Process of Thermal Denaturation of Xylanase (XynB) from Clostridium stercorarium F-9

Masayuki Fukumura, Akiyoshi Tanaka, Kazuo Sakka, and Kunio Ohmiya

Faculty of Bioresources, and * Department of Chemistry, Faculty of Education, Mie University, Tsu 514, Japan
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The thermal denaturation process of Clostridium stercorarium F-9 xylanase (XynB) was studied by monitoring remaining activity and recovered activity of the enzyme. At pH 5.5, aggregation occurred rapidly after the thermal denaturation initiated. The aggregated protein could be dissolved in 8 M urea solution, and the enzyme activity was recovered by diluting the urea. The extent of the recovered activity was gradually decreased with two phases as the reaction time of the thermal denaturation became longer. These results suggested the thermal denaturation process to be as follows:

\[ \text{N} \xrightarrow{k_1} \text{D1} \xrightarrow{k_2} \text{D2} \quad \text{and} \quad \text{D2} \xrightarrow{k_3} \text{D3} \]

where N is the native state of the enzyme; D1 is the denatured state of the enzyme that is formed rapidly after the reaction started and can be renatured by the urea treatment, and D2 and D3 are the denatured states of the enzyme that cannot be renatured even by the urea treatment. The rate constants were \( k_1 = 9.2 \), \( k_2 = 0.33 \), and \( k_3 = 0.57 \), and \( k_3 = 0.13 \) (in min\(^{-1}\) unit).

Xylan, a major component of hemicellulose in plant cell walls, is the most abundant form of biomass second to cellulose and is now regarded as a natural resource that is convertible to biofuels, chemicals, and value-added compounds by microbial fermentation or enzymatic processes. In recent years, thermostable xylanases have received considerable attention for potential applications in biomass conversion strategies and in biobleaching of pulp, i.e., mild and safe procedures for pulp improvement by xylanase treatment. Study of the molecular events that take place in proteins on heating should be of great practical interest.

The xynB gene encoding a xylanase XynB was cloned from a thermophilic, strictly anaerobic, cellulolytic, and xylanolytic bacterium, Clostridium stercorarium F-9. The deduced amino acid sequence of XynB showed that the protein consists of 387 amino acids containing no cysteine residue with a molecular weight of 44,377. The gene product translated in a recombinant Escherichia coli showed both cellulolytic and xylanolytic activities in the same manner as the enzymes in family F. The purified XynB with a molecular weight of 41,000, which was processed at the N-terminal by host protease(s) to release 12 amino acids, showed maximum activity at 85°C but no activity at 100°C and pH 6.5. However, the enzyme in the absence of substrate recovered more than half of its initial activity when it was chilled in ice water. This phenomenon was not observed at pH 5.5.

This paper reports the process of the thermal denaturation of XynB.

Materials and Methods

**Enzyme source.** The recombinant XynB was purified from E. coli JM109 (pMF6) containing the xynB gene as described previously (M. Fukumura, manuscript submitted to this journal); XynB was purified from a total cell-free extract by ammonium sulfate precipitation and DEAE-Toyopearl 650S (Tosoh Co., Ltd., Tokyo, Japan), Butyl-Toyopearl 650S (Tosoh), and the TSK-gel G3000SW (Tosoh) column chromatographies. The purest preparation, homogeneity of which was confirmed by SDS-polyacrylamide gel electrophoresis and isoelectric focusing gel electrophoresis, was used for study of thermostability of XynB. The protein concentration of the enzyme was measured using the extinction coefficient \( E_{280nm}^{1\%} \) of 1.5.

**Enzyme assay.** XynB is active on p-nitrophenyl-β-D-cellobioside (PNP; Sigma Chemical Co., St. Louis, U.S.A.) as well as xylan, and the strength of its cellobiosidase activity is parallel to that of xylanase activity. Therefore, the enzyme activity was measured using PNP. The reaction mixture (0.4 ml) containing 20 mM PNP in 50 mM sodium phosphate-12 mM sodium citrate buffer (pH 6.3, PC buffer) was incubated at 60°C for 10 min and the release of p-nitrophenol from PNP was measured spectrophotometrically at 405 nm after addition of 0.6 ml of 1 M sodium carbonate.

**Evaluation of \( K_a \) and \( V_{max} \).** Steady state kinetics parameters, the Michaelis constant \( K_a \) and the maximum velocity \( V_{max} \), for the hydrolysis of PNP were evaluated from suitable weighted \( 1/s \) vs. \( 1/v \) plots, where \( s \) is the substrate concentration and \( v \) the initial velocity.

**Calorimetry.** Differential scanning calorimetry (DSC) was done with an adiabatic scanning microcalorimeter, DASM-4 (Biopirron, Moscow, USSR) with an NEC personal computer, at a scan rate of 1.0 K⋅min\(^{-1}\). Before the measurements, the protein solutions were dialyzed against 50 mM phosphate buffer (pH 7.0) or 50 mM succinate-NaOH buffer (pH 5.5) at 4°C three times. The protein concentration in each measurement was 1.1 mg/ml. The dialysate was used for reference.

**Measurement of remaining activity.** XynB solutions (500 μl, 13 μg/ml) at various pHs dispensed in microfuge tubes were incubated at 100°C for various periods. After the XynB solutions were quickly chilled in ice water, the enzyme activity was measured at 60°C with PNP as a substrate, which is referred as the “remaining activity” throughout this study.

At pH 7.0 of 50 mM phosphate buffer and pH 5.5 of 50 mM succinate-NaOH buffer, solutions of the enzyme at a protein concentration of 13 μg/ml were incubated at 100°C. Samples were periodically withdrawn and were chilled in ice water.

**The effects of urea and guanidine hydrochloride on aggregated XynB and measurement of recovered activity.** When XynB (500 μl, 0.4 mg/ml) was kept at pH 5.5 and 100°C, aggregates of XynB were formed. The resulting aggregates were collected by centrifugation and dissolved in 500 μl of denaturant solutions such as urea and guanidine hydrochloride (GdmHCl). The mixtures were diluted 30-fold to lower the concentration of denaturants and the enzyme activity was measured. This enzyme activity is referred as...
the "recovered activity".
Dependence of the recovered activity on the incubation time at 100°C was observed as follows: solutions of the enzyme at a protein concentration of 0.4 mg/ml were incubated at 100°C and pH 5.5. Samples were periodically withdrawn and chilled in ice water. Resulting aggregates of XynB were collected by centrifugation at 4000×g for 10 min and were dissolved in 8 M urea at pH 6.4. The enzyme thus obtained was diluted 30-fold for assay of recovered activity.

Far UV CD spectra. The far UV CD spectra of the native XynB and the thermally-denatured XynB were measured with a Jasco spectropolarimeter, Model j-720 (Japan Spectroscopic Co., Ltd, Tokyo, Japan) at a protein concentration of 0.4 mg/ml in the absence and presence of 8 M urea with far UV light in a 1-mm path length cell. The spectra were recorded at a scanning speed of 10 nm min⁻¹. The reported C.D. values were the average of at least three independent measurements.

Results
pH dependence of thermostability of XynB
The remaining activity of XynB was measured after incubation at 100°C for 10 min at various pHs. As shown in Fig. 1, about 50% of the original activity was observed at pH 6.1 and 60% from pH 7.0 to 9.0. XynB was completely inactivated below pH 5.8.

Figure 2A shows the courses of the remaining activity observed at pH 5.5 (○) and 7.0 (●). At pH 5.5, the enzymatic activity was abolished within 30 s. In contrast, the XynB activity was gradually lost at pH 7.0. The inactivation of XynB at pH 7.0 was not explained by a first-order reaction, as shown in Fig. 2B, where the semilogarithmic plot of the remaining activity vs. incubation time is depicted.

Enzymatic activity at 100°C
Figure 3A shows effects of temperature on activities of XynB. XynB was optimally active around 80°C and inactive at 100°C. Figure 3B shows DSC traces of XynB obtained at pHs 5.5 and 7.0. The shape of the DSC trace did not depend on pH. A fairly large excess specific heat due to the unfolding reaction of XynB was observed between 80°C and 90°C. These results indicate that XynB begins to denature at around 80°C and is completely denatured at 100°C, and that the remaining activity detected at 60°C after incubation in the pH range of 6.1 to 9.0 at 100°C is due to the activity of the enzyme that recovered its native structure by correct protein refolding from the denatured state during chilling in ice water.

Fig. 1. pH Dependence of Thermostability of XynB at 100°C.
XynB (13 μg/ml) was incubated at 100°C at the given pHs for 10 min followed by chilling on ice. The remaining activity of XynB was measured at 60°C using PNPC as a substrate. Buffers used: pH 5.5 and 5.8, 50 mM succinate-NaOH buffer; pH 6.1, 50 mM sodium phosphate-sodium citrate buffer; pH 7.0, 8.0, 9.0, and 10.0, 50 mM Britton–Robinson’s universal buffer.

Fig. 2. (A) Course of Remaining Activity of XynB at pH 7.0 (●) and 5.5 (○). (B) the Semilogarithmic Plot of Remaining Activity of XynB at pH 7.0.
XynB (13 μg/ml) was incubated at 100°C. Samples were periodically withdrawn and chilled in ice water. Remaining activity was assayed at 60°C using PNPC as a substrate. Solid line is a theoretical curve drawn based on Eq. (1).

Fig. 3. Effects of Temperature on XynB.
(A): Effects of temperature on the activity of XynB. The enzyme reaction was done at pHs 5.5 (●) and 7.0 (○), at given temperatures for 10 min using PNPC as a substrate.
(B): DSC curves observed with XynB (1.1 mg/ml) at pHs 7.0 and 5.5.

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* Maximum value of the excess specific heat was 7.9 J K⁻¹ g⁻¹, and the enthalphy change of the unfolding reaction was estimated to be 26 J g⁻¹ at 85°C.
Thermal Denaturation of Xylanase

**Fig. 4.** Effects of Denaturants on the Activity Recovery of the Thermally Aggregated XynB.

Aggregates formed by heating for 5 min at 100°C and pH 5.5 were dissolved with GdnHCl and urea. The solution was diluted 30-fold to measure the recovered activity. Denaturant used: GdnHCl at pHs 6.4 (●) and 4.6 (▲), urea at pH 6.4 (○), urea at pH 5.5 (△).

**Fig. 5.** Far UV CD Spectra of Native XynB and XynB Resolved with Urea.

(a) native XynB of 0.4 mg/ml in 50 mM succinate-NaOH buffer, pH 5.5.
(b) XynB (0.4 mg/ml) dissolved in 8 M urea at pH 6.4 after thermally aggregated at pH 5.5. The mixture was centrifuged at 15,000 × g for 15 min and then filtered to remove residual aggregates.

**Urea treatment of the denatured and aggregated XynB**

When a XynB solution (0.4 mg/ml) of pH 5.5 was kept at 100°C for 5 min, a large quantity of precipitate without any activity was formed. After centrifugation at 4000 × g for 10 min, no XynB was detected in the supernatant by protein assay. The aggregated XynB was dissolved in various concentrations of urea and GdnHCl, diluted 30-fold with PC buffer, and the recovered enzyme activity was measured. As shown in Fig. 4, up to 50% of the enzyme activity was recovered by the urea or GdnHCl treatment. The effect of urea was dependent on pH (cf. ○ and △), while that of GdnHCl was independent of pH (cf. ● and ▲). Even lower concentrations of GdnHCl had some effects on the aggregated XynB (Fig. 4).

Although the aggregated XynB could be dissolved in 8 M urea solution, it showed no activity in 8 M urea, but recovered its activity immediately after the dilution of urea. Figure 5 shows the far UV CD spectra of native XynB (a) and the enzyme which was thermally aggregated and dissolved in 8 M urea solution (b). The structure of XynB in 8 M urea solution is far from the native structure, showing that the aggregated XynB is dissolved by urea treatment. These results suggest that its native structure is restored by rapid refolding after dilution of urea.

**Extent of the recovered activity on the reaction time of thermal denaturation of XynB**

XynB solution was kept at pH 5.5 and 100°C for various periods. The aggregated enzyme collected were treated with 8 M urea to dissolve, and the recovered enzyme activity was measured. The recovered activity gradually diminished as incubation time at 100°C became longer. The extent of the recovered activity on the reaction time of thermal denaturation does not obey first order kinetics as shown in a semilogarithmic scale in Fig. 6. This course of the recovered activity is similar to that of the remaining activity observed at pH 7.0 shown in Fig. 2.

**K_m and V_max values**

For XynB that was kept at 100°C and pH 5.5 for various periods and was treated with 8 M urea, K_m and V_max values were measured. Table shows K_m and V_max values. V_max values decreased gradually from 67 to 19 nmol · μg⁻¹ · min⁻¹ in proportion to the incubation period at 100°C, while the K_m was constant.

**Discussion**

Renaturation of the aggregated enzyme dissolved by urea treatment was dependent on pH as shown by Fig. 4 (○ and △). At low pHs, XynB with a pI of 4.26 loses electrical charge, and heating at 100°C seems to allow XynB to expose its hydrophobic regions and to aggregated by probably hydrophobic interaction. Although the aggregated XynB can be dissolved with 8 M urea even at pH 5.5 as shown
by Fig. 4 (Δ), it does not recover the enzyme activity, suggesting that some of the electrical change(s) on a peptide region of XynB are necessary for correct refolding to the native structure. Since GdnHCl is strong electrolyte and electrostatic interaction has little or no importance in concentrated GdnHCl, the influence of pH on the refolding of XynB may be negligible.

XynB thermally aggregated at acidic pHs also recovered its enzyme activity completely by urea treatment (Fig. 6).

It is very interesting that some part of the enzyme activity was restored by the urea treatment. For example, although the enzyme kept at 100°C for 0.5 min had no activity at pH 5.5 (Fig. 2), 78% of the original activity was restored by the urea treatment. The ratio of the restored activity decreased as incubation time at 100°C increased. This indicates that the inactive enzyme species that is formed rapidly after the thermal inactivation initiated can be renatured by urea treatment, and this enzyme species converts slowly into other denatured states which cannot be renatured by the urea treatment.

Open circles in Fig. 6 show two phases. From the figure, the apparent rate constant of the first faster phase, \( k_{\text{rapid}} \), was evaluated to be 0.90 min\(^{-1}\), and that of the second slower phase, \( k_{\text{slow}} \), to be 0.0425 min\(^{-1}\). A minimum and possible mechanism that can explain the result shown in Fig. 6 and Fig. 2A (●) is as follows (at 100°C):

\[
N \xrightarrow{k_1} D1 \xrightarrow{k_2} D2 \xrightarrow{k_3} D3
\]

where N is the native enzyme, D1 is a denatured state of the enzyme which is formed rapidly after the thermal denaturation starts and can be renatured by the urea treatment; D2 and D3 are denatured states of the enzyme that cannot be renatured by the urea treatment; \( k_1 \), \( k_2 \), \( k_3 \) and \( k_4 \) are the rate constants as indicated in Eq. (1). In this model, the remaining activity at pH 5.5 demonstrated in Fig. 2 corresponds to the relative amount of N, and the recovered activity in Fig. 6 corresponds to the amount of D1. Since the remaining activity at incubation time 0.5 min is less than 0.01%, the value of \( k_1 \) is greater than 9.2 min\(^{-1}\).

The values of rate constants \( k_2 \), \( k_3 \), and \( k_4 \) were evaluated by the least squares method to be 0.33 min\(^{-1}\), 0.57 min\(^{-1}\), and 0.13 min\(^{-1}\), respectively, by assuming that the first step in Eq. (1) is sufficiently faster than the other steps. The solid line in Fig. 6 is a theoretical value of the amount of D1 calculated based on Eq. (1) using the values of these rate constants thus obtained. The experimental values of the recovered activity and the theoretical value of the amount of D1 agreed, indicating that the equation 1 is one of the most probable models for explaining the thermal denaturation of the enzyme. If we can suppose that the second step is faster than the third step, two apparent rate constants \( k_{\text{rapid}} \) and \( k_{\text{slow}} \) can be expressed using \( k_2 \), \( k_3 \), and \( k_4 \) as follows:

\[
k_{\text{rapid}} (=0.90 \text{ min}^{-1}) = k_2 + k_3
\]

\[
k_{\text{slow}} (=0.0425 \text{ min}^{-1}) = k_2 \cdot k_3 / (k_2 + k_3)
\]

Three rate constants \( k_2 \), \( k_3 \), and \( k_3 \) obtained above also satisfy Eqs. (2) and (3).

It is reasonable from Eq. (1) that \( V_{\text{max}} \) decreased with increasing incubation time at 100°C as shown in the Table, since \( V_{\text{max}} \) is proportional to the concentration of the active (native) enzyme, and the concentrations of D2 and D3, which cannot be renatured by the urea treatment, increase with longer incubation time. \( K_m \), which is usually regarded as the dissociation constant of the enzyme-substrate complex, was independent of the incubation time, suggesting that the structure of the renatured enzyme by the urea treatment is the same as that of the native enzyme.

Equation (1) is also valid for the denaturation mechanism at pH 7.0, by regarding D1 as the enzyme species that is denatured and inactive at 100°C, but is easily renatured by lowering the temperature. Assuming again that the first step is sufficiently faster than the second or the third step, the values of \( k_2 \), \( k_3 \), and \( k_4 \) for the thermal denaturation at pH 7.0 can be evaluated by the least squares method to be 0.42, 1.01, and 0.13 min\(^{-1}\), respectively. The solid line in Fig. 2 is the theoretical value of the ratio of D1 calculated by using these values.

In conclusion, the XynB aggregated by heat treatment at pH 5.5 could be dissolved in 8 M urea solution, and the enzyme activity was recovered by diluting the urea. The extent of the recovered activity was gradually decreased with two phases as the reaction time of the thermal denaturation longer, indicating that the aggregated XynB has at least 3 species of denatured state.

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References