PR2 Direct observation of the proton or protonation in a protein molecule by neutron and high resolution X-ray joint analysis

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OBJECTIVES: Protein sciences are accelerated with use of a neutron and an X-ray beam to clarify the tertiary and quaternary structural information. Proton and/or protonation of the amino acids in the protein molecule affect a chemical reaction in the biological macromolecule, neutron is well useful to isolate findings of such reaction mechanisms and can determine a precise position and roles of hydrogen or protonation in active amino acids. Drugs, as reagents, anti-cancer compounds, play important roles in a host-protein molecule with electrons or protons, hydrogen (deuterium) atoms should be determined clearly to design a compound molecule. X-ray analysis is good method to find a whole molecule and neutron is sensitive and selective to hydrogen (deuterium), the both complementary usage or joint refinement of the protein molecule is very powerful technique to analyze a precise reaction mechanism with atomic resolution.

I have called for members to carry out the examination of this purpose.

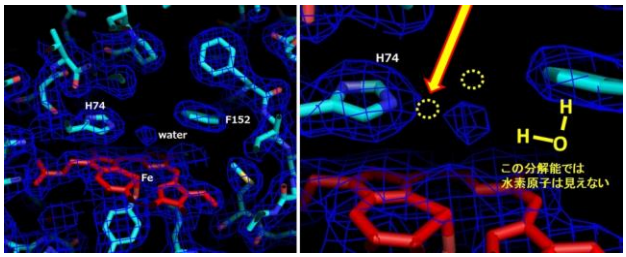
(1) Structural investigation of the 20S/26S Proteasome in the highly structural organization, Y.Morimoto
(2) Improved Techniques for Neutron Biology. T.Chatke
(3) Proteolytic enzymes derived from *NephilaClavata*. Y.Hidaka

RESULTS:

26P2-1

T. Murakami, M. Unno, I. Hisatome, K.Hosokawa, H. Yamaguchi and Y. Morimoto reported “Degradation control and structural motion of the 20S/26S proteasome elucidated by genetic mutation and atomic force microscope”. A newly inhibitor of the 20S proteasome and its complex is described. Since the wild-type 20S proteasome has gradual decrease against drug concentration, Y170A, Y170L and Y170F has no clear decreasing. Drug A has an aromatic ring in its structure, the aromatic ring interacts the Y(tyr) ring but F(phe) has also a ring structure. 26S whole structure has been investigated by the AFM. The 26S has two large components (19S) at the bothe ends of 20S particle. The AFM images show the size of the whole particle of 26S, but a single-capped particles are also observed with self-rotating images. We have tried to detect when a substrate molecule, 6 x His tagged protein generated by expression system of the *E coli*, enter into the 20S particle by the AFM. The *in situ* detection of the degradation will make clear *in vivo* mechanism in the living cell.

Other research of the structure analyses, catalase from bovine liver has been crystallized in deuterium solution. Diffraction data of the Spring-8 showed clear electron densities at an active site, heme, and water passing tunnel (following figure). However, the resolution 2.5 Å is not enough to determine hydrogen atoms.



An ultra-high resolution (~0.9A) analysis of the lysozyme crystallized under deuterium solution has been investigated by thermal turbulence refinement of carbon and/or nitrogen atoms with use of the Photon Factory data.

26P2-2

T. Chatake, S. Fujiwara, I. Tanaka, Y. Yanagisawa reported “Development of Improved Techniques for Neutron Biology” of the result for the three interesting waorks. The first is a newly structure determination method of the neutron crystallography by use of a H/D contrast density mapping, like to be a solvent flattening of the X-ray determination. The second describes a fully deuterated protein production method by overexpression system, and modification of a cultivation and purification of the kinase. The last is interesting view point on the neutron scattering or diffraction, by use of dynamic polarization technique for a neutron biology.

26P2-3

Y. Hidaka, M. Fujiwara, T. Nakanishi, S. Shimamoto, and M. Miyazawa reported “Identification and characterization of proteolytic enzymes derived from *NephilaClavata”* focusing to the spider protease digestting amyloid fibers including pathogenic β-amyloid, such as amyloid fibrils, associated with the development of Alzheimer's disease. The N-terminal amino acid sequence of spider protease was determined by Edman degradation. Spider genes were amplified using generating primers, base on the N-terminal amino acid sequence, by RT-PCR and cloned into pBR-SKII(-).The cloning of spider protease genes is in progress.

採択課題番号 26P2　　蛋白質分子内のプロトン・プロトン化の直接観察　　プロジェクト
（京大・原子炉）森本幸生

PR2-1 Degradation control and structural motion of the 20S/26S proteasome elucidated by genetic mutation and atomic force microscope

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INTRODUCTION: Proteasomes are the multicatalytic protein complexes with huge molecular weight. It is well known that the ubiquitin proteasome system plays an important role in regulated proteolysis. Proteasomes are known to act as antigen-processing enzymes responsible for the generation of peptide ligands presented by major histocompatibility complex (MHC) class I molecules. In the 20S proteasome each of 1, 2 and 5 β subunits has different enzyme activities; 1 has caspase-, 2 tryptic- and 5 chymotryptic-like activities. It was found recently that inhibition of 20S proteasome activity resulted in decrease and disappearance of cancer cells. Since weak interaction of compounds to active amino acid residues we found, processing or degradation assay have been clarified by use of genetic mutations of the yeast 20S proteasome. The whole particle, 26S proteasome, including such 20S core is very important in the actual living cell for a degradation or stabilization on the cell circulation. Especially, a motion or behavior of the 26S proteasome when a regulatory particles bound should be clarified at atomic- or nano-scale resolution, we have tried to visualize such behavior as a motion capture of the atomic force microscopy (AFM) technique.

EXPERIMENTS: Genetic modification such as Y170A, Y170L and Y170F are succeeded in the β5 subunit of the yeast. Wild and mutant cells were homogenized by glass beads and the crude extracts were purified by M2 affinity chromatography and Mono-Q anion exchange one. When the 26S particle was isolated, gel-filtration chromatography was continuously applied with a buffer solution including ATP. Isolated 20S proteasome was concentrated by ultrafiltration, and co-crystallized with small ligand and inhibitor molecules. AFM measurements of the 26S particle were carried out in the Research Institute of Biomolecule Metrology in Tsukuba.

RESULTS: The electron densities with a resolution of 2.85Å have been observed at the active site in the β-ring. Binding site of inhibitor is closed to Tyr170 and Thr1 of the β5 subunit. It is considered that an aromatic ring of the inhibitor interacts with Y170 by ring-stacking force. Genetic mutation for Y170 would have activities because of a lack of aromatic ring in the amino acid residue in the Ala and Leu, binding ability of the inhibitor might be lost. Typical activity assay for wild-type 20S shows reduction (Fig.1), but its concentration is slightly high rather than a clinical usage.

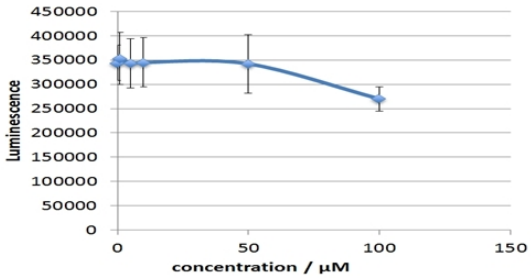

Fig.1 Activities of the 20S proteasome with inhibitor concentration.

The AFM measurement shows whole structure of the 26S particle, and analyses of resultant images describe a shape and size, and also a structural motion (Fig.2).

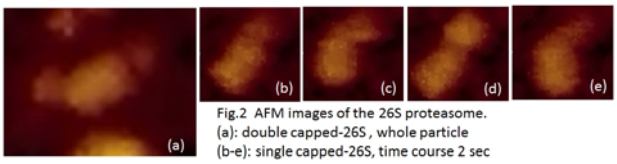

Fig.2 AFM images of the 26S proteasome.
(a): double capped-26S , whole particle
(b-e): single capped-26S, time course 2 sec

Fig.2a shows a double-capped whole 26S particle composed of central 20S and the both ends (19S regulatory particles). Those ends, however, are same direction like square brackets. Fig.2b-e shows a single-capped 26S, it means that preparation of the 26S is insufficient or scarce of ATP. Fig.2b to 2e is spent six seconds, in the meantime 19S part seems like to bow twice. In this case, since a preparation buffer includes no ATP reagents, it is interesting in such a motion by random movement or biologically functional changes. On the other hand the images are also considered the single-capped particle to be rotated in a solution, physical fixation of the 20S core to a basal plate should be required and the subject for a future study. This work was supported by a grants-in-aid for the Customer Care Plan Foundation (YM). The synchrotron radiation experiments were conducted under the approvals 2013AB6856, 2014A1855 and 2014AB6956, of SPring-8.

採択課題番号 26P2-1　酵母プロテアソームの高次構造形成と活性動作機構の解明　プロジェクト
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PR2-2 Development of Improved Techniques for Neutron Biology

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INTRODUCTION: The neutron diffraction technique is undoubtedly one of important methods for elucidating behavior of hydrogen atoms in biological systems, as well as neutron scattering technique. Nevertheless, neutron diffraction analysis is not a popular method for structural biology yet, in comparison to a large number of X-ray diffraction analyses. The main reason of the limitation of neutron diffraction experiments is, of course, the lower flux of neutron beam against those of X-ray sources. On the other hand, several technical problems, which derive from to the lack of experimental experience, become obstacles for this forefront science. In this project, the following three themes were studied for neutron structural biology.

(1) A new technique for neutron structure determination.
(Fujiwara and Chatake).

A new technique for the deuterium/hydrogen (D/H) contrast map (the neutron scattering length of D and H atoms is largely different.) in neutron macromolecular crystallography is in developing by us. Our results suggested that this type of neutron map had advantages on the neutron structure determinations.

(2) An improved technique for production of deuterated biomolecule in bacteria.
(Yanagisawa and Chatake)

Deuteration of protein has been the important technique for neutron biology, and the most popular method is the overexpression of the target protein using genetically engineered E. coli. Meanwhile, in food industry, a considerable number of materials and supplements are produced in native bacteria. In this project, the protocols of producing deuterated biomacromolecules in Bacillus subtilis natto have been developed and improved.

(3) The basic research of the application of dynamic neutron polarization (DNP) for neutron protein crystallography (NPC).
(Tanaka and Chatake)

DNP is the sophisticated technique in neutron science. In DNP, polarized neutron alters its coherent and incoherent scattering depending on the nuclear spin of the polarized hydrogen. DNP is already applied for neutron scattering except biomolecules. In this project, the basic experiments essential for DNP were carried out.

EXPERIMENTS and RESULTS:

(1) A new technique for neutron structure determination.

In the previous study, D_2O- and H_2O- solvent crystals of bovine pancreatic ribonuclease A were prepared (21P9-6), and their neutron diffraction images were collected at BIX-3 at JRR-3M (JAEA). In this fiscal year, The D/H contrast neutron map was calculated in real space, and discrete peaks could be observed at the positions of D/H exchangeable atoms inside and outside of the protein [1]. The detailed investigation of the hydration of the protein is in progress.

(2) An improved technique for production of deuterated biomolecule in bacteria.

In the previous study, nattokinase (NK) and menaquinone-7 (MK-7) from Bacillus subtilis natto were purified, and the non-hydrogen structure of NK was determined by X-ray diffraction technique [2]. In addition, D_2O-resist Bacillus subtilis natto was obtained by successive cultivation in D_2O medium [3] (21P9-3, 22P6-2, 23P2-2,3 and 24P5-4). NK and MK-7 are expected for good samples for neutron biology, NK neutron crystallography, MK-7 neutron small angle scattering. In order to perform these neutron experiments, deuterated NK and MK were necessary. In this fiscal year, the improvement of the amount of production was tried. The temperature of the cultivation was estimated to be 42 degree. In addition, hipolypeptone was not good for the cultivation, although hipolypeptone was superior to polypeptone in the cultivation of native Bacillus subtilis natto in H_2O medium.

(3) The basic research of the application of dynamic neutron polarization (DNP) for neutron protein crystallography (NPC).

2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) is a kind of radical, which is widely used for DNP. In the previous study, TEMPO was introduced in crystals of hen egg white lysozyme, and was confirmed in physicochemical method [4]. Recently, death-associated protein kinase (DAPK) was expressed and purified for neutron crystallography, and its X-ray structure was reported [5].

REFERENCES:

[1] T. Chatake, S. Fujiwara, to be published.
[2] Y. Yanagisawa *et al.*, Acta Crystallogr. F66 (2010) F66 (2010) 1670-1673.
[3] Yanagisawa, Y. *et al.*, J. Synchrotron Rad. 20 (2013) 875-879.
[4] Tanaka, I. *et al.*, J. Synchrotron Rad. 20 (2013) 958-61.
[5] Yamaguchi, A. *et al.,* JPS Conference Proceedings, in press.

採択課題番号26P2-2 納豆菌由来生理活性物質をはじめとした生体分子の中性子実験の基礎研究プロジェクト
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PR2-3 Identification and characterization of proteolytic enzymes derived from *NephilaClavata*

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INTRODUCTION: Spiders hunt insects using a web net. The fact that they eat them without chewing, indicate that spiders possesses highly efficient digestive enzymes [1]. Our previous studies suggest that spider's proteolytic enzymes are able to digest synthetic spider dragline amyloid fibers [2,3]. Thus, the spider protease has the potential ability to digest amyloid fibers including pathogenic β-amyloid, such as amyloid fibrils, that are associated with the development of Alzheimer's disease [1]. Therefore, we purified, identified, and characterized the enzymes derived from *Nephila Clavata*. The sequence analyses of the blotted protein on a PVDF membrane showed N-terminal amino acid residues. Based on this sequence information, we tried to clone the protease gene.

EXPERIMENTS: The spiders (*Nephila Clavata*) were collected in the field of Kyoto University Research Reactor Institute and the spider saliva including proteolytic enzymes was obtained from *Nephila Clavata* (50 heads) by electrical stimulation. The extracts were stored in a deep freezer until use.

Casein protease assay [4] of the crude spider enzymes was performed, as previously reported [1]. Peptide substrates for the assay of the protease activity of spider enzymes were chemically synthesized by the Boc solid phase method, treated with hydrogen fluoride, and purified by reversed phase HPLC [1, 4].

The N-terminal protein sequence was determined by the Edman degradation. First, the spider saliva was applied to SDS-PAGE, and the proteins bands were electrically transferred to a PVDF membrane. The electro-blotting of spider proteases was performed using the semi-dry blotting method then applied to then-terminal sequence analysis

Based on the N-terminal sequence, we prepared generated primers for the gene cloning and RT-PCR was performed using mRNA's from gut cells of *Nephila Clavata.*

RESULTS: Spider's digestive fluid that includes proteolytic enzymes was obtained from *Nephila Clavata* by electrical stimulation using a micropipet. The extracts were applied to SDS-PAGE and the enzymatic activity of the protein bands was estimated by a casein protease assay [4]. Two protein bands showed protease activities and their molecular weights were estimated to be approximately 21.9 and 19.5 kDa, based on the SDS-PAGE analysis [1]. To characterize the enzymes, FLGLPA-KADA-NH_2 and SIFKTLRTIA-NH_2 were synthesized as model peptide substrates and treated with the crude spider protease. The results indicated that the spider protease can be classified as a Ca^{2+}-dependent carboxypeptidase.

To further characterize the spider protease, the N-terminal amino acid sequence was determined by Edman degradation using blotted protein bands on PVDF membrane after SDS-PAGE separation. The results suggested the N-terminal amino acid sequence of the spider protease. Based on the amino acid sequence, generated primers for the gene cloning of the protease were prepared and RT-PCR was performed using mRNA's from gut cells of *Nephila Clavata.* Three clones were isolated and provided second PCR to produce cDNA's of spider proteases. The purified candidate cDNA's were inserted pBluescript SKII(-) and sequence analyses were performed. However, the sequence analysis of the cloned spider protease gene revealed that the cloned genes were non-specifically amplified during RT-PCR.

In conclusion, Spider protease was extracted from its digestive fluid and exhibited strong protease activity. The protease can be classified as a Ca^{2+}-dependent carboxypeptidase, based on the results of protease inhibition assays. The N-terminal amino acid sequence of spider protease was determined by Edman degradation. Spider genes were amplified using generating primers, base on the N-terminal amino acid sequence, by RT-PCR and cloned into pBR-SKII(-). However, DNA sequence analysis indicated that the cloned DNA was non-specifically amplified during RT-PCR. The cloning of spider protease genes is in progress.

REFERENCES:

[1] M. Fujiwara *et al.*, *Peptide Science*, **2013,** 447-448 (2014).

[2] S. Zheng, *et al.*, *Appl. Spectrosc.,* **43**, 1269-1272 (1989).

[3] Y. Hidaka, *et al.*, *Biopolymers*, **96**, 222-227 (2011).

[4] C. Dicko, *et al.*, *Biochemistry*, **43**, 1269-1272 (2004).

採択課題番号26P2-3　　ジョロウグモ由来消化酵素の機能評価　　プロジェクト

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