2.7.1 Laboratory : Enzyme Chemistry

Member :	Professor	Inouye, Kuniyo, PhD
	Associate Professor	Yasukawa, Kiyoshi, PhD
	Assistant Professor	Takita, Teisuke, PhD
	Assistant Professor	Kojima, Kenji, M
	Doctor's program	3
	Master's Program	7
	Undergraduate	3
	Other	4
	Program-Specific Researcher	2
	Researcher	1

A. Research Activities (2009.4-2010.3)

A-1. Main Subjects

a) Studies on proteolytic enzymes.

(i) Molecular mechanism of the activity of thermolysin. We found that the activity of thermolysin, a typical thermophilic proteinase, is greatly enhanced up to over 20 times in the presence of high concentration (2-5 M) of neutral salts. Thermal stability of the enzyme is also enhanced in the presence of the salts. We expect that the molecular mechanism of the activity of thermolysin can be revealed by understanding the halophilicity of this enzyme. Involvement of tyrosyl residues at the active site and charged groups on the surface of the enzyme in the enhancement of the enzyme activity has been suggested. Site-directed mutagenesis and chemical modification have been applied to reveal the roles of tyrosy1, tryptophyl, lysyl, aspartic, and glutamiac residues in the halophilicity of thermolysin.

(ii) Enzymatic properties of MMP-7. The metalloproteinase MMP-7, which contains a zincion essential for enzyme activity, plays an important role in tumor invasion and metastasis with proteolysis of extracellular matrix proteins. We have compared enzymatic properties of MMP-7 with those of thermolysin. In addition, we have studied on naturally occurring MMP-7 inhibitors which could be useful for cancer therapy.

b) Studies on aminoacyl-tRNA synthetases.

Reaction mechanism of lysyl-tRNA synthetase (LysRS) of mesothermophilic bacteria. Aminoacyl-tRNA synthetases guarantee the fidelity of translation of the genetic information into the structure of a protein by their substrate recognition mechanisms. We purified LysRS to homogeneity from Bacillus stearothermophilus. Interactions of the substrates (L-lysine and ATP) and their analogues with LysRS were studied by a combination of several enzyme-activity assays, fluorescence titration, equilibrium dialysis, stopped-flow method etc. The order of binding of the substrates to LysRS and some features of substrate recognition by the enzyme were revealed. We cloned the LysRS gene of Bacillus stearothermophilus, deduced total amino acid sequence, and established the overexpression system by using E. coli. For further details of the recognition mechanism of LysRS, we have applied site-directed mutagenesis to the LysRS gene and tried to evaluate the 3D-structure by X-ray crystallographic analysis. In addition, we have cloned aminoacyl-tRNA synthetase cDNA from hyperthermophilic archaeon Aeropyru pernix Kl and tried its expression in E. coli.

c) Studies on carbohydrate hydroxylases and their inhibitors.

Stabilization of bacterial amylases against thermal denaturation has been examined by mutagenesis and improvement of the reaction conditions. The thermal stability was much improved by introducing negatively-charged residues into the calcium-ion binding sites. In the cases of Bacillus amylases, enhancement in the stabilization has been desired for glucose-production industry, whereas attenuation in the stability for the baking industry. The results obtained might be useful for these purposes. On the other hand, we have purified a protein amylase inhibitor named 0.19AI from the albumin fraction of wheat proteins, and examined the inhibition against porcine pancreas alpha-amylase (PPA). It was revealed that a single electrostatic interaction is essential for the interaction between 0.19AI and PPA. Currently, we are in progress for identifying the residues involved in the electrostatic interaction by chemical modification. The fruits of this study might be useful for prevention and therapy of obesity and diabetes.

d) Studies on application of monoclonal antibodies.

(i) The application to immunoassays. Monoclonal antibodies have been used widely in diagnoses and analysis of bioactive substances. There are some points to be improved in enhancement of the sensitivity and simplification in the operation. We would solve these points by the use of active fragments, and bispecific antibodies in place of the native monoclona1 antibodies. Liposome assay and fluorometric assay might be examined for development of homogeneous enzyme immunoassays. In order to increase a sensitivity of the enzyme immunoassays, we have developed an assay system using synchronization of multiple enzymes containing an alkaline phosphatase conjugated with a second antibody. We have also studied an enzyme immunoassay for histamine, which is important in food analysis and a test

for allergy.

(ii) Catalytic antibodies. Monoclonal antibodies which catalyze the hydrolysis of ester derivatives of chloramphenicol are examined from the view of enzyme kinetics and spectrophotometric analysis.

e) Studies on the application of soy proteins and whey proteins.

Soy proteins especially defatted ones are not utilized well. In this project, a potentiality of the soy proteins as food staffs are examined. We have developed a method of deodorization of soybean proteins by physicochemical processing with hydrophobic resins. In addition, we have developed a new method to make a bean curd with proteinases. We are studying the aggregation process of soy proteins induced by various proteinases with physicochemical methods. On the other hand, whey protein especially gluten is also an important food protein but it has unique amino acid composition so that its proteolytic digestion in vitro and in vivo proceeds hardly. Presently, we are in process to develop an effective method for whey protein degradation to amino acids.

f) Studies on reverse transcriptase

Reverse transcriptase (RT) is an enzyme which is indispensable as a tool for research in molecular biology and diagnosis of RNA virus. Although RTs from avian myeloblastosis virus (AMV) and Moloney murine leukaemia virus (MMLV) have been the most extensively used due to their high catalytic activity, thermal stability, and fidelity, those with higher activity and stability have been desired. We have compared their enzymatic properties and attempted to improve their activity and stability by site-directed mutagenesis.

A-2.Publications and presentations

a) Publications

Books

- Inouye, K.: Protease-catalyzed digestion of soy-proteins and modification of their protein chemical characterization. Technology and Market of Enzymes 2009 (CMC Publishers Editorial Office. ed.) p. 36-44, 2009 (Japanese)
- Hasegawa, N., and Inouye, K.: Application of bacterial amylases to starch industry, and its problems and future perspectives. Technology and Market of Enzymes 2009 (CMC Publishers Editorial Office. ed.) p. 19-27, 2009 (Japanese)
- Inouye, K. (Editor): Applied technology and the latest trend of industrial enzymes (Supervised Editor: Inouye, K.), p. 1-345, CMC, Tokyo, 2009 (Japanese)
- Inouye, K.: General review: Past, present, and future of research on industrial enzymes (Editor: Inouye, K.), p. 1-14, CMC, Tokyo, 2009 (Japanese)

- Inouye, K., Hashida, Y., Kusano, M., and Yasukawa, K.: Application and improvement of thermolysin (Editor: Inouye, K.), p. 58-68, CMC, Tokyo, 2009 (Japanese)
- Yasukawa, K.: Method for nucleic acid amplification (Editor: Inouye, K.), p. 194-202, CMC, Tokyo, 2009 (Japanese)
- Inouye, K. (Editor) Food Proteomics - Application Techniques of Food Enzymes, p. 1-243, CMC, Tokyo, , 2009 (Japanese)

Original Papers

- Asaoka, K., Yasukawa, K., and Inouye, K. Coagulation of soy proteins induced by thermolysin and comparison of the coagulation reaction with that induced by subtilisin Carlsberg. Enz. Microb. Technol., 44; 229-234, 2009
- Inouye, K., Nakano, M., Asaoka, K., and Yasukawa, K. Effects of thermal treatment on the coagulation of soy proteins induced by subtilisin Carlsberg. J. Agric. Food Chem., 57; 717-723, 2009
- Inouye, K., Yasumoto, M., Tsuzuki, S., Mochida, S., and Fushiki, T. The optimal activity of a pseudozymogen form of recombinant matriptase under the mildly acidic pH and low ionic strength conditions. J. Biochem., 147; 485-492, 2010
- Kojima, K., Tsuzuki, S., Fushiki, T., and Inouye, K. Role of the stem domain of matriptase in the interaction with its physiological inhibitor, hepatocyte growth factor activator inhibitor type I. J. Biochem., 145; 783-790, 2009
- Kojima, K., Tsuzuki, S., Fushiki, T., and Inouye, K. The activity of a type II transmembrane serine protease, matriptase, is dependent solely on the catalytic domain. Biosci. Biotechnol. Biochem., 73; 454-456, 2009
- Kusano, M., Yasukawa, K., and Inouye, K. Insights into the catalytic roles of the polypeptide regions in the active site of thermolysin and generation of the thermolysin variants with high activity and stability. J. Biochem., 145; 103-113, 2009
- Kusano, M., Yasukawa, K., and Inouye, K. Synthesis of N-carbobenzoxy-L-aspartyl-L-phenylalanine methyl ester catalyzed by thermolysin variants with improved activity. Enz. Microb. Technol., 46; 320-325, 2010
- Kusano, M., Yasukawa, K., and Inouye, K. Effects of the mutational combinations on the activity and stability of thermolysin. J. Biotechnol., 147; 7-16, 2010
- Miyake, Y., Yasumoto, M., Tsuzuki, S., Fushiki, T., and Inouye, K. Activation of a membrane-bound serine protease matriptase on the cell surface. J. Biochem., 146; 273-282, 2009
- Miyake, Y., Yasumoto, M., Tsuzuki, S., Fushiki, T., and Inouye, K. The role of asparagine-linked glycosylation site on the catalytic domain of matriptase in its zymogen

activation. Biochim. Biophys. Acta, 1804; 156-165, 2010

- Miyake, Y., Tsuzuki, S., Fushiki, T., and Inouye, K. Matriptase does not require hepatocyte growth factor activator inhibitor type-1 for activation in an epithelial cell expression model. Biosci. Biotechnol. Biochem., 74; 848-850, 2010
- Miyake, Y., Tsuzuki, S., Yasumoto, M., Fushiki, T., and Inouye, K. Requirement of the activity of hepatocyte growth factor activator inhibitor type 1 for the extracellular appearance of a transmembrane serine protease matriptase in monkey kidney COS-1 cells. Cytotechnology, 60; 95-103, 2009.
- Mizuno, M., Yasukawa, K., and Inouye, K. Insight into the mechanism of the stabilization of Moloney murine leukaemia virus reverse transcriptase. Biosci. Biotechnol. Biochem., 74; 440-442, 2010
- Mochida, S., Tsuzuki, S., Yasumoto, M., Inouye, K., and Fushiki, T. Secreted expression of pseudozymogen forms of recombinant matriptase in Pichia pastoris. Enz. Microb. Technol., 45; 288-294, 2009
- Murai, N., Miyake, Y., Tsuzuki, S., Inouye, K., and Fushiki, T. Identification of the basolateral sorting signal of a type II transmembrane serine protease matriptase. Cytotechnology, 59; 169-176, 2009
- Narita, Y. and Inouye, K. Kinetic analysis and mechanism on the inhibition of chlorogenic acid and its components against porcine pancreas a-amylase isozymes I and II. J. Agric. Food Chem., 57; 9218-9225, 2010
- Sakurama, H., Takita, T., Mikami, B., Itoh, T., Yasukawa, K., and Inouye, K. Two crystal structures of lysyl-tRNA synthetase from Bacillus stearothermophilus in complex with lysyladenylate-like compounds: insights into the irreversible formation of the enzyme-bound adenylate of L-lysine hydroxamate. J. Biochem., 145; 555-563, 2009
- Tsukiyama, T., Lee, J., Okumoto, Y., Teraishi, M., Tanisaka, T., and Inouye, K. Gene cloning, bacterial expression, and purification of a novel rice (Oryza sativa L.) ubiquitin-related protein, RURM1. Biosci. Biotechnol. Biochem., 74; 430-432, 2010
- Tsuzuki, S., Miyake, Y., Inouye, K., and Fushiki, T. The occurrence of matriptase C-terminal fragments on the apical and basolateral sides of Madin-Darby canine kidney epithelial cells. Biosci. Biotechnol. Biochem., 73; 2538-2540, 2009

- Yasukawa, K., Agata, N., and Inouye, K. Detection of cesA mRNA from Bacillus cereus by RNA-specific amplification. Enz. Microb. Technol., 46; 391-396, 2010

- Yasukawa, K., Konishi, A., and Inouye, K.: Effects of organic solvents on the reverse transcription reaction from avian myeloblastosis virus and Moloney murine leukaemia virus. Biosci. Biotechnol. Biochem., in press, 2010

- Yasukawa, K., Mizuno, M., and Inouye, K. Characterization of Moloney murine leukaemia virus/avian myeloblastosis virus chimeric reverse transcriptases. J. Biochem., 145; 315-324, 2009

Reviews

- Okumura, S. and Inouye, K. A miracle of crystal proteins produced by Bacillus thuringiensis. Kagaku-to-Seibutsu, 47; 670-672, 2009

- Kohno, H., Shimizu, S., and Inouye, K. Hydrogen and ethanol production by bio-conversion of Laminaria japonica. Annual Reports of the Nippon University Bioengineering Research Center, H-21; 7-14, 2009

- Inouye, K. (Co-translator). Biochemistry, 4th Edition. The Molecular Basis of Life (T. McKee and J. McKee), p. 171-120, Kagaku-Dojin, Kyoto (March, 2010)

Patents

- Inouye, K., Murakami, Y., and Kanaya, M. Preparation method of water-soluble hemi-celluolose. Patent application number: 2009-169711 (July 21, 2009)

b) Conference and seminar papers presented

- Annual Meeting of the Japan Society (2009) for Bioscience, Biotechnology, and Agrochemistry: 19 papers
- Joint Meeting of Kansai, Chushikoku, and Nishinihon Branches of the Japan Society for Bioscience, Biotechnology, and Agrochemistry, Kyushu and Okinawa Branches of Japan Society of Nutrition and Food Science, and Nishinihon Branch of the Japanese Society for Food Science and Technology: 6 papers
- 463th Meeting of Kansai Branch of the Japan Society for Bioscience, Biotechnology, and Agrochemistry: 1 paper
- 56th Annual Meeting of Kinki Branch of the Japanese Biochemical Society: 4 papers
- 82th Annual Meeting of the Japanese Biochemical Society: 9 papers
- 56th Annual Meeting of the Japanese Society for Food Science and Technology: 1 paper
- 61th Annual Meeting of the Japanese Society for Biotechnology: 1 paper
- 9th Annual Meeting of the Food Enzyme Chemistry Forum, 15th Annual Meeting of Akita Applied Life Science Forum: 2 papers
- 14th Meeting of the Japanese Society for Proteases in Pathophysiology: 1 paper
- 13th Meeting of the Nippon Kinoko Society: 1 paper

A-3.Off-campus activities

Membership in academic societies

- Inouye, Kuniyo, PhD : The Japanese Biochemical Society (Councilor, Councilor of the Kinki Branch; Annual Meeting Advisory Member) , Japan Society for Bioscience, Biotechnology, and Agrochemistry (Director, Chief of the Kansai Branch, Councilor of the Kansai Branch), The Japanese Society for Food and Technology (Councilor of the Kansai Branch), Japanese Association of Animal Cell Technology (Councilor), Academic Meeting of the Food Enzyme Chemistry Forum (Chair), Japanese Association of Food Analysis (Councilor), The Japanese Society for Proteases in Pathophysiology (Councilor)
- Yasukawa, Kiyoshi, PhD : Japan Society for Bioscience, Biotechnology, and Agrochemistry (Organizer of General Affairs)

Research grants

1. Grants-in-aid for Scientific Research(KAKENHI)

- Scientific Research (B) : Inouye, Kuniyo, PhD : Protein engineering and reaction control engineering of thermolysin aiming at expansion of use of thermolysin in food industry
- Scientific Research (C) : Yasukawa, Kiyoshi, PhD : Evaluation of the cDNA synthesis and RNA amplification with reverse transcriptase with high performances and expansion of their use

2.Other Research Grants

- NEDO Grants for Innovation Practicing Support : Inouye, Kuniyo, PhD : Development of a carriable immuno-analyzing system
- JST Grant, Research for Promoting Technological Seeds : Yasukawa, Kiyoshi, PhD : Generation of MMLV RT with high performances and its application to rapid diagnosis
- Iijima Memorial Foundation, Grants : Yasukawa, Kiyoshi, PhD : Detection of emetic Bacillus cereus in processed grain food by the RNA amplification with reverse transcriptase

A-4.International cooperation and overseas activities

Membership in academic societies

- Inouye, Kuniyo, PhD: Sixth General Meeting on the International Proteolysis Society, Surfers Paradise Marriott Resort and Spa, Gold Coast, Australia (International Advisory Board member) , Biotechnology Annual Review (Elsevier) Vols. 1-15 (Editor) , New Biotechnology (Elsevier) (Review Editor) , Enzyme and Microbial Technology (Elsevier) (Editor) , Food Science and Biotechnology (International Editorial Board Member)

International meetings(country,roles)

- Inouye, Kuniyo, PhD：8th International Conference on Protein Stabilization, Graz University of Technology, Graz, Austria (Invited speaker) (April, 2009)
, Sixth General Meeting on the International Proteolysis Society, Surfers Paradise Marriott Resort and Spa, Gold Coast, Australia (International Advisory Board member; Speaker) (October, 2009) , 4th International Conference on Polyphenols and Health, Harrogate International Centre, Harrogate, UK (Co-speaker) (December, 2009)

International joint research, overseas research surveys

- Proteases on bacterial infection, diagnosis, and therapy, Inouye, Kuniyo, PhD (University of Michigan, USA)
- Characterization of soy and wheat protein, Inouye, Kuniyo, PhD (University of Wageningen, the Netherlands)
- Role of MMPs in digestive diseases, Inouye, Kuniyo, PhD (University of Leiden and University of Groningen, the Netherlands)
- Reaction mechanism of MMPs, Inouye, Kuniyo, PhD (Imperial College, London, UK)
- Biotechnology of enzymes and antibodies, Inouye, Kuniyo, PhD (University of Tromso, Norway)
- Research on structure-function relationship of thermophilic enzyme, Inouye, Kuniyo, PhD (Seoul National University, Yonsei University and Gwanju Institute of Technology, Korea)
- Enzyme reaction mechanism, Inouye, Kuniyo, PhD (Warwick and Exceter, UK)
- Research on reaction mechanism of amylase and proteinase and their application to food science and technology, Inouye, Kuniyo, PhD (Seoul, Korea)
- Application of amylases and proteinases, Inouye, Kuniyo, PhD (University of Guelph, Canada)
- Collaborative study on the stabilization of proteins, Inouye, Kuniyo, PhD (Graz Technical University, BOKU, and Austrian Research Center for Science and Technology, Austria)

B.Educational Activities(2009.4-2010.3)

B-1.On-campus teaching

a) Courses given

- Undergraduate level： Fundamental Chemical Experiments (Kojima), Food Biochemistry II (Inouye, Yasukawa), Enzymes: Function and Application (Inouye, Yasukawa), Enzyme Chemistry (Inouye, Yasukawa), Introduction to Research I (Inouye, Yasukawa), Laboratory Course

in Enzyme Chemistry and Biochemistry (Yasukawa, Takita, Kojima).

- Graduate level: Advanced Course of Enzyme Chemistry (Inouye, Yasukawa), Enzyme Chemistry Seminar (Inouye, Yasukawa, Takita, Kojima), Experimental Course of Enzyme Chemistry (Inouye, Yasukawa, Takita, Kojima).

B-2.Off-campus teaching etc.

Part-time lecturer

- Inouye, Kuniyo, PhD: Iwate University, Special Seninar(Faculty of Agriculture), University of Ryukyus, Special Seninar(Faculty of Agriculture)
- Inouye, Kuniyo, PhD: Visiting Professor of Toyo University, Visiting Researcher of the Nippon University Biotechnology Research Center

B-3.Overseas teaching

International students

- International students : Master 1 (Kenya) Research Students 4 (Zimbabwe 1, China 3)

Lectures and seminars

- Inouye, Kuniyo, PhD

Enzyme Chemistry(Lecturer) : Department of Food and Nutrition, Yonsei University(South Korea)

C.Other Remarks

- Inouye, Kuniyo, PhD: Member of the Institute of Science and Technology Policy of the Ministry of Education, Culture, Sports, Science and Technology, Kyoto Municipal Bio-industrial Business Promotion Forum, Member of Advisory Committee for Kyoto Municipal Project of the Cooperation of Medicine, Engineering, and Life Sciences, Member of the Judging Committee for Member of the Ministry of Economy, Trades, and Industry, Member of the Judging Committee for Industrialization by Minor Enterprises, Member of the Science Committee of the Iijima Foundation, Member of the Judging Committee for JSPS Fellows of the Japan Society for the Promotion of Science, Member of the Kansai Science Forum Working Group