature on hemagglutinating activity and thermostability was tested. The result suggested that lectin was still active up to 75 to 80°C.

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ISOLATION OF LECTIN FROM RHIZOMES OF *Curcuma longa* L. WITH ANTIFUNGAL ACTIVITY

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ABSTRACT

Plant lectin was isolated from rhizomes of *Curcuma longa* L. The procedures involved extraction with Tris-HCl buffer saline, pH 7.4 and precipitation with 80% ammonium sulfate. The crude extract had the specific activity of 702.621 HU/mg protein. The lectin was purified by affinity chromatography on ConA Sepharose column and eluted with 2.0 M methyl α-D-glucopyranoside. It had the specific activity of 64,566 HU/mg protein with the yield of 41.2% of total protein. The molecular weight of the lectin was estimated by 15% SDS-PAGE to be 17.3 kDa. The purified *C. longa* lectin was shown to be able to inhibit the growth of *Fusarium oxysporum*, *Exserohilum turicicum* and *Colectrotrichum cassiicola*.

Keywords: Lectin, ConA sepharose, *Curcuma longa*, Antifungal activity

INTRODUCTION

Lectins are proteins or glycoproteins of non-immune origin that specifically and reversibly bind to carbohydrates of glycoconjugates. These proteins are ubiquitous in nature, and can be found in animals, bacteria, fungi, plants and viruses. Most lectins play a crucial role in diverse biological processes, particularly in host defense mechanisms, inflammation, and metastasis [1, 2]. In recent years, plant lectins have received more attentions, mainly due to the discovery of their potent biological activities [3]. However, it has been proposed that plant lectins act as storage proteins and play a crucial role as host defense proteins [4]. Turmeric (*Curcuma longa* L.) is of special importance to humans with the discovery that its rhizome powder, when added to various food preparations, preserves food freshness and imparts a characteristic flavour. Turmeric, which belongs to a group of aromatic spices, was originally used as food additive in curries to improve the storage condition, palatability and preservation of food [5]. In this report, we describe the purification of lectin from rhizomes of *C. longa* and its anti-fungal activity.

MATERIALS AND METHODS

Plant material

Rhizomes of *C. longa* were purchased from the local market (Bangkok, Thailand). A voucher specimen (BK60689) has been deposited at the Bangkok Herbarium (BK) of The Plant Variety Protection Division, Department of Agriculture, Thailand.

Isolation of lectin from rhizomes of *C. longa*

Rhizomes of *C. longa* (400g) were homogenized in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl using a blender. The homogenate was extracted with isolation buffer (1L) by stirring overnight at 4^{o}C then filtered through cheesecloth followed by centrifugation at 14,336 g at 4^{o}C for 30 min (Kubota 6500, Japan). The supernatant was then brought to 80% saturation with ammonium sulfate, and stirred overnight at 4^{o}C. The suspension was collected by centrifugation at 14,336 g at 4^{o}C for 30 min and the supernatant was discarded. The precipitate was redissolved and desalted by dialysis with deionized water at 4^{o}C over 24 h.

Affinity chromatography

Crude solution (200 ml) was applied to a ConA sepharose (1.6x20 cm) installed in an AKTA prime instrument (GE Healthcare, Sweden) and equilibrated with buffer A (20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl). The column was washed with 100 ml of buffer A to remove unbound proteins. The bound proteins were then eluted with buffer B (buffer A containing 2.0 M methyl α-D-glucopyranoside) at a flow rate of 1.5 ml/min, and was dialyzed against distilled water for 24 h at 4^{o}C followed by freeze dried.

Protein concentration

The protein content of the samples obtained during the purification process was determined using the Bradford assay [6], using bovine serum albumin (BSA) as a standard. During the column chromatographic separation, the elution profiles of the proteins were determined by measuring the absorbance at 280 nm.

Hemagglutination assay

Two-fold serial dilution method was used for heamagglutination assay. The lectin solution (50 µl) was placed in the first well and serially diluted into the successive wells with 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. Then, 50 µl of 2% rabbit erythrocyte suspension was added to the wells. Heamagglutination was visualized in the plate after 1 h of incubate at 37^{o}C [7]. The titer was defined as the reciprocal of the highest dilution exhibiting heamagglutination, and was considered to be equal to one heamagglutination unit. Specific activity was expressed as the number of unit per mg of lectin [8].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed in reducing condition (in the presence of β-mercaptoethanol) in 15% separating gel (pH 8.8) and 5% stacking gel (pH 6.8). Prior to electrophoresis, protein samples were re-dissolved in buffer, and boiled for 5 min at 100^{o}C [9]. Electrophoresis was performed at 20 mA per gel at room temperature using a Mini-Gel Electrophoresis Unit. The gels were stained with Coomassie blue R-250 concentrate for 1 h, and then de-stained in a solution containing 10%methnol 10% acetic acid in water until the protein bands appeared.

Assay of antifungal activity

This was carried out in 90x15 mm petri plates containing 10 ml potato dextrose agar. After the mycelial colony developed, steriled blank filter paper disks (0.625 cm in diameter) were placed at a distance of 1 cm away from the rim of the mycelial colony. The samples were dissolved in 20 mM Tris-HCl buffer pH 7.4 containing 0.15 M NaCl. The plates were incubated at 25^{o}C until mycelial growth

developed peripheral disks for the control and formed crescents of inhibition around the paper disks containing samples of the purified lectin. The fungal species used in this study including *Exserohilum turicicum*, *Fusarium oxysporum*, and *Colectrotrichum cassiicola*. The petri dishes were incubated at 25^{o}C for 5-day period, at the end of which the diameter of the clear zone of inhibition surrounding the sample was measured.

RESULTS AND DISCUSSIONS

After saline extraction, ammonium sulfate precipitation and dialysis, the crude extract exhibited the specific activity of hemagglutination at 702.621 HU/mg proteins. Then, the crude protein of *C. longa* was purified by affinity chromatography using ConA Sepharose column. The bound fraction eluted with 2.0 M methyl α-D-glucopyranoside had the specific activity of 64,566 HU/mg protein (Table 1).

Table1. Purification of the lectin from rhizomes of *C. longa*.

Purification step	Total protein (mg)	Total activity (HU)	Specific activity (HU/mg)	Yield (%)	Purification (fold)
Crude extract	10,930.5	$7.68x10^{6}$	702.6	100	1
80% $(NH_4)_2SO_4$ precipitation	3131.8	$8.192x10^{6}$	2,615.7	106.7	3.7
Con A Sepharose (bound fraction)	49.1	$3.17x10^{6}$	64,566	41.2	91.9

The molecular mass of the *C. longa* lectin as estimated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was approximately 17.3 kDa (Fig. 1). In agreement with the previously published biochemical characteristics of other plant lectins which showed the molecular mass of 17 to 20 kDa [10–11].

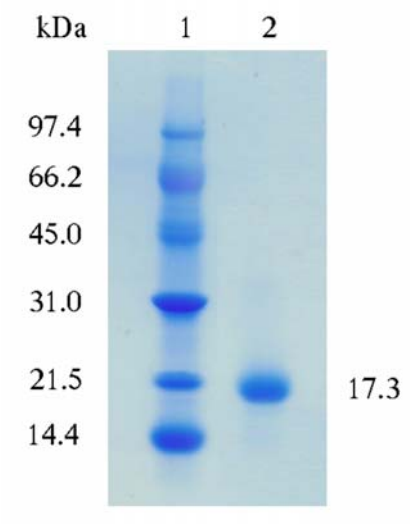

Fig. 1 Electrophoresis pattern of purified *C. longa* lectin on 15% SDS–PAGE. Lane1: molecular weight markers: phosphorylase b (97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and α-macroglobulin (14.4 kDa). Lane 2: Purified lectin from bound fraction of the ConA sepharose column.

The purified *C. longa* lectin at 47 µg and 94 µg showed antifungal activity against various fungal species of the phytopathogenic fungi namely *Exserohilum turicicum*, *Fusarium oxysporum*, and *Collectrotrichum cassiicola* (Fig. 2). Similarly, lectin from *Annona muricata* seeds inhibited the growth of *F. oxysporum*, *F. solani* and *Colletotrichum musae* at a concentration of 100 µg [12], and lectin from *Astragalus mongholicus* exterted antifungal activity against *F. oxysporum, Colletorichum* sp. and *D. turcia* at the concentration of 100 µg [13].

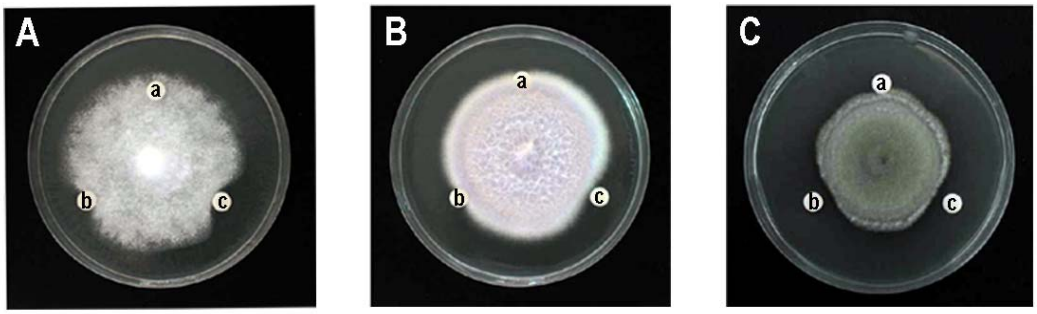

Fig. 2 Inhibitory effect of *C. longa* lectin on antifungal protein toward *E. turicicum* (A), *F. oxysporum* (B) and *C. cassiicola* (C). The negative control containing 10 µl of 20 mM Tris-HCl buffer pH 7.4 (a); 47 µg *C. longa* lectin (b); and 94 µg *C. longa* lectin (c).

CONCLUSIONS

In the present study, the purified lectin of *C. longa* was isolated by affinity chromatography using a Con A sepharose column. The protein showed molecular mass of 17.3 kDa upon SDS-PAGE. The purified *C. longa* lectin strongly inhibited the growth of *C. cassiicola*.

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A LECTIN FROM THE RHIZOMES OF *Curcuma amarissima* Roscoe AND ITS ROLE AS ANTICANCER ACTIVITY

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ABSTRACT

From rhizomes of *Curcuma amarissima* Roscoe, a lectin was purified by extraction, fractionation with 80% ammonium sulphate, followed by a combination of ConA- Sepharose affinity chromatography and Superdex G-75 gel filtration chromatography, respectively. The molecular mass of the purified lectin was 32.4 kDa as estimated by SDS-PAGE. Furthermore, *C. amarissima* lectin showed cytotoxic effect against breast cancer cell lines at IC_{50} about 21.18 μg/ml.

Keywords: Lectin, ConA–Sepharose, *Curcuma amarissima*, anticancer activity

INTRODUCTION

Lectins are proteins in the group of glycoproteins or oligomeric proteins from non-immune origin with one or more sugar-binding site(s) per unit, and it binds reversibly with specific sugars [1]. Lectins were found in all living organisms, ranging from viruses through bacteria and plants to animals [2], and were most found in the Plantae kingdom where they can be found in seeds, leaves, barks, bulbs, rhizomes, roots and tubers depending on the plant species [3–7]. Lectins have many biologically significant activities such as the ability to agglutinate cells, for examples, red blood cells, lymphocytes, fibroblasts, spermatozoa, fungal, bacterial, and plant cells [8–10]. Such vast biological activities have *rendered lectins* numerous applications in diverse fields of clinical interest, such as typing of blood cells, drug delivery, mitogens, and as epidemiological and taxonomic markers of specific microorganism [11].

Several works have previously been reported on Curcuma plants, for examples, the studies of their effects on bladder cancer cells [12], and antitumor effect of β-elemene isolated from *Curcuma wenyujin* [13]. However, the lectin from Curcuma plants was barely mentioned. In 2007 a mannose-binding lectin was found in Zingiberaceae member, *C. Zedoaria* Rosc and was identified to be similar to a mannose-binding lectin from *Epipactis heleborine*, a member of the Orchidaceae [14]. Moreover, Sangvanich and colleagues [15] reported in 2007 that hemagglutinating activity of Curcuma plants on Khamindum or *C. amarissima*, a member of Zingiberacea family which was used to treat amoebic dysentery, enteritis, and vermicide. However, there is no report of lectin from *C. amarissima*. This study addresses the purification of lectin from the rhizomes of *C. amarissima* and its anti-cancer activity.

MATERIALS AND METHODS

Plant material

Fresh rhizomes of *C. amarissima* were purchased from Jatujak market, Bangkok, Thailand.

Extraction of lectin

One kilogram of crushed and washed *C. amarissima* rhizomes were blended in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (1.5 kg/L), stirred overnight at 4 ^{o}C and filtered through cheesecloth. The filtrate was centrifuged at 22,400 g for 30 min and the supernatant was precipitated with 80% ammonium sulfate saturation. Subsequently, it was centrifuged at 22,400 g for 30 min and the precipitated was dissolved in the same buffer, dialyzed and freeze dried.

Purification of lectin

The freeze dried sample was dissolved in de-ionized water and centrifuged at 22,400 g for 10 min. The supernatant was loaded on a ConA-Sepharose column (1.6 x 20 cm). The unbound was eluted with equilibrating buffer (20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl), and the bound proteins were eluted with 2.0 M methyl-α-D-glucopyranoside in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl. The samples from the ConA–Sepharose column was loaded on a Superdex G-75 column (1.6 x 60 cm), and eluted with 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl.

Protein determination

The protein concentration was determined following the Bradford assay [16] using bovine serum albumin as a standard. Absorbance was measured at 595 nm. During the column chromatographic separations the elution profiles of proteins were determined by measuring the absorbance at 280 nm.

Hemagglutination assay

Serial two–fold dilutions of purified lectin in 20 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (50 µl) were incubated with 50 µl of rabbit erythrocyte suspension in U-shaped microtiter plates and the agglutination was stored at room temperature for 1 h. The hemagglutination unit (HU) was expressed as the reciprocal of the highest lectin. Dilution showing detectable visible erythrocyte agglutination and the specific activity was calculated as HU/mg protein.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The gel was prepared with 2% SDS in 15% separating gels and 8% stacking gels. Tris-glycine buffer pH 8.3 containing 2% SDS was used as the electrode buffer. Discontinuous SDS-PAGE under reducing conditions was performed according to the procedure of Laemmli [17]Samples to be analyzed were treated with sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was performed at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. High and low molecular weight standards were used to determine the subunit molecular weight of the enzyme. After electrophoresis, proteins in the gel were visualized by staining with Coomassie Brilliant Blue G-250 R-250.

Cytotoxicity test

Lectin binding to breast cancer cell line has been investigated. The cells were trypsinized before seeded at a density of 1×10^4 cells/µl in 96 well plates for 24 h, at 37 ^oC in an atmosphere of 5% CO_2. Serial concentrations of the purified lectin were added before further incubation for 72 h. MTT (3-[5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium bromide) solution (5 mg/ml) was then added, followed by further incubation for 4 h. Absorbance at 540 nm was measured using microtiter plate reader after incubation with dimethyl sulfoxide (DMSO; 150 µl/well) for 30 min. Reagents and controls were included with the absence of cells or the crude extract, respectively.

RESULTS AND DISCUSSIONS

The present study describes purification and characterization of a lectin from the rhizomes of *C. amarissima*. Crude extract from the rhizomes of *C. amarissima* was precipitated with 80% ammonium sulfate followed by affinity chromatography. For affinity chromatography, the crude protein extract was loaded on a ConA-Sepharose column, un-bound and bound peaks were observed. The un-bound peak did not show hemagglutinating activity, but the bound peak had hemagglutinating activity. The bound peak was loaded onto a Superdex G–75 colum, resulting in three distinguish peaks. Only the last peak was found to habor hemagglutinating activity. The amount of purified protein and its activity are summarized in Table 1.

Table 1. Purification of the lectin from rhizomes of *C. amarissima*.

Purification step	Total protein (mg)	Total activity (HU)	Specific activity (HU/mg)	Yield (%)	Purification (fold)
Crude extract	3,960	49,200	12.42	100	1
80% $(NH_4)_2SO_4$ precipitation	41,900	805,056	19.21	1,636.29	1.55
ConA-Sepharose (bound fraction)	6.04	6,671	1,103.45	13.56	88.84
Superdex G-75 (last peak)	1.28	1,299	1,014.76	23.80	81.70

The lectin from gel filtration chromatography was checked for its purity using 1D-SDS-PAGE. A single band was seen on SDS-PAGE (Fig. 1). Under dissociating conditions (15% SDS–PAGE), the purified lectin was resolved into a major band of 34.2 kDa. Similar results were obtained for *Phaseolus vulgaris* [18].

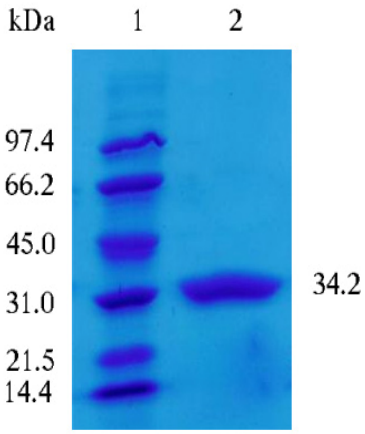

Fig. 1 Electrophoresis pattern of purified *C. amarissima* lectin from gel filtration on 15% SDS-PAGE. Lane1: molecular weight markers: phosphorylase b (97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and α-macroglobulin (14.4 kDa). Lane 2: Purified lectin from Superdex G75.

The purified lectin was measured for its cytotoxicity. The results of MTT assays of *C. amarissima* lectin against breast cancer cell lines showed IC_{50} of 21.18 μg (Fig. 2) which might relate to sugar-binding activity of lectins. The specific carbohydrate chain, which was limited on the surface of tumor cells, could act as the receptor of lectins [19]. There were several kinds of plant lectins reported to have antiproliferative effect on tumor cell lines. These include *Cratylia mollis* lectin [20], and *Viscum album* lectin [21].

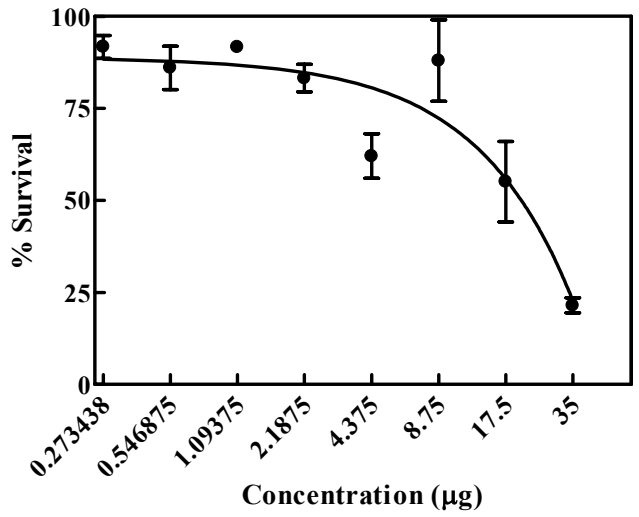

Fig. 2 Inhibition of *C. amarissima* lectin against breast cancer cell lines.

CONCLUSION

In this study, a lectin from the rhizomes of *C. amarissima* was purified by affinity and gel filtration chromatography. The molecular mass of the purified lectin was estimated to be 32.4 kDa. Lectin from *C. amarissima* showed cytotoxicity against breast cancer cell lines with an IC_{50} of 21.18 μg/ml.

ACKNOWLEDGEMENTS

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PURIFICATION AND CHARACTERIZATION OF MYOGLOBIN FROM ASIAN SWAMP EEL (*MONOPTERUS ALBUS*)

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ABSTRACT

Myoglobin (Mb) from Asian swamp eel (Monopterus albus) muscle was extracted with a buffer solution and isolated by salting-out using saturated ammonium sulfate (75%w/v). Sephadex G-75 gel-filtration and DEAE-Cellulose ion exchange chromatographic methods were used to separate and purify the fish Mb. Its molecular weight was determined by SDS-PAGE and found to be about 16 kDa. The isoelectric point of the purified Mb was 6.4 and 7.12, indicating the protein existed in two isoforms. The UV-Visible absorption spectrum and fluorescence spectrum of the protein are corresponding to the heme protein property. Partial peptide sequences of this Mb showed that it is identical to Mb from several fish species.

Keywords: myoglobin, Asian swamp eel, partial peptide sequence.

INTRODUCTION

Myoglobin (Mb) is an oxygen binding protein present in a wide variety of species and is important for proper oxygen supply near the oxidative capacity of muscle [1]. Certainly, the richest sources of Mb are found in aerobic muscle and heart ventricle of mammals such as whales and seals that undertake extended breath-hold dives [2]. Moreover, this protein is also found in reptiles, amphibians, teleosts chondrichthid fish and lower animal's species [3]. Mb from various animals living in their different surroundings show differences in the physico-chemical properties [3-5]. The aim of this research is to investigate structural and functional properties of Mb from Asian swamp eel (*Monopterus albus*). This fish is found in mud underneath a dried up pond. Most of fish species can breathe air, allowing them to survive in deoxygenated water for long periods [6]. Some of swamp eel fish behaviors are related to Mb functions. Therefore, the physico-chemical properties of this fish Mb were investigated.

MATERIALS AND METHODS

Chemicals

Sodium dodecyl sulfate (SDS), tris(hydroxymethyl)–aminomethane, acrylamide, bisacrylamide, ammonium sulfate, bovine serum albumin (BSA), N,N,N',N'–tetramethylethylenediamine and DEAE-Cellulose were purchased from Sigma (Saint Louis, Missouri, USA). β-mercaptoethanol was purchased from Acros (New Jersey, USA). Horse heart myoglobin was purchased from Fluka (Buchs,

Switzerland). Sodium chloride, sodium hydroxide, acetic acid, methanol and ethanol were purchased from Carlo Erba (Milano, Italy). Sephadex G-75® was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Fish sample

Asian swamp eel fish (*Monopterus albus*) with an average body weight of 300 - 400 g was bought from a local market in Khon Kaen, Thailand.

Extraction, isolation and purification of myoglobin

Methods for extraction, isolation and purification of Mb were modified from the method of Chaijan et al. [7]. Mb was extracted from red muscle tissue by homogenization in cool buffer (10 mM Tris-HCl, pH 6.8) using a Moulinex™ (Moulinex, France) blender (wt/vol ratio = 1:2). The crude extract obtained was centrifuged (10,000×g, 10 min, 4 °C). Crude Mb in the supernatant was precipitated with saturated ammonium sulfate. The protein precipitate obtained with 55–75% salt saturation was used for separation on Sephadex G-75 column (90 × 1.5 cm. i.d.) pre-equilibrated with the starting buffer and run with a flow rate of 0.3 mL/min and 2 mL/fraction. The main fractions containing Mb were further isolated and concentrated by Amicon® (Millipore, USA) with molecular weight cut off 30 and 10 kDa, respectively. The concentrated Mb fraction was applied onto DEAE-cellulose column (50 × 1.5 cm. i.d.) also equilibrated with the starting buffer with a flow rate of 0.3 mL/min and 2 mL/fraction. The main Mb fractions with the highest absorbance at 408 nm were assayed for the purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using a Mini Protean II unit (Bio-Rad Laboratories, USA).

Molecular weight determination

Molecular weight (MW) of the purified fish Mb was approximately determined by SDS–PAGE (4% stacking gel, 15% separating gel, 150V applied voltage) using a set of low molecular weight makers (Amersham, Sweden). The MW of the Mb was estimated by plotting the logarithm of MW of the protein standards versus their relative mobility as previously described [7].

Isoelectric point determination

Isoelectric point (pI) of the purified fish Mb which was verified by a horizontal slab gel isoelectric focusing (5.5% acrylamide) with a pH gradient from 3.5–10 ampholytes (Amersham, Sweden) was used to determine the pI value of Mb [8]. About 7 µL (~150 ppm) of the Mb was applied on the gel and higher voltages were applied (100 V for 15 min, 200 V for 15 min and 800 V for 30 min). To determine the pI of the isolated Mb, its position was compared with that of the standard pI marker (Amersham, Sweden).

Absorption measurement

The absorption spectra of Mb were taken using a UV-visible Spectroscopy System (8453 Agilent, Germany) according to the method as described [9]. The spectra were recorded from 250 to 600 nm using 20 mM Tris-HCl buffer, pH 6.8 as a blank.

Tryptophan fluorescence measurement

Fluorescence measurements were performed with a RF-5301PC spectrofluorophotometer (Shimadzu, Japan) according to the method as described [10]. One mL of the protein concentration (~16 ppm) of the Mb was used in a 1.0 cm path length cell. Excitation (280 nm) and emission (325 nm) wavelengths for tryptophan fluorescence intensity of the Mb were used.

Partial peptide sequence analysis

The protein band obtained from SDS-PAGE was excised from the gel and then determined using in-gel digestion with trypsin and analyzed the peptide fragments by LC-MS/MS performed at the Genome Institute, BIOTEC, NSTDA, Pathumthani, Thailand.

RESULTS AND DISCUSSION

Purification of myoglobin

The main fractions of Mb showed the UV-Visible absorption ratio of 280/408 nm (Fig. 1a), resulting in two main peaks. The high concentration of the Mb was found as the later peak. However, there were still few protein bands with MW of ≤ 14.4 kDa to < 67 kDa as shown in Fig. 1b.

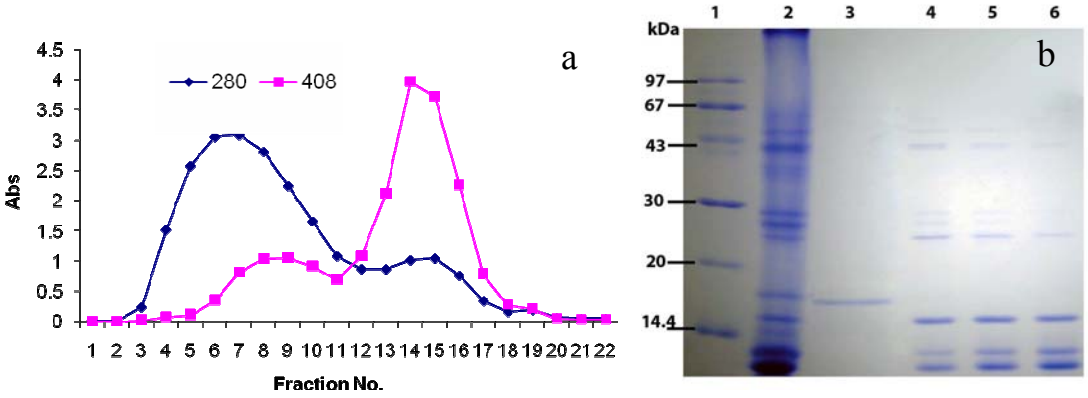

Fig. 1 (a) Sephadex G-75 elution profile of crude extract proteins (b) SDS-PAGE of fractions which exhibited high absorption at 408 nm (the Soret peak) (fraction No.13: lane 4, fraction No.14 : lane 5 and fraction No.15 : lane 6) compared with protein low molecular mass standard (lane 1), horse heart Mb (lane 3) and crude protein extract (lane 2).

The main fractions containing Mb after concentrating were further purified by a DEAE-Cellulose column (Fig. 2a). Fractions No. 17-23 gave strong UV-Visible absorption at 408 nm (the Soret peak) and SDS-PAGE showed a single band with MW about 16 kDa (Fig. 2b), indicating that the protein is the purified fish Mb.

Characterization of myoglobin

UV-Visible spectrum of the purified Mb exhibited maximum adsorption at 280 and 408 nm, compared with those of horse heart Mb solution and bovine serum albumin (BSA) as shown in Fig. 3a. Tryptophan fluorescence intensity of Mb, which is normally quenched by the heme group, was also studied. The fluorescence spectrum of this fish Mb gave a low intensity at 325 nm similar to that of horse heart Mb solution (Fig. 3b). Slab gel isoelectric focusing of this fish Mb showed that the protein has two isoforms with pI around 7.12 and 6.4 (Fig. 4).

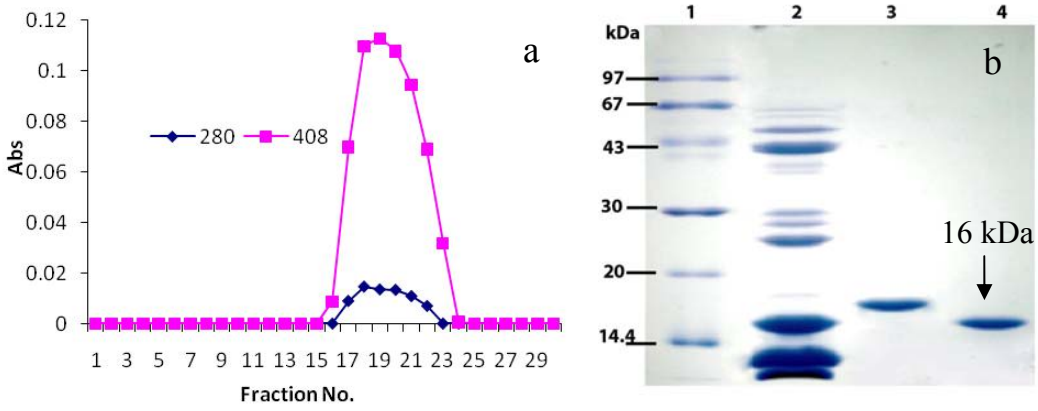

Fig. 2 (a) The elution profile of fish Mb obtained from DEAE-Cellulose ion-exchange chromatography. (b) SDS-PAGE of the fractions eluted from DEAE-Cellulose column; lane 2: fraction containing Mb after eluted from Sephadex G-75 column, lane 4: fraction No.19 compared with protein low molecular mass standard (lane 1), horse heart Mb (lane 3).

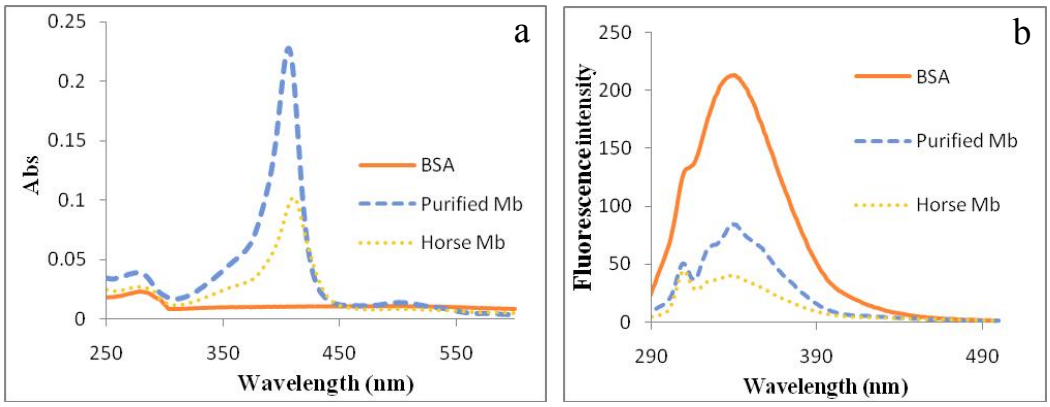

Fig. 3 (a) UV-Visible absorption spectra of the purified fish myoglobin comparing with those of horse heart myoglobin and bovine serum albumin (BSA) (b) Tryptophan fluorescence spectra of the purified fish myoglobin comparing with those of horse heart myoglobin and bovine serum albumin.

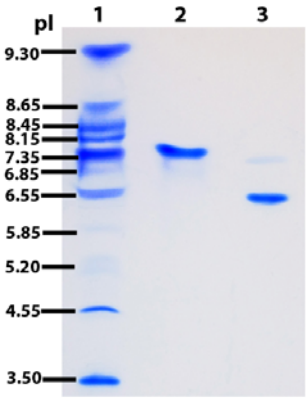

Fig. 4 Slab isoelectric-focusing gel of the purified myoglobin (lane 3) compared with pI value maker (lane 1) and horse heart myoglobin (lane 2).

Partial peptide sequences of the purified Mb were obtained from LC-MS/MS with trypsin digestion, resulting in six peptide fragments obtained (Table 1). In addition, when matching each peptide fragment obtained from these data with the protein database [11], it corresponds to Mb from various fish species as shown in Table 2.

Table 1 Sequence tags of the purified fish myoglobin obtained from LC- MS/MS with in-gel trypsin digestion

No	Sequence tags from LC-MS/MS	No	Sequence tags from LC-MS/MS
1	AKGSHAAILKPLANSHATK	4	IPINNFR
2	GSHAAILKPLANSHATK	5	LFTEHPETQK
3	HKIPINNFR	6	LFTEHPETQKLFPK.

Table 2 Identification of protein sequence tags obtained from the purified fish myoglobin, using LC-MS/MS and database search

Sequence tags from LC-MS/MS	The matching amino acid residues against the protein database	Identities	Protein identification
RLF+EHPETQKLFPK **LLKAKGSHAAILKPLANSH ATKHKIPINNF**	MADFDAVLKC WGPVEADYTT IGGLVLT**RLF** K**EHPETQKLF PK**FAGIAQAD IAGNAAVSAH GATVLKKLGE **LLKAKGSHAA ILKPLANSHA** **TKHKIPINNF** KLISEVLVKV MHEKAGLDAG	44/142 (30.9%)	Myoglobin from North Pacific bluefin tuna*(Thunnus orientalis)*
RLFTEHP+TQKLFPKF KAKGSHAAILKPLANSHAT KHKIPINNF	LVLKCWGPVE ADYAAYGSLV LT**RLFTEHP**D **TQKLFPKF**AG IAQGDMAADA GISAHGATVL RKLGELL**KAK GSHAAILKPL ANSHATKHKI** **PINNFR**LITE VIGKVMGEKT GLDAAGQQAL RNVMAIVVAD MEADYKLLGF TG	44/142 (30.9%)	Myoglobin from Sea raven fish (*Hemitripterus americanus)*
RLF++HPETQKLFPK KA+G+HAAILKPLATTHA ++HKI++NNFR	MHDAELVLKC WGGVEADFEG TGGEVLT**RLF** KQ**HPETQKLF PK**FVGIASNE LAGNAAVKAH GATVLKKLGE LL**KARGDHAA ILKPLATTHA** NT**HKI**AL**NNF R**LITEVLVKV MAEKAGLDAG GQSALRRVMD VVIGDIDTYY KEIGFAG	36/147 (24.4 %)	Myoglobin from Common carp *(Cyprinus carpio)*
RLF++HP+TQKLFPK AKG+HAAIL+P+A++HA +KHKI++NNF R	MADYERFLKC WGAVEADYTG NGGEVLT**RLF** KA**HPDTQKLF PK**FKGISQSE LAGNALVAAH GATVLKKLGE LLR**AKGDHAA ILHPMATTHA** N**KHKI**TL**NNF R**LITEVLVEV MKEKAGLDSA GQGALKRIMD CIIHDIDRYY KEIGFAG	32/147 (21.7%)	Myoglobin from Goldfish *(Carassius auratus)*

CONCLUSION

Myoglobin from Asian swamp eel had its molecular weight of 16 kDa with two isoforms of pI values around 6.4 and 7.12. Spectral properties of this Mb were corresponding to the heme protein properties. The peptide sequences of the purified Mb were found to be similar to those of myoglobins from other fish species.

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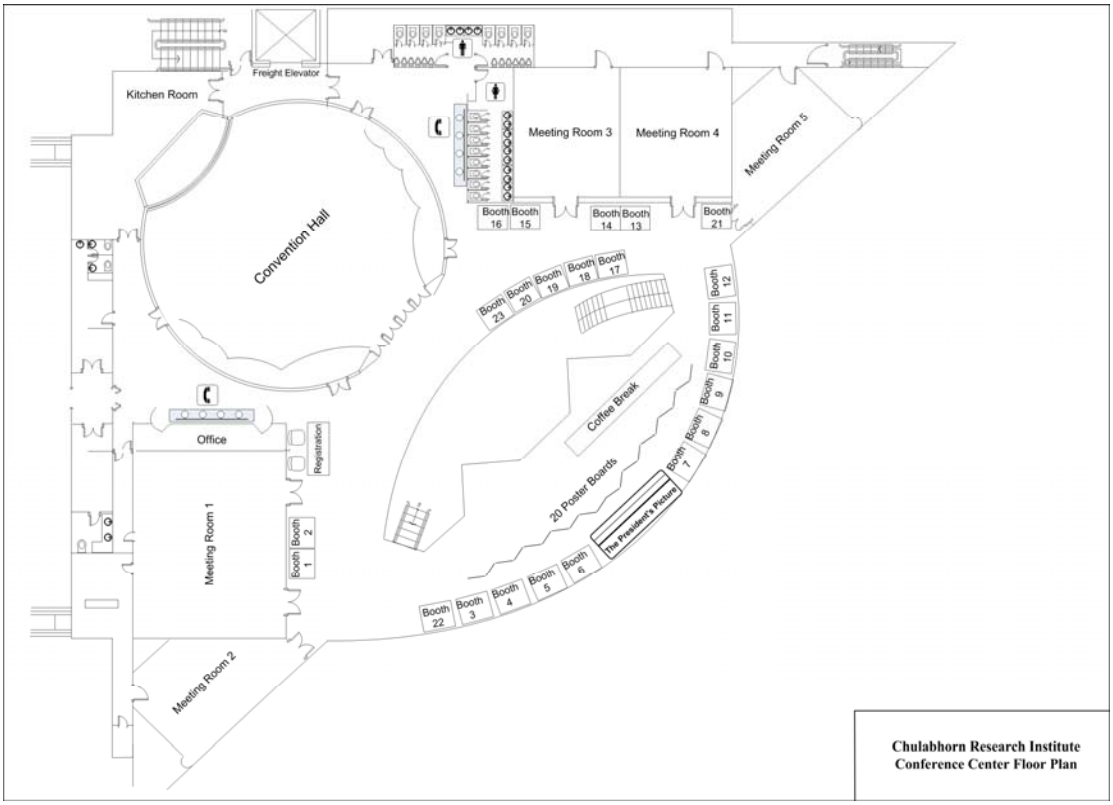

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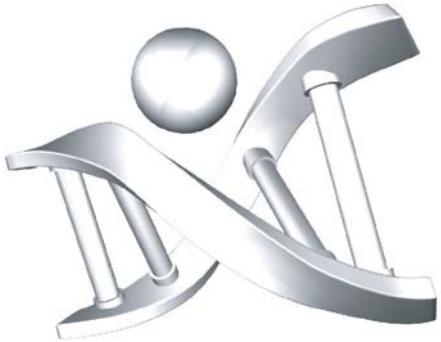

Gene Systems. Co., Ltd.

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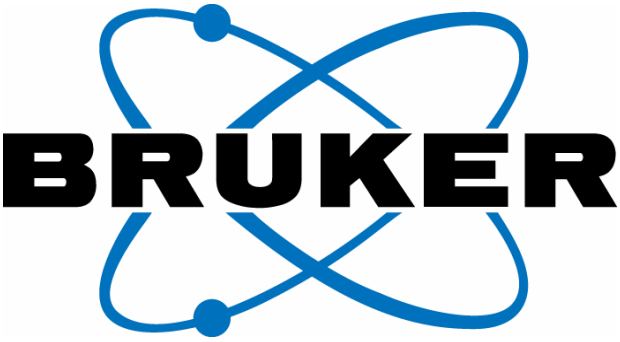

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