Alkaline serine proteinase from *Thermomonospora fusca* YX

Stability to heat and denaturants

Magnus M. KRISTJANSSON and John E. KINSELLA*

Institute of Food Science, Cornell University, Ithaca, NY 14853, U.S.A.

The serine proteinase isolated from *Thermomonospora fusca* YX shows considerable thermal stability up to 80 °C, and progressive inactivation occurs at higher temperatures. Lyotropic salts affected the thermal stability of the enzyme at 85 °C, suggesting that disruption of hydrophobic interactions play an important role in the decreased thermal stability of the enzyme above 80 °C. Thermal stability is highly pH-dependent; above pH 6.0–6.5 there is a sharp decrease in the stability of the enzyme, reflecting increased autolysis. Although some stabilization occurs upon increasing ionic strength, Ca²⁺ binding does not appear to play a role in thermal stability. Denaturants, i.e. 8 M-urea, 6 M-guanidinium chloride or 1 % SDS, had no significant effect on the activity of the enzyme after 24 h at 25 °C.

INTRODUCTION

Proteins from thermophilic micro-organisms are of special interest because of their intrinsic thermal stability and, in addition, because they provide experimental material for studying thermophily and stability at elevated temperatures. Thermostable enzymes are also of considerable commercial interest for some industrial applications [1–3]. Commercially, proteinases are probably the most important class of industrial enzymes, and proteinases from thermophilic micro-organisms, because of their high thermal stabilities, are of interest for several potential biotechnological applications [1,4]. To aid both basic and industrial applications, knowledge of the stability characteristics of these enzymes under different conditions is required. Furthermore, studies of thermal stability under different conditions provides information concerning the molecular forces involved in stabilizing enzymes. In a previous paper [5] we described some of the properties of YX-proteinase, an extracellular proteinase from the thermophilic bacteria *Thermomonospora fusca* YX. This enzyme is an alkaline serine proteinase belonging to the chymotrypsin family of serine proteinases [5]. In the present study we determined the thermal stability characteristics of YX-proteinase under different conditions. The effects of pH and uni- and bi-valent salts were studied to determine whether ionic interactions influenced stability. Because Ca²⁺ binding stabilizes some thermostable serine proteinases [6–9], the effect of Ca²⁺ on the thermal stability of the enzyme was assessed. In order to estimate the contribution of hydrophobic interactions to the stability of the enzyme, the thermal stability of the proteinase in the presence of common different lyotropic salts was determined. The stability of the enzyme in the presence of common denaturants is also reported.

EXPERIMENTAL

Materials

YX-proteinase was isolated and purified as described previously [5,10]. CaCl₂, MgCl₂, KCl, NaH₂PO₄, Na₂HPO₄, Na₂SO₄, sodium acetate, NaNO₃ and urea were all purchased from Mallinkrodt (St. Louis, MO, U.S.A.). Trizma base, NaSCN, guanidinium chloride, guanidine thiocyanate were obtained from

Sigma Chemical Co. (St. Louis, MO, U.S.A.), and EDTA and SDS were obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). All these reagents were of analytical grade.

Thermal-stability measurements

The time-dependence of thermal inactivation of the proteinase at 75–90 °C was determined by heating enzyme solutions (20 µg of proteinase/ml, dissolved in 25 mM-Tris/acetate buffer, pH 6.2, containing 0.3 M-NaCl and 10 mM-CaCl₂) in sealed tubes at the different temperatures. After a specific heating time the samples were cooled on ice and assayed for remaining proteolytic activity using succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide (Suc-AAPF-NA) as described previously [5]. The residual activity was determined by comparing the enzyme activity after heating to that of freshly made unheated enzyme solutions. The activation parameters for the thermal inactivation of the proteinase at these temperatures were calculated from the first-order inactivation plots. The inactivation rate constants were obtained from the slopes of the plots and were used to calculate the activation free energies of inactivation ($\Delta G^\ddagger$) at each temperature according to the equation [11]:

$$\Delta G^\ddagger = -2.303RT \left[\log \left(\frac{k_i}{T} \right) - \log \left(\frac{R}{Nh} \right) \right] \quad (1)$$

where k_i is the first-order rate constant, R is the universal gas constant, N is Avogadro's number, h is Planck's constant and T is the absolute temperature. The activation energy (E_a) for the thermal inactivation of the enzyme was obtained from the slope of the Arrhenius plot [$E_a = -R(\text{slope})$] [2]. Activation enthalpies ($\Delta H^\ddagger$) at each temperature were then calculated using the relationship $\Delta H^\ddagger = E_a - RT$ [3], and from the known values of $\Delta G^\ddagger$ and $\Delta H^\ddagger$, the activation entropies at each temperature were calculated from:

$$\Delta S^\ddagger = \Delta H^\ddagger - \Delta G^\ddagger / T \quad [4,11]$$

The thermal stability of the proteinase in presence of different concentrations of CaCl₂, MgCl₂, KCl and NaCl was determined by heating the enzyme dissolved in 25 mM-imidazole buffer, pH 6.2, containing specific concentrations of each salt in sealed tubes for 30 min at 80 °C. Enzyme activity was measured before

Abbreviation used: Suc-AAPF-A, succinyl-L-Ala-L-Ala-L-Pro-L-Phe *p*-nitroanilide.

* To whom correspondence and reprint requests should be sent.

and after heating to determine residual activity at each salt concentration.

The pH-stability profile of the enzyme was studied using the following buffers: 25 mM-sodium acetate, pH 3.65–5.2; 25 mM-sodium phosphate, pH 5.7–8.1; 25 mM-Tris/acetate, pH 6.0–7.5; and sodium borate, pH 8.3–9.8. All buffers were made up to 1.0 l with NaCl. The pH measurements (at 80 °C) were made with a Corning pH-meter (model 120). Thermal stability at each pH was assessed by incubating the enzyme (20 $\mu\text{g}\cdot\text{ml}^{-1}$) in the appropriate buffer for 30 min at 80 °C, and enzyme activity was measured before and after heating to determine residual activity.

Thermal stability of YX-proteinase in the presence of different lyotropic salts was measured as described above. The enzyme concentration was 20 $\mu\text{g}\cdot\text{ml}^{-1}$, as determined by the Lowry method [12], and the buffer was 25 mM-Tris/acetate at either pH 6.2 or 7.7 (at 85 °C) containing 10 mM- CaCl_2 and 0.6 M of the specific lyotropic salt (NaSO_4 , NaCl, sodium acetate, NaNO_3 or NaSCN).

To determine the stability of the proteinase towards denaturants, the enzyme (30 $\mu\text{g}\cdot\text{ml}^{-1}$) was incubated with 6 M-guanidinium chloride, 8 M-urea or 1% SDS, in sealed tubes for 24 h at 25 °C. The buffer used was 0.1 M-Tris/HCl, pH 7.0. A control without added denaturant was also incubated for the same extent of time. The activity of the enzyme against Suc-AAPF-NA was measured before and after the incubation period to determine remaining activity.

Thermal-transition curves for the unfolding of YX-proteinase in the presence of guanidine thiocyanate was determined by monitoring the decrease in fluorescence intensity of the emission spectra of the enzyme at 330 nm, after incubation for 1 h at 25 °C in the presence of different concentrations of guanidine thiocyanate in 50 mM-sodium phosphate, pH 6.0. The excitation wavelength was 286 nm and the fluorescence-emission spectra of the enzyme solutions and of the corresponding blanks between 320 and 370 nm was recorded with a Perkin-Elmer 650-40 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.). The transition curves were normalized according to the equation:

$$f_n = \frac{(y - y_u)}{(y_n - y_u)} \quad (2)$$

where f_n is the fraction of the enzyme in its native state, y is the fluorescence intensity of the sample at 330 nm (minus blank) at a given guanidine thiocyanate concentration and y_u and y_n are the values for the unfolded and native enzyme extrapolated to the concentration of guanidine thiocyanate in the transition region.

RESULTS AND DISCUSSION

Thermal stability of proteinase

The thermal inactivation of YX-proteinase at 75, 80, 85 and 90 °C is depicted in Fig. 1. Inactivation followed first-order kinetics at all these temperatures, suggesting that, under the conditions used in these experiments, thermal unfolding of the protein, rather than autolysis, was primarily responsible for the inactivation. The slope of the Arrhenius plot (Fig. 1), constructed from the first-order rate constants at each temperature, corresponded to an activation energy of 389.5 $\text{kJ}\cdot\text{mol}^{-1}$, reflecting high thermal stability of the proteinase. Other calculated activation parameters are listed in Table 1. Over the temperature range between 75 and 90 °C, neither $\Delta H^\ddagger$ nor $\Delta S^\ddagger$ were significantly affected by temperature, whereas $\Delta G^\ddagger$ decreased linearly with temperature, in accordance with the equation:

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger.$$

Fig. 1. First-order plots for the thermal inactivation of YX-proteinase

The enzyme (20 $\mu\text{g}\cdot\text{ml}^{-1}$) was incubated at the appropriate temperature in 25 mM-Tris/acetate, pH 6.2, containing 0.3 M-NaCl and 10 mM- CaCl_2 . Aliquots were withdrawn to determine remaining activity expressed relatively to that of an unheated sample. The inset shows an Arrhenius-plot for thermal inactivation of YX-proteinase.

Table 1. Thermodynamic activation parameters for thermal inactivation of YX-proteinase

T (°C)	$\Delta G^\ddagger$ ($\text{kJ}\cdot\text{mol}^{-1}$)	$\Delta H^\ddagger$ ($\text{kJ}\cdot\text{mol}^{-1}$)	$\Delta S^\ddagger$ ($\text{kJ}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$)
75	115.9	386.6	0.78
80	112.3	386.5	0.78
85	108.6	386.5	0.78
90	104.5	386.4	0.78

According to these results, the thermal inactivation of YX-proteinase can be attributed to a temperature effect, i.e. input of thermal energy increases structural fluctuation in the protein, weakens or disrupts non-covalent bonds (such as hydrogen bonds and Van der Waals forces in the transition state), facilitating denaturation of the enzyme. It has been proposed that kinetic thermal stability should be used for defining thermostable enzymes [6]. According to this definition, a thermostable enzyme has a $\Delta G^\ddagger \geq 104.6 \text{ kJ}\cdot\text{mol}^{-1}$ (25.0 $\text{kcal}\cdot\text{mol}^{-1}$), at 70 °C, under optimal conditions of pH, ionic strength etc. [6]. By extrapolation of the Arrhenius plot (Fig. 1) and using eqn. (1), a value of G of 121.1 $\text{kJ}\cdot\text{mol}^{-1}$ was obtained

Fig. 2. Effect of increasing ionic strength on thermal solubility of YX-proteinase

The enzyme ($20 \mu\text{g} \cdot \text{ml}^{-1}$) dissolved in 25 mM-imidazole buffer, pH 6.2, containing the different concentrations of the salts, was heated at 80°C for 30 min before assaying for remaining activity. Residual activity is expressed as relative to that of unheated sample. Symbols: ●, CaCl_2 ; □, MgCl_2 ; △, NaCl; ○, KCl.

for the stability of YX-proteinase at 70°C . This value is almost identical with what has been reported for thermolysin under similar conditions and about $8.4 \text{ kJ} \cdot \text{mol}^{-1}$ higher than that of the thermostable serine proteinase thermomycolase [6]. The YX-proteinase is also considerably more stable than either subtilisin Carlsberg or BPN [6].

Effect of CaCl_2 and other salts

In contrast with observations with many microbial serine proteinases, e.g. subtilisin [6,7], thermitase [9] and thermomycolase [6–8,13], the specific binding of Ca^{2+} did not enhance the thermal stability of YX-proteinase. When stability of the enzyme was measured at 80°C in the presence or absence of 15 mM- CaCl_2 (I 0.2 M set with NaCl), no effect of added CaCl_2 was observed when thermal stability was measured in low-ionic-strength ($<0.3 \mu\text{M}$) buffers. Some degree of stabilization was observed upon the addition of CaCl_2 . A comparable effect was exerted by similar ionic strengths using MgCl_2 , NaCl or KCl (Fig. 2). It was therefore concluded that the stabilizing effect was not specific for CaCl_2 , but could be attributed to a charge-shielding effect by the salts. Thus, by interacting with surface charges on the protein, the ions may block repulsive charge interactions that contribute to destabilization of the enzyme. Hence, shielding of these charges may enhance the stability of the enzyme.

Effect of pH

The thermal stability of YX-proteinase at 80°C is highly pH-dependent (Fig. 3). Above pH 4, the stability reaches a plateau until above pH 6.5, where a sharp decrease in thermal stability occurred. When the enzyme was incubated under identical conditions (as in Fig. 3) in the pH range 6–8, a rapid increase in the rate of autolysis was observed (results not shown). Furthermore, the conformational stability of the proteinase, inhibited by phenylmethanesulphonyl fluoride (to prevent autolysis), was not diminished at pH 8.0 compared with pH 6.0 (as determined by denaturation of the enzyme by guanidine thiocyanate at

Fig. 3. pH-stability profile of YX-proteinase at 80°C

Samples were incubated for 30 min before assaying for remaining activity relative to that of an unheated sample. Buffer concentrations were 25 mM and I was adjusted to 0.1 with NaCl. △, sodium acetate; ●, sodium phosphate; ○, Tris/acetate; □, sodium borate.

25°C), indicating that autolysis rather than diminished conformational stability is responsible for the decrease in thermal stability of the proteinase between pH 6 and 8.

Effect of lyotropic salts

To assess the contribution of hydrophobic interactions to stabilization of the proteinase, the effect of different lyotropic salts on the kinetic thermal stability of the enzyme was studied at 85°C . If perturbation of hydrophobic interactions is involved in the sharp decrease in thermal stability of the enzyme occurring above 80°C (Fig. 1), it is expected that lyotropic salts would affect the stability of the enzyme at these temperatures, i.e. chaotropes (such as SCN^-) would be expected to decrease the stability, whereas cosmotropes (like SO_4^{2-}) should stabilize the enzyme [14–17]. Lyotropic salts at a concentration of 0.6 M had a significant effect on the stability of YX-proteinase at 85°C and pH 6.2 (Fig. 4). The salts tested affected the stability of the enzyme in the order: $\text{SO}_4^{2-} > \text{CH}_3\text{COO}^-$ (acetate) $> \text{Cl}^- > \text{NO}_3^- > \text{SCN}^-$, in accordance with their ranking in the lyotropic salt or Hofmeister series. A comparable effect of the salts were observed at pH 7.7 (results not shown). An estimate of the stabilizing/destabilizing effect of the salts may be obtained from the difference in activation free energies of thermal inactivation in presence or absence of salts, according to the expression:

$$\Delta(\Delta G^\ddagger) = \Delta G_0^\ddagger - \Delta G_s^\ddagger \quad (5)$$

where $\Delta G_0^\ddagger$ and $\Delta G_s^\ddagger$ are the activation free energies in the absence or presence of salts respectively, calculated according to eqn. (1) above. The calculated values of $\Delta(\Delta G^\ddagger)$ at both pH 6.2 and 7.7, at 0.6 M-lyotropic salt, are listed in Table 2. The salts had a similar effect on the stability of the proteinase at both pH values, although a somewhat smaller effect was observed at pH 7.7. The rates of inactivation, however, were between 2.4 and 3.3 times higher at pH 7.7 than at pH 6.2, corresponding to about 2.6 – $3.6 \text{ kJ} \cdot \text{mol}^{-1}$ difference in stability of the enzyme at these two pH values. This was consistent with the pH-stability profile of the proteinase (Fig. 3).

The mechanism by which lyotropic salts affect protein stability

Fig. 4. Effect of lyotropic salts (0.6 M) on thermal stability of YX-proteinase, at 85 °C at pH 6.2

The proteinase ($20 \mu\text{g}\cdot\text{ml}^{-1}$) was dissolved in 25 mM-Tris/acetate buffer, containing 10 mM- CaCl_2 and the different salts at 0.6 M. Samples were heated at 85 °C and assayed at intervals for activity. Residual activity is expressed as relative to that of an unheated sample. Symbols: ●, no added salt; ○, Na_2SO_4 ; ▲, sodium acetate; □, NaCl; △, NaNO_3 ; ■, NaSCN.

Table 2. Effect of lyotropic salts on thermal stability of YX-proteinase at 85 °C and at pH 6.2 or pH 7.7

Salt (0.6 M)	pH...	$\Delta(\Delta G^\ddagger)$ ($\text{kJ}\cdot\text{mol}^{-1}$)	
		6.2	7.7
None		0	0
NaSCN		3.97	3.11
NaNO_3		0.90	0.47
NaCl		-1.62	-1.51
Sodium acetate		-2.37	-
Na_2SO_4		-	-6.10

has been explained by their effect on water structure and hence on the strength of hydrophobic interactions [14,16]. The stabilizing effect of lyotropic salts has been correlated with their ability to increase the surface tension of water [17–19]. Melander & Horvath [17] suggested that the effect of lyotropic salts on hydrophobic interactions is best described by their effect on the surface tension of water and is quantified by their molal surface increment, a parameter that, according to these authors, forms a logical basis for the lyotropic series. The correlation between hydrophobic interactions and the surface tension of water is expected if hydrophobicity arises from the energy required to make a cavity for exposed non-polar groups of proteins in water [20]. The free energy associated with the formation of such a cavity in the solvent ($\Delta G_{\text{cav.}}$) can be expressed by the relationship [17]:

$$\Delta G_{\text{cav.}} = [NA + 4.8N^{\frac{1}{3}}(k^e - 1)V^{\frac{2}{3}}]\gamma^0 + [NA + 4.8N^{\frac{1}{3}}(k^e - 1)V^{\frac{2}{3}}]\sigma m \quad (6)$$

where N is Avogadro's number, A is the molecular surface area of the protein molecule, V is the molar volume of the solvent, γ^0

Fig. 5. Relationship between the relative change in free energy of activation $\Delta(\Delta G^\ddagger)$ for thermal inactivation of YX-proteinase, at 85 °C in the presence of 0.6 M lyotropic salts and the molar surface tension increments of the salts

The $\Delta(\Delta G^\ddagger)$ values are from Table 2 and the values for σ were obtained from [17]; ●, pH 6.2; △, pH 7.7. Note: 1 dyn = 10^{-5} N (SI unit).

is the surface tension of pure water, m is the molality of the salt and σ is the molal surface tension increment and is a constant for a given salt. k^e corrects the macroscopic surface tension of the solvent to molecular dimensions [17]. Assuming that A , k^e and V are unaffected by the presence of salt, it can be expected from [6] that, at a given salt concentration, $\Delta G_{\text{cav.}}$ should be directly proportional to σ , the molal surface tension increment of the salt. If hydrophobicity arises from the free energy required to form a cavity for the non-polar groups in the solvent, it follows that, if the salts are affecting the hydrophobic interactions of the protein, the observed stabilization effect should be directly proportional to σ . In the present study a plot of $\Delta(\Delta G^\ddagger)$ in the presence of salts and their respective surface tension increments (Fig. 5) gave a linear relationship. This result indicates that the primary effect of the lyotropic salts on the thermal stability of YX-proteinase can be ascribed to their effects on hydrophobic interactions. The marked lyotropic effect strongly suggest the significant contribution of hydrophobic interactions to the stability of this enzyme in the thermal-transition region around 85 °C.

Stability towards denaturants

No significant loss of activity resulted from incubation of YX-proteinase with 8 M-urea or 6 M-guanidinium chloride for 24 h at 25 °C, and only a negligible activity loss resulted from incubation with 1% SDS under the same conditions. In the presence of 5.6 M-guanidine thiocyanate, however, a complete inactivation of the enzyme occurred within 10 min. The stability of the proteinase towards guanidine thiocyanate was further investigated by determining the unfolding of the enzyme by this denaturant. A normalized transition curve for the unfolding (monitored by changes in fluorescence intensity), at pH 6.0 and 25 °C, is shown in Fig. 6. The midpoint of the unfolding transition was at 2.9 M-guanidine thiocyanate, but concentrations as high

Fig. 6. Guanidine thiocyanate (GuSCN) denaturation of YX-proteinase at 25 °C and pH 6.0

The buffer used was 50 mM-sodium phosphate.

as 4 M were required to denature fully the enzyme after 1 h incubation under those conditions.

Although there is no simple generally accepted mechanism for the mode of action of the different denaturants, it is most likely that they diminish the strength of hydrophobic interactions [20]. The remarkable stability of YX-proteinase towards denaturants is a further indication of the importance of hydrophobic interactions for the stability of this proteinase. High stability towards denaturants has also been reported for some other microbial proteinases, such as subtilisin [21,22], proteinase B from *Streptomyces griseus* [23] and the thermophilic proteinases caldolyisin [24] and aqualysin I [25].

On the basis of its anomalous elution from a gel-filtration column YX-proteinase may possess a compact globular structure [10]. It may be that a tight folding of the protein, facilitating dense packing of hydrophobic residues, as well as a possible exclusion of water molecules from contact with those hydrophobic residues in the protein interior, may contribute to stabilization of the enzyme. A high glycine content of the proteinase [5] is noteworthy in this respect. Glycine, because of its small size and possible unusual dihedral angles, can feed the main chain through tight segments in the protein molecule [26], thus giving rise to a possibility of closer packing of the polypeptide chain.

This work was supported in part by the Cornell Biotechnology Program.

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Received 14 November 1989/5 March 1990; accepted 9 March 1990