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[Original paper]

Hydroxyl groups of threonines contribute to the activity of Ca^{2+}-depdendent type II antifreeze protein

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Type II antifreeze protein from herring and smelt (AFPII) exhibits thermal hysteresis (TH) activity when the protein is saturated with Ca^{2+}. The ice-binding site of AFPII consists of Thr96, Thr98, and two Ca^{2+}-coordinating residues. Here we examined TH activity of AFPII from Japanese smelt (*Hypomesus nipponensis*) and its mutant proteins, T96S, T96V, T96A, T98S, T98V, and T98A. Note that T96S denotes a mutant whose Thr96 is replaced with Ser. It appeared that substitution of Thr96 with Ser and Val retained 90% and 45% of TH activity, respectively, while it becomes inactive when substituted with Ala. For Thr98, Ser substitution had no significant influence on TH, whereas it becomes less than 20% by Val and Ala substitutions. These results suggest that hydroxyl group rather than methyl group of Thrs are essential for TH activity of AFPII, which is quite distinct from the other types of AFP.

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INTRODUCTION

Antifreeze protein (AFP) identified from body fluids of various cold-tolerant organisms can bind to a seed ice crystal emerged in the process of water freezing, which results in depression of non-colligative freezing temperature. A difference between the freezing and melting temperatures of AFP solution is generally termed thermal hysteresis (TH), which has been used as an indicator of the strength of ice-binding ability of AFP [1].

Type II AFP is a globular protein of approximately 14 kDa and adopts a tertiary fold similar to a carbohydrate recognition domain of Ca^{2+}-dependent (C-type) lectin [2,3]. This type of AFP is subdivided into two species, one requires Ca^{2+} but the other does not for the TH activity [4-6]. In the present study, AFPII denotes the Ca^{2+}-requiring species of type II AFP. AFPII has been isolated from herring (*Clupea harengus,* hAFP), rainbow smelt (*Osmerus mordax,* rsAFP), and Japanese smelt (*Hypomesus nipponensis,* jsAFP) [5-8]. Sequence identity among AFPIIs is

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approximately 80%. All AFPIIs contain five Ca^{2+}-coordinating residues (Gln92, Asp94, Glu99, Asn/Asp113, and Asp114) corresponding to those located in a Ca^{2+}-binding region of C-type lectin [3,9]. Site-directed mutagenesis for hAFP has proven that the Ca^{2+}-binding site is involved in an ice-binding surface of AFPII [10,11]. In addition, X-ray and mutation studies on hAFP indicated that ice-binding site of AFPII consists of Thr96, Thr98, and the two Ca^{2+}-coordinating residues (Asp94 and Glu99), all of which are conserved in the other species of AFPII [3].

It is a common knowledge that Thr residues play a critical role for ice binding in various types of AFP. For example, α-helical AFPI protrudes 4 Thrs on one side of the molecule to interact with ice surface. Interestingly, Val substitutions of these Thrs caused no significant change of TH, so that it was speculated that methyl group of 4 Thrs have significant contribution to TH rather than their hydroxyl group [12-15]. For β-helical insect AFP, both methyl and hydroxyl groups of Thrs on one flat face of the molecule fit to the oxygen atom positions of ice prism plane [16,17]. For AFPII, however, contribution of side-chain groups of Thr residues to TH was not clarified yet.

The aim of our study is to clarify TH dependence on the side-chain groups of Thr96 and Thr98 of AFPII. We initially prepared a recombinant protein of jsAFP using yeast *Pichia pastoris*, and examined whether TH activity of recombinant protein is indistinguishable from that of native protein. We then generated T96S, T96V, T96A, T98S, T98V, and T98A mutants of jsAFP. As these mutations caused no significant change on the Ca^{2+}-affinity, we could discuss TH dependence on the types of side-chain group.

MATERIALS AND METHODS

Purification of native jsAFP

Native jsAFP was purified from the muscle of Japanese smelt as described previously [7] with the following modifications. After 35-60% ammonium sulfate fractionation, the precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer. The precipitate formed was removed by centrifugation at 15000×*g* for 15 min at 4°C and the supernatant was purified using Sephadex G-75 (Amersham Biosciences) equilibrated with 20 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$. The fraction containing jsAFP was loaded to High Q column (Bio-Rad Laboratories) equilibrated with 20 mM potassium phosphate buffer (pH 7.4), and jsAFP was eluted by a linear NaCl gradient from 0 to 0.4 M. The reverse-phase HPLC using C_{18} column (TOSOH, Tokyo, Japan) was further applied to purify jsAFP, which was eluted by a liner gradient of 40-55% acetonitrile with 0.1% trifluoroacetic acid.

Digestion of native jsAFP with N-glycosidase

After dissolving the lyophilized native jsAFP with 20 mM sodium phosphate buffer (pH 7.8) to be a 50 μM protein sample, *N*-glycosidase F (Roche Diagnostics) was added to 4 μl of the sample. After incubation of the reaction mixture for 24 h at 37°C, its molecular weight was checked by 16% poly-acrylamide gel SDS-PAGE.

Construction of a synthetic jsAFP gene

A gene encoding jsAFP was designed by utilizing the codon usages of *P. pastoris* with *Xho* I and *Xba* I recognition sites at 5' and 3' termini, respectively. This gene was prepared from 10 pairs of oligonucleotide, which were separately annealed and phosphorylated at 5' terminal in a reaction mixture containing 1 mM of ATP and 1 unit of T4 polynucleotide kinase (TaKaRa). After being incubation for 1 h at 37°C, the kinase was inactivated by heating (95°C) for 5 min. Each oligonucleotide mixture was then heated at 75°C for 5 min and cooled slowly to 30°C to form complementary strands. All phosphorylated oligonucleotide pairs were mixed and annealed in

a reaction mixture containing 40 mM NaCl, 10% PEG 6000, and 1 unit of T4 DNA ligase (TaKaRa). To prevent *N*-linked glycosylation, codon for Asn12 in the obtained gene was substituted with that for Asp by polymerase chain reaction (PCR). The amplified DNA was ligated into pZErO-1 (Invitrogen), the resultant plasmid was named jsAFPN12D/pZErO-1. The mutation at positions 96 and 98 was performed by primer directed PCR, for which jsAFPN12D/pZErO-1 was used as a template.

Recombination of jsAFP and mutants

Plasmid jsAFPN12D/pZErO-1 was initially digested with *Xho* I and *Xba* I, and the obtained DNA fragment was ligated into *P. pastoris* expression vector pPICZα (Invitrogen). This plasmid was linearized by *Sac* I, and then introduced into methylotrophic yeast *P. pastoris* X-33 strain (Invitrogen). The transformant was incubated using high cell-density fermentation [18] with following modifications. The medium pH was maintained at 5.5 and methanol was fed at 23°C for 4 days. After fermentation, the medium was centrifuged at 10000×*g* for 30 min at 4°C, and the supernatant was 5-times diluted with 20 mM sodium phosphate buffer (pH 7.4) containing 500 mM NaCl. Recombinant jsAFP was purified by Ni-Sepharose High Performance coulmn (Amersham Biosciences) followed by gel-filtration chromatography (Sephadex G-75) [8]. Approx. 1.6 mg of purified recombinant jsAFP was obtained from 1 liter of fermentation medium. Purity of the sample and the proper cleavage of α-factor secretion signal sequence were confirmed by reverse-phase HPLC and N-terminal amino acid sequence analysis, respectively. The concentration of both jsAFP and mutants was determined by measuring the absorbance at 280 nm using the extinction coefficient of 4.25×10^4 M^{-1} cm^{-1}.

Fluorescence Spectroscopy

We used XFLUOR4 (TECAN) fluorescence spectrophotometer for 0.2 mM protein sample with an optical cuvette of 10 mm pathlength. The sample buffer was 20 mM Tris-HCl (pH 7.5) containing less than 1 mM $CaCl_2$. All samples were excited at 280 nm, and the emission was monitored from 300 to 400 nm at 1 nm intervals.

Ca^{2+}-induced change of the relative intensity (f) was fitted to the following equation:

$$f = [(K[Ca^{2+}]+KC+1) + \{(K[Ca^{2+}]+KC+1)^2 - 4K^2[Ca^{2+}]C\}^{1/2}] /2KC$$

where $[Ca^{2+}]$ = Ca^{2+} concentration, C = protein concentration, K = Ca^{2+}-binding constant.

TH measurements

TH measurement was carefully performed by observation of an ice crystal prepared in the sample by using our in-house photomicroscope system with a cooling rate of 0.01 °C/min [19]. The non-equilibrium freezing point (T_f) of the sample was defined as the temperature where rapid growth of the ice crystal was observed along the c-axis direction. TH value of the sample was subsequently determined as a difference between equilibrium melting temperature (T_m) and T_f of the ice crystal. This TH measurement was repeated for 3 times using fresh sample, and averaged value was evaluated with error. The protein samples were dissolved in 20 mM Tris-HCl (pH 7.5) with or without 10 mM $CaCl_2$ to give a final protein concentration of 0.05 ~ 1.0 mM. For the measurements of Ca^{2+} dependent change of TH, 0 ~ 5 mM $CaCl_2$ was added to 1.0 mM of the sample solution.

RESULTS AND DISCUSSION

N-linked glycosylation of native jsAFP

For native jsAFP, molecular weight of 16.8 kDa was determined by mass spectroscopy [7]. This value is not, however, consistent with the

estimated amount of 14.4 kDa based on the 130-residue primary sequence determined from cDNA. We hence examined glycosylation of native jsAFP prior to the construction of the gene expression system. Figure 1 shows SDS-PAGE of *N*-glycosidase-treated and native jsAFP. As shown, molecular weight of the treated jsAFP is ~14 kDa, which is consistent with the estimation from the primary sequence (14.4 kDa). In contrast, the non-treated jsAFP is estimated to be a 17 kDa protein, which is consistent with the result of mass spectroscopy (16.8 kDa). These results suggest that *N*-linked glycosylation occurs for native jsAFP. When primary sequence was analyzed for native jsAFP, N-terminal 40-residue sequence is in accordance with that deduced from cDNA analysis, excepting unidentified Asn12, which was while identified in the previous report [7]. Since Asn12 corresponds to a glycosylation site of the consensus signal sequence (Asn12-Gly13-Thr14) for *N*-linked glycosylation, it could be assumed that the *N*-linked glycosylation occurs at Asn12.

Similar to jsAFP, the *N*-linked glycosylation was identified for rsAFP, but not for hAFP. In addition, TH activity is indistinguishable between rsAFP and hAFP. Hence, one can speculate that glycosylation has no significant effect on TH [20-21].

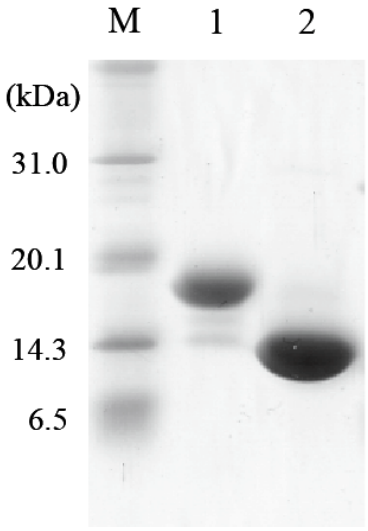

Fig. 1. SDS-PAGE of purified jsAFP. Lane M: molecular size marker, Lane 1: native jsAFP, Lane 2: jsAFP treated with *N*-glycosidase.

Comparison of TH activity

Figure 2a compares photomicroscope images of an ice crystal observed for solutions of native and recombinant (de-glycosylated) jsAFPs in the presence and absence of Ca^{2+} ion. As shown, two proteins inhibit the crystal growth strongly only in the presence of Ca^{2+}, as evidenced by formation of an ice bipyramid along the *c*-axis direction.

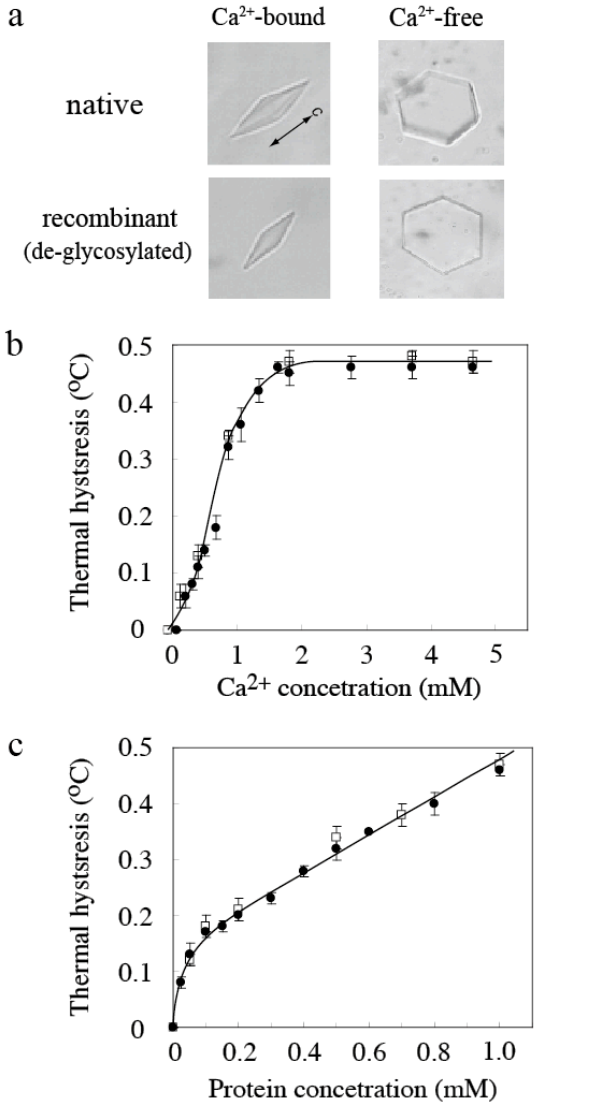

Fig. 2. Antifreeze activity of native and recombinant jsAFP. (a) Comparison of ice crystal morphology. (b) Ca^{2+} dependence of TH of native (□) and recombinant jsAFP (●). (c) Concentration dependence of TH of native (□) and recombinant jsAFP (●) in the presence of 10 mM of Ca^{2+} ion. Each point represents the mean of three measurements.

Figure 2b plots Ca^{2+} dependence of TH for native and the recombinant jsAFPs (1mM). It appeared that the obtained curves are perfectly identical, which gives 2.5 x 10^5 M^{-1} of the Ca^{2+}

binding constant for both proteins. Figure 2c plots TH dependence on the concentration of native and recombinant jsAFPs in the presence of excess amount of Ca^{2+} (10 mM). As shown, no significant difference between the two proteins was detected in a whole concentration range (0 ~ 1.0 mM). All these results indicate that TH activity of the recombinant jsAFP without glycosylation is indistinguishable from that of native protein. The observed bipyramidal ice crystal and TH strength of the recombinant jsAFP (e.g. TH = ~0.3°C at 0.5 mM protein) are also comparable to those of other AFPIIs [11].

Ca^{2+}-binding property of the mutants

We generated T96S, T96V, T96A, T98S, T98V, and T98A mutants of de-glycosylated jsAFP by site-directed mutagenesis. Neither Thr96 nor 98 are involved in Ca^{2+}-coordination, but are located in the vicinity of Ca^{2+}-coordinating residues [3]. We hence examined influence of the mutations on Ca^{2+}-binding before evaluation of TH, since Ca^{2+}-binding is the primary request for the TH activity. We monitored Ca^{2+}-induced spectral change of intrinsic tryptophan fluorescence, and found for all mutants that a peak observed at 353 nm changes its intensity as a function of $[Ca^{2+}]/[protein]$. The intensity change generally reflects a fraction of Ca^{2+}-bound form of a protein; i.e., a position of the peak (353 nm) is attributable to exteriorized Trp residues [22]. For native jsAFP, relative change profile of the fluorescence intensity was perfectly identical to Fig.2b, indicating that our employed fluorescence experiments can determine the Ca^{2+}-binding constant of recombinant jsAFP and its mutants. For T96S, T96V, T96A, and T98S mutants, the Ca^{2+}-binding constant of 2~2.5 x 10^5 M^{-1} was evaluated, indicating that the mutations have no significant influence on the Ca^{2+}-binding property. The binding constant of approximately 6 x 10^4 M^{-1} was while evaluated for T98V and T98A mutants, indicating a slight decrease of Ca^{2+}-affinity of the proteins. In the following TH measurements, we added excess amount (10mM) of Ca^{2+} to all mutants to saturate their Ca^{2+}-binding site, so that we can neglect the mutation effect on the Ca^{2+}-binding and focus the effect on TH activity.

TH activity of the mutants

TH activity of T96S, T96V, and T96A are plotted in Figure 3a, and that of T96S, T96V, and T96A in Figure 3b, respectively. In both figures 'Wild-type' indicates the data of the recombinant de-glycosylated jsAFP.

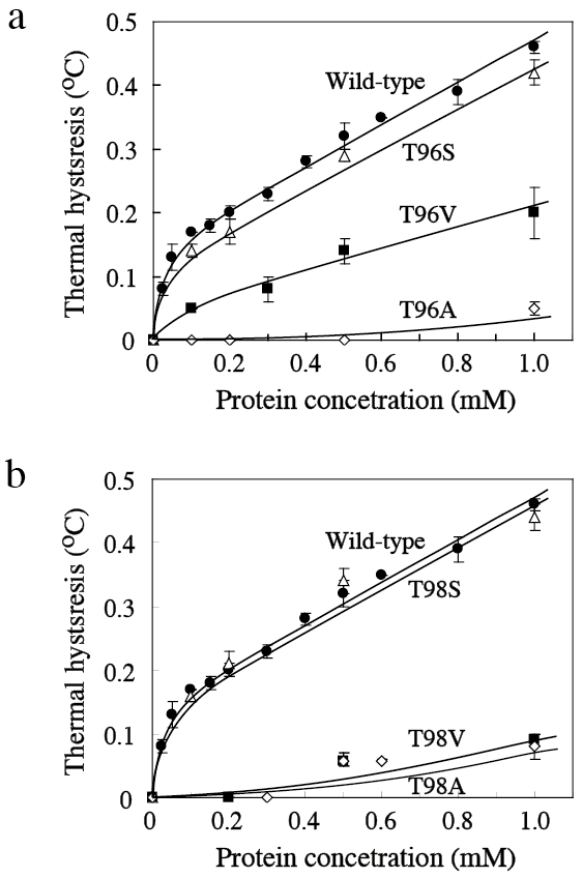

Fig. 3. TH dependence on the concentration of jsAFP mutants. (a) T96S (△), T96V (■) and T96A (◇), (b) T98S (△), T98V (■), and T98A (◇). Wild-type (●) represents the data for the recombinant de-glycosylated jsAFP. The samples are dissolved in to a buffer contains 20 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$.

It was found from Fig. 3 that substitution of Thr96 with Ser and Val retains approximately 90% and 45% of the TH activity of wild-type, respectively, while it becomes inactive when substituted with Ala. For Thr98, Ser mutant also

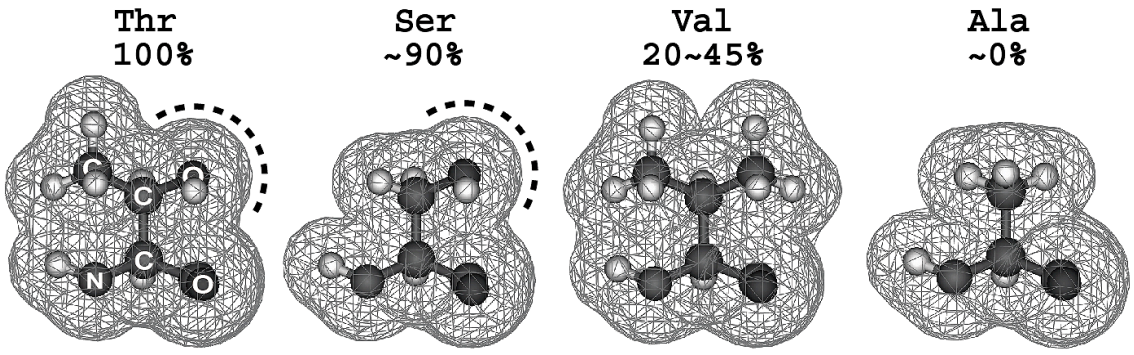

Fig. 4. Illustrations of van der Waals surface of Thr, Ser, Val, and Ala residues. The percentage (%) shows TH activity evaluated from Fig. 3.The sidechain group is drawn toward the upper side of the figure. Broken lines indicate polar surface of the sidechain. The illustrations were produced with Discovery Studio 2.0, Accelrys Software Inc.

retains TH activity, whereas it becomes less than 20% by substitutions with Val and Ala residues, respectively.

In general, Thr residues are located at a surface of various types of AFP, and involved in the binding to the surface of an ice crystal. As for the structurally simplest α-helical AFPI, Thr residues are spaced 16.5 Å apart at 2, 13, 24, and 35 positions of the 37-residue AFPI sequence [1]. Since this interval almost ideally matches the oxygen atom spacing of 16.7 Å in the target ice plane $\{2\,0\,\bar{2}\,1\}$, an earlier model assumed that both methyl- and hydroxyl-groups of the four Thr residues (denoted TTTT) is the key determinant of ice binding of AFPI [13]. However, site-directed mutagenesis showed in later that only methyl group but not the hydroxyl group of threonines are the principal requirement for the ice binding; i.e., the residual TH activity of 0, 0, 100, and 30~50% were obtained for AFPI mutants of TSST, SSSS, VVVV, and AAAA, respectively [12-15]. The most striking result was the detection of full activity for VVVV, which has no potential of hydrogen bonding in their sidechains. Similar results were also obtained for type III AFP (AFPIII), in which Ala substitution of the ice binding residues, Thr15 and Thr18, retains 70~80% of TH activity [23].

The present results obtained for AFPII are largely distinct from those for AFPI and III. As shown in Fig. 3, the full TH activity was not preserved in the Val and Ala mutants, while it was almost perfectly retained in the Ser mutants. There exists polar surface in Thr and Ser, while it does not in Val and Ala (Fig. 4). These results suggest that hydroxyl groups in the sidechains of Thr96 and 98 are the principal requirements for the ice binding of AFPII. In other words, hydrogen bonding of the two threonines is crucial for the activity of AFPII. These results are consistent with the recent ice-binding model for herring AFPII [3]. Liu et al. (2007) showed that the ice-binding residues, Thr96, Thr98, Asp94, and Glu99 are able to form hydrogen bond with ice [3], which is further strengthened by the bound Ca^{2+} ion through the coordination with oxygen atom of the ice lattice.

To summarize, we succeeded expression of AFPII from Japanese smelt (jsAFP), and found that TH activity is indistinguishable between the de-glycosylated (recombinant) and glycosylated (native) jsAFPs. Substitutions of the two ice binding residues, Thr96 and Thr98, with Ser, Val, and Ala do not significantly lower the Ca^{2+}-affinity of jsAFP, while decrease TH activity differently in the presence of excess amount of Ca^{2+}. Since no significant TH change is detected only for the Ser substitutions, it is concluded that hydroxyl groups rather than methyl groups of the Thr residues located in the ice binding surface may significant for the ice binding of AFPII.

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