

VIII- II -1. Project Research

Project 7

PR7 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 molecule, neutron is well useful to isolate findings of such reaction mechanisms and can determine a precise position 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 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. Therefore a plan of this study, which is based on the last project (Hydrogen/Deuterium Exchanges in the Protein molecule), has been started in our institute.

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

- (1) Structural investigation of the 20S Proteasome and Anesthetic agents, Y.Morimoto
- (2) Deuterium treatment of DNA molecules in vitro synthesis. T.Chatke
- (3) Ligninase purification for a low carbon energy system by plant-type organic resources. Y.Hidaka
- (4) Deuterium culture of bacterial cells and purification of a deuterated kinase. Y.Yanagisawa

There are the following research activities in this year.

RESULTS:

24P7-1

Y. Morimoto, T. Murakami, Y. Morita, M. Unno, I. Hisatome and H. Yamaguchi reported "Structural Insights of Anesthetic Agents in the Yeast 20S Proteasome". A newly inhibitor of the 20S proteasome, so called an anesthetic agent, binds to the S1-pocket inside the whole 20S particle. The compound is packed among two or three β -strand of the 20S proteasome, and interacts with some amino acids of this 20S proteasome. And the other compounds have been determined and

checked with 20S whole structure complexes. Common structural characters are found in such compounds whose aromatic ring interacting a surrounding amino acid residues in the 20S proteasome subunits. They are good points in a drug designing of a compounds.

24P7-2

T. Chatake, S. Fujiwara, Y. Morimoto reported "Time of flight neutron experiment of Z-DNA hexamer d(CGCGCG)" of the result for the newly time of flight diffractometer in the JPARC. The neutron analysis demonstrated hydrogen and hydration structure of Z-DNA, but some hydrogen networks and water molecules were not well defined. Time of flight neutron experiment (TOF) using high flux of pulsed neutron source is expected to provide better neutron data, which enable to obtain detailed structural information of hydrogen-bonding networks and hydration of Z-DNA.

24P7-3

Y. Hidaka, Y. Morimoto, H. Yamaguchi, Y. Nozu, K. Hosokawa, J. Ishibashi, M. Fujiwara, S. Shimamoto reported "High Efficient Lignin- Degradation System of *Pleurotus Eryngii* Versatile Peroxidase VPL2" focusing to a low carbon energy world. They herein report on the preparation of *Pleurotus eryngii* VPL2 using the *E. coli* expression system. The recombinant VPL2 was produced as a soluble form in *E. coli* cells and purified with several types of chromatography. However, the enzymatic activity of the recombinant VPL2 was too low under the typical condition. Therefore, the recombinant VPL2 was applied to in vitro folding reaction in the presence or absence of heme.

24P7-4

T. Chatake, Y. Yanagisawa, Y. Morimoto reported "Production of deuterated nattokinase from D₂O resistance strain of *Bacillus subtilis natto*". They established purification and crystallization protocols of nattokinase, and its non-hydrogen structure by X-ray crystallography, in order to accomplish neutron crystallography. In this project, we obtained D₂O resistance *Bacillus subtilis natto* by successive cultivation rounds, to obtain better neutron data using smaller crystals. Here, we report production and purification of deuterated NK from D₂O resistance *Bacillus subtilis natto*.

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INTRODUCTION: Proteasomes are widely distributed in eukaryotes, ranging from human to yeasts. In higher eukaryotes, 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. The 20S proteasome forms barrel shape and consists of 4 protein rings arranged each other in parallel plain. Upper two rings are arranged symmetrically with lower two rings and they are called α - and β -rings from outer to inner side. 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. Therefore the inhibitor of 20S proteasome serves as a new anticancer reagent. Here we report on the preparation of yeast 20S proteasome, the enzymatic activities with inhibitor, crystallization, and also on initial determination of the structure.

EXPERIMENTS: Yeast 20S proteasome tagged with affinity peptides was prepared in yeast. Cells were homogenized by glass beads and the crude extracts purified by M2 affinity chromatography and Mono-Q anion exchange one. Isolated 20S proteasome was concentrated by ultrafiltration, and co-crystallized with small ligand molecules. Vapor diffusion method was applied. Crystals are isomorphous as described in the previous paper and belong to the space group $P2_1$. Initial phases were determined by molecular replacement method, and the structure model without ligand was refined by a program *Refmac*.

RESULTS: Fig.1 shows a previous complex model with binding the compound. The compound, such as anesthetic agents, has two kinds of aromatic rings. One of common structure is a benzene ring, it is strongly hydro-

phobic and interacts with Phe, Tyr and Trp amino acid residues existing in a subunit or subunits cleft.

The S1 pocket of the 20S proteasome particle forms an active site degrading unnecessary proteins. This is located inside 20S whole particle. 20S whole structure is composed of 28 subunits and has many crevices within neighboring subunit. A chymotryptic-like active site is composed of Tyr(135), Gly(128) and VAL(129).

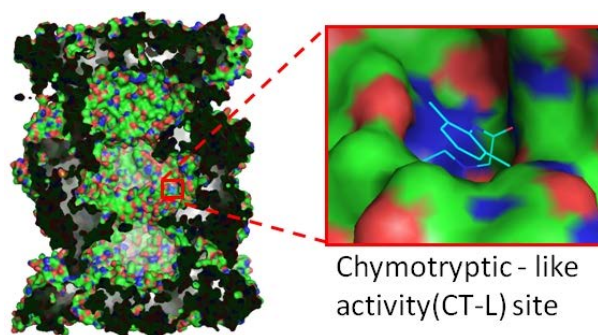


Fig. 1 Structure of 20S proteasome and a ligand complex left: whole 20S proteasome with cross-section, right: a ligand stick model in space filling S1 pocket of the 20S

Such an aromatic ring interacts with those amino acid residues by ring-stacking force. It may confirm the other compound reagents bind on the same place or position, so another three compounds have been determined in the 20S complex analysis by synchrotron radiation sources.

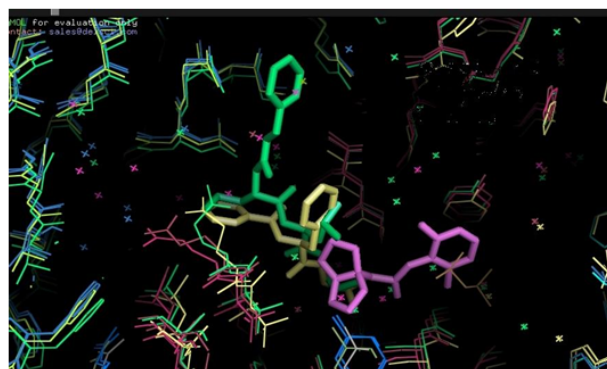


Fig.2 Three kinds of compound held at the 20S proteasome active site by each host particle. Structures are superimposed by a least-square fitting as minimum root-mean square distances.

Results show possible inhibition with proteasome activities are found in biological assay and actual complex crystal structures. It may suggest the drug designing of such compounds led by use of the result, and more we are preparing crystals by means of co-crystallization or inhibitor-soaking crystal method as *in vivo* situation. Analysis of their structure is now under progress.

PR7-2 Time of Flight Neutron Experiment of Z-DNA Hexamer d(CGCGCG)

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INTRODUCTION: DNA is a flexible biopolymer, which changes its helical conformation depending on its environment [1]. Z-DNA is a left-handed double helical structure of DNA, which was found in CG-rich DNA sequence and/or high-salt condition [2]. So far, we determined neutron structure of Z-DNA d(CGCGCG) obtained from D₂O solution using a monochromatic neutron experiment [3]. Although the neutron analysis demonstrated hydrogen and hydration structure of Z-DNA, some hydrogen networks and water molecules were not well defined. Time of flight neutron experiment (TOF) using high flux of pulsed neutron source is expected to provide better neutron data, which enable to obtain detailed structural information of hydrogen-bonding networks and hydration of Z-DNA. In addition, data collection of undeuterated crystal of Z-DNA would be available by the combination of high-flux neutron and TOF method. In the present study, crystallization of Z-DNA hexamer d(CGCGCG) in H₂O solution was carried out using temperature control technique [4], and a preliminary neutron experiment was performed.

EXPERIMENTS: DNA hexamer d(CGCGCG) was purchased from Hokkaido Science C.C. 1 ml solution containing 4 mM Z-DNA, 20 mM sodium cacodylate buffer (pH 7.0), 20 mM NaCl, 20 mM MgCl₂, 12 mM spermine tetrahydrochloride and 10% MPD, was sealed in a 1.5 ml micro tube, and placed in a heat block. The solution was incubated at 348K overnight, and cooled to 293K with the rate of 2.5 K/day. A largest crystal was sealed in a NMR capillary ($\phi=4$ mm) with crystallization solution, and the capillary was transported in a vacuum bottle from KUR to J-PARC at Tokai.

Neutron experiment was carried out with the iBIX diffractometer at J-PARC [5], operating at 280 kW. Thirty WLSF area detectors were used in this experiment. The range of wavelength were adjusted from 1.2 - 5.2 Å. The exposure time was 15.5 hours. The neutron data was visualized and analyzed using the program STARGazer [6]

RESULTS: The largest Z-DNA crystal obtained from undeuterated crystallization solution was a hexagonal plate-like crystal with the volume of about 0.5 mm³. The hexagonal plane corresponded to an *ab* plane of its unit cell. Although the edges of the crystal were sharp, some cracks were observed with a microscope. Fig. 1 shows a diffraction image on a detector of iBIX. In this picture, the integrated intensities were sliced into several dozens along temporal axis, in order to clarify each diffraction spot. This images was approximately parallel to *ab* plane,

and each diffraction had a good shape, suggesting the quality of this crystal was quite good as for *a* and *b* directions of the unit cell. In this direction, the highest resolution, where the diffraction could be detected, was 1.85 Å (a left picture in Fig. 2). This value was comparative to the highest resolution of the previous neutron experiment of deuterated Z-DNA crystal (1.8 Å). On the other hand, in *c* direction of the unit cell, the diffraction quality was not enough to integrate intensities. As shown in a right picture of Fig. 2, an unique diffraction was split into a few spots, indicating that the present crystal was polycrystal. In order to solve this problem, there are two strategies; (1) to cut the crystal into several single crystals, and (2) to obtain better crystals by further purification of Z-DNA prior to crystallization.

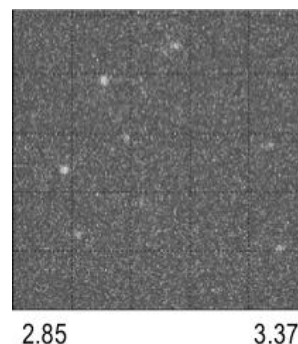


Fig. 1. A diffraction image of the Z-DNA crystal in the range of medium resolution.

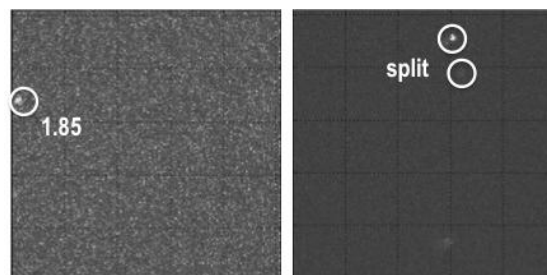


Fig. 2. Diffraction images at the highest resolution (left), and where an unique diffraction spot was split (right).

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INTRODUCTION:

Lignin peroxidase plays an important role in the biodegradation of the plant cell wall, consisted of lignin. Versatile oxidase (VP) has been recently reported as a new family of lignolytic peroxidase, together with lignin-peroxidase and manganese peroxidase, *pleurotus eryngii* produces a versatile peroxidase which is able to oxidize both Mn²⁺ and aromatic compounds. Thus, the most interesting feature of VP is that it possesses substrate specific characteristics of the other peroxidase families. Therefore, it is very useful to degrade phenolic, non-phenolic lignin dimer, and a numerous aromatic compounds to obtain low molecular organic compounds as an energy source.

To obtain recombinant VPL2, we herein report on the preparation of *Pleurotus eryngii* VPL2 using the *E. coli* expression system [1,2]. The recombinant VPL2 was produced as a soluble form in *E. coli* cells and purified with several types of chromatography. However, the enzymatic activity of the recombinant VPL2 was too low under the typical condition [1]. Therefore, the recombinant VPL2 was applied to in vitro folding reaction in the presence or absence of heme.

EXPERIMENTS:

The cDNA encoding *Pleurotus eryngii* VPL2 was chemically synthesized and cloned into the pET17b and pET32b expression vectors between the *NdeI* and *XhoI* sites. *E. coli* cells, transformed with the VPL2 expression vector, were incubated at 37C in LB media supplemented with ampicillin (50 mg/L) for 18hr. The cells were harvested, washed with Tris/HCl buffer (pH7.5), sonicated, centrifuged (15,000 x g, 15min), and applied to SDS-PAGE. The recombinant VPL2 was purified by a Ni-affinity chromatography. The purified recombinant VPL2 was refolded in the presence or absence of heme

and the enzymatic activity was estimated for lignin-peroxidase and Mn²⁺ peroxidase activity.

RESULTS:

To obtain a soluble and active form of *Pleurotus eryngii* VPL2, several types of VPLS fusion proteins were expressed in *E. coli* cells. We found that Thioredoxin-fused VPL2 was well expressed as a soluble form in *E. coli* BL21(DE3) T7 Shuffle cells, in which DsbC protein is over-expressed for the disulfide exchange system, as shown in Fig. 1. Therefore, the recombinant protein was over-expressed and purified by Ni-affinity chromatography.

The enzymatic ability of the recombinant VPL2 was estimated by lignin-peroxidase and manganese peroxidase assays. VPL2 from *Pleurotus eryngii* showed a strong activity for the assay but the recombinant VPL2 possessed only a weak activity for each assay. Therefore, we are currently investigating the refolding condition of the recombinant VPL2 in the presence or absence of heme to obtain its fully active form.

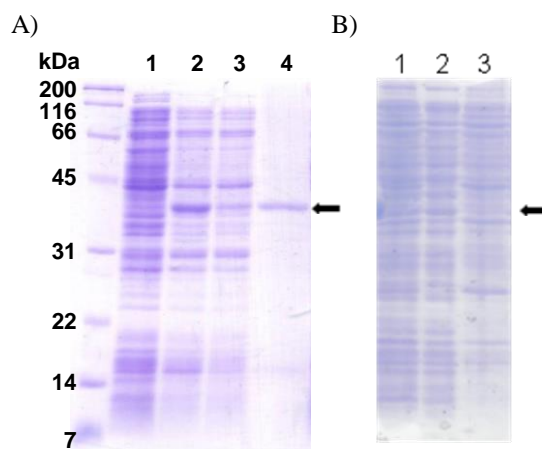


Fig. 1 SDS-PAGE of the recombinant VPL2 expressed in BL21(DE3) (A) and T7 Shuffle (B) cells. A: lane1, *E. coli* cells without the expression plasmid; lane 2, total cells; lane3, supernatant; lane4, precipitates. B: lane 1, total cells; lane2, supernatant; lane3, precipitates.

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INTRODUCTION: Nattokinase is fibrinolytic enzyme, which is produced in abundance by *Bacillus subtilis natto* [1]. This enzyme is contained in Japanese traditional fermented food 'natto'. Nattokinase belongs to the subtilisin family, but it has substrate specificity different from popular subtilisin such as subtilisin BPN' and subtilisin Carlsberg [2]. Molecular simulation predicted the importance of hydrogen environments around Ser221 at the active site [3]; therefore neutron protein crystallography is expected to provide valuable information about enzymatic mechanisms of this enzyme. So far, we established purification and crystallization protocols of nattokinase, and its non-hydrogen structure by X-ray crystallography [4], in order to accomplish neutron crystallography. In this project, we obtained D₂O resistance *Bacillus subtilis natto* by successive cultivation rounds [5], to obtain better neutron data using smaller crystals. Here, we report production and purification of deuterated NK from D₂O resistance *Bacillus subtilis natto*.

EXPERIMENTS: *Bacillus subtilis natto Miyagino* (BSNM) was used as the starter culture. The precultured medium was transferred into 5 ml of liquid medium containing 2% polypeptone S, and 3% glycerol BSNM, and incubated at 310 K. The activity of NK was assessed using the fibrin plate method. The BSNM medium, which has the highest activity of NK in the assessment, was used for the next starter culture. Contamination of H₂O into medium was checked using neutron transmission [6], because all cultivations were carried out in an open system. Neutron transmission was measured using the 4CND at KUR. The results of the neutron experiment indicated that the contamination was negligible.

Deuterated NK was purified from the cultured medium of D₂O resistance strain of BSNM. Undeuterated NK from native BSNM was also purified for comparison. The supernatant of 11 ml cultured medium was filtered, and then concentrated to 1 ml. At first, the solution was partially purified on a gel filtration (Sephadex G10). The second purification step involved hydrophobic interaction chromatography using a butyl-Sepharose FF column with a gradient from 2 to 0 M ammonium sulfate. Final purification was performed using a Sephacryl-S100 column and the resulting solution was concentrated to 45 µl.

RESULTS: The concentration of D₂O was gradually increased in successive cultivations. Finally, D₂O resistance strain of BSNM (BSNM-D₂O) that grew in 100% deuterated medium and produced sufficiently good yields of NK was obtained after 7 and 8 passage cultiva-

tion steps.

Purification was succeeded as shown in Fig. 1. Major peaks in the both charts of final chromatography corresponded to NK. Molecular weight was confirmed by 15% SDS electrophoresis. The electrophoresis also suggested self-digestion of NK, which sometimes happen in NK production. Fig. 2 is a photograph of fibrin plate 18 hour after applying deuterated and undeuterated NK, indicating that both of the NKs kept fibrinolysis activity after the present purifications.

In conclusion, deuterated NK could be obtained from NSBM-D₂O by small-scale cultivation, and purification could be in success.

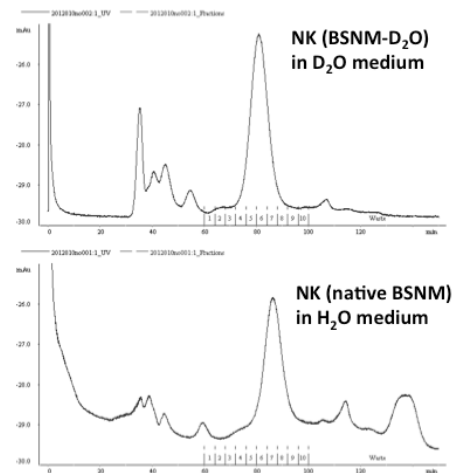


Fig. 1. Chromatography charts of the final purification of deuterated NK from BSNM-D₂O (upper), and undeuterated NK from native BSNM (lower).

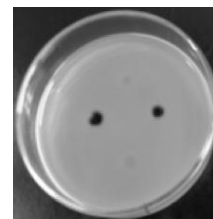


Fig. 2. The result of the fibrin plate test of deuterated NK (left) and undeuterated NK (right).

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