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Differential scanning calorimetric studies of the thermal unfolding of acid proteinase A from *Aspergillus niger* at various pHs[1]

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Abstract

The thermal unfolding of acid proteinase A, isolated from *Aspergillus niger*, was studied by differential adiabatic scanning calorimetry at various pHs ranging from 2 to 9. The temperature of the maximal excess heat capacity was strongly dependent on pH and highest at a pH around 3, whereas the enthalpy change of the unfolding was maximal at a pH around 5. The excess heat capacity curve at a pH below 6 showed a single asymmetric peak. In contrast, the unfolding at a pH above 6 exhibited an excess heat capacity curve which is characterized by two sharp and broad peaks. The broad peak observed at about 40°C at a pH above 6 was found to be unchanged irrespective of the pH of the solution. Curve resolution revealed that the unfolding at a pH range between 5 and 6 is characterized by a simple two-state transition, with dissociation to the light and heavy peptide chains.

Keywords: DSC; Proteinase A; *Aspergillus niger*

1. Introduction

Acid proteinase A is a non-pepsin type proteinase isolated from *Aspergillus niger* var.

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macrosporus and is composed of two peptide chains with 173 (heavy chain) and 39 (light chain) amino acid residues [1]. The enzyme is formed by the processing of a single chain proenzyme [2] and the two chains are bound non-covalently. The amino acid composition is known to be quite different from that of typical acid proteases classified as the pepsin family, and the amino acid sequence has approximately 50% homology with that of acid proteinase B from *Scytalidium lignicolum* [3] which belongs to the same subclass of the non-pepsin type acid proteinases [2].

The thermal denaturation of pepsin has been previously studied by Privalov et al. [4] and Makarov et al. [5]. Privalov and his co-workers reported in their DSC study that pepsin has the native structure with four domains at neutral pH. This work was attempted to obtain quantitative information about the structural stability of this enzyme. The thermal unfoldings were carefully studied by the DSC technique at various pHs and the thermodynamic properties of the enzyme are discussed on the basis of the excess heat capacity curve observed during heating.

2. Experimental

Acid proteinase A was prepared according to the method described previously [1,6]. The purified enzyme was dialyzed against the appropriate buffer solutions overnight at ice-cold temperature. After appropriate dilution by the buffer dialyzates, the protein concentration was adjusted on the basis of specific absorptivity at 280 nm of 1.63 $cm^2\ mg^{-1}$. The buffers employed were phosphate (pH 2.1–3.0 and 6.3–7.5), citrate (pH 3.0–3.5), acetate (pH 4.5–5.8), cacodylate (pH 6.0–6.7), pyrophosphate (pH 8.1) and carbonate (pH 9.2) at a concentration of 0.05 M and containing 0.2 M NaCl, unless otherwise specified.

2.1. Differential scanning calorimetry

The thermal unfolding of the enzyme was measured by a differential adiabatic scanning microcalorimeter, DASM-4 (Mashpriborintorg, Moscow) designed by P.L. Privalov [7,8] equipped with a data processing unit. The measurements were made immediately after the preparation of the sample solution by dialysis. The buffer dialyzates were used as a reference solution for DSC measurements. The protein concentration used for the DSC measurement was 3 mg ml^{-1} unless otherwise specified. The heating rate employed was usually 1 K min^{-1}.

3. Results and discussion

Typical excess heat capacity curves observed at various pHs are shown in Fig. 1. From the figure it is obvious that the thermal unfolding of acid proteinase A is strongly dependent on pH, and the mid-point temperature of unfolding, T_m, as judged from the peak temperature, is maximal at a pH around 3. In contrast, the enthalpy change of the unfolding evaluated from the area under the excess heat capacity curve after establishing

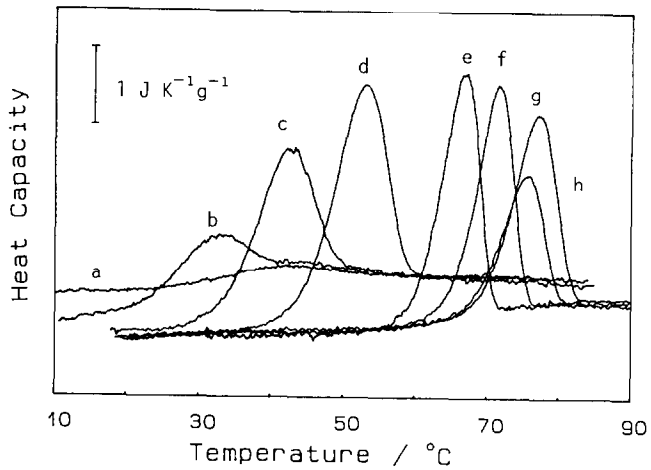

Fig. 1. DSC tracings of acid proteinase A observed at various pHs. (a) pH 7.0; (b) pH 6.6; (c) pH 6.0; (d) pH 5.6; (e) pH 4.8; (f) pH 4.5; (g) pH 3.0; (h) pH 2.1.

the appropriate baseline has a maximal value at a pH around 5. The fact that the maximal T_m does not agree with the temperature at which the enthalpy change of the unfolding becomes maximal is reflected in the observation at an acidic pH of 4.8 and below where the total area under the curve becomes smaller as the peak temperature becomes higher.

Because the unfolding of the enzyme is obviously associated with the positive change in heat capacity as known from the baseline change before and after the unfolding at any

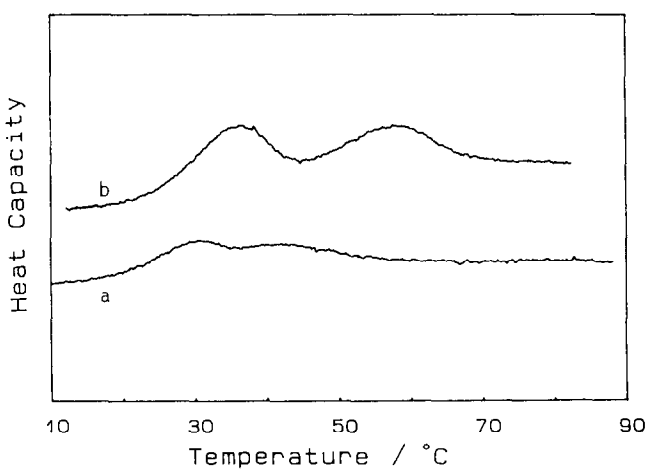

Fig. 2. DSC tracings of acid proteinase A in phosphate buffer at pH 6.7 containing (a) 0.2 M and (b) 1 M NaCl.

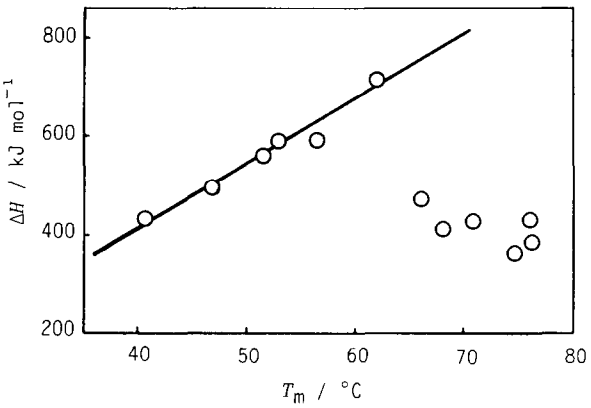

Fig. 3. Plot of the enthalpy change of the thermal unfolding of acid proteinase A as a function of the mid-point temperature of transition in the pH range of 3–6. Solid line was drawn with the least-squares method by using the data obtained at pH 5–6.

pH studied, the above finding seems to indicate that the unfolding at acidic pH might differ from that at a pH above 5. This point is considered later on the basis of Fig. 3.

Another interesting finding is that the unfolding at pH 6 and above shows two peaks; a typical example is seen in trace (b) of Fig. 1 where the main peak is at around 34°C and a relatively broad peak, which is observed as a shoulder, exists at around 40°C. This situation becomes more evident when the unfolding is observed at higher salt concentrations. Typical excess heat capacity curves observed for the enzyme solutions containing 1.0 and 0.2 M NaCl at pH 6.7 are shown in Fig. 2. From this result, it may be concluded that acid proteinase A still has a certain tertiary structure and unfolds via a three-state mechanism.

Interestingly, the first peak having a comparatively high amplitude which is observable at pH 6 and above shifts towards a lower temperature range as the pH is raised, whereas the T_m of the second broad peak remains unchanged, being independent of pH. Furthermore, the higher the pH, the weaker is the amplitude of the main peak, the latter not being present at a pH above 7. The result was also obtained that at pH 6–7 the first peak observable with the enzyme solution containing 0.2 M NaCl becomes weaker as the storage time of the solutions at 4°C becomes longer. However, with the solution containing 0.6 M NaCl, the DSC curve observed a week after its preparation was the same as that observed immediately after the preparation, indicating that the enzyme is sensitive to electrostatic conditions and stabilized at high salt concentrations.

The apparent enthalpy changes of the unfolding observed at 12 different pHs from 3 to 6 were plotted against T_m and are shown in Fig. 3. In the figure, the data points taken at a pH above 6 are eliminated from the plot, because they showed the two peaks. As expected, in the pH range between 5 and 6, a good linear relationship was found to exist and from the slope of the plot, the heat capacity change of the unfolding was determined to be $\Delta C_{pd} = 13.0 \pm 1.3$ kJ K^{-1} mol^{-1}. This value is in good agreement with the value of 12.8 ± 0.9 kJ K^{-1} mol^{-1} determined for the observed excess heat capacity curves after establishing the appropriate baselines. The unfolding enthalpy obtained from the area

under the DSC curves was of the same order of magnitude as that known for many other globular proteins.

On the other hand, as known from the figure, the data points taken at a pH below 4.8 were not found to fall on the same line. This seems to imply that the unfolding at acidic pH is of a different nature and involves additional processes characterized by an exothermic nature. In fact, at an acidic pH below 4.8, the unfolding was irreversible and the precipitation of the unfolded proteins was found to take place. Because of this, no quantitative discussion was provided on the unfolding at acidic pH.

Because the thermal unfolding of the enzyme was quantitatively reversible at pH 5–6, the DSC results observed at this pH range were analyzed on the basis of the van't Hoff equation using the curve resolution technique [9,10]. The observed DSC curves were found to be well characterized by the two-state transition with dissociation to the light and heavy chains. The van't Hoff enthalpy of the unfolding derived for the process associated with the dissociation to the two peptide chains was found to agree with the calorimetric enthalpy which was determined from the area under the excess heat capacity curve observed, indicating that the unfolding is characterized by a two-state transition with dissociation to the light and heavy chains. A typical example of the DSC resolution is shown in Fig. 4 in the form of a curve fitting, where the experimentally observed excess heat capacity values are given as open circles and calculated values as a solid line. The dissociation of the two peptide chains during the unfolding was also shown by the fact that at a pH above 4 the apparent peak temperature of unfolding shifts towards a higher temperature range as the protein concentration is increased, as is the case with the other globular proteins where the unfoldings are associated with the dissociation to their subunits [9,11].

The reheating experiments conducted at pH 6–7 showed that the unfolding is even

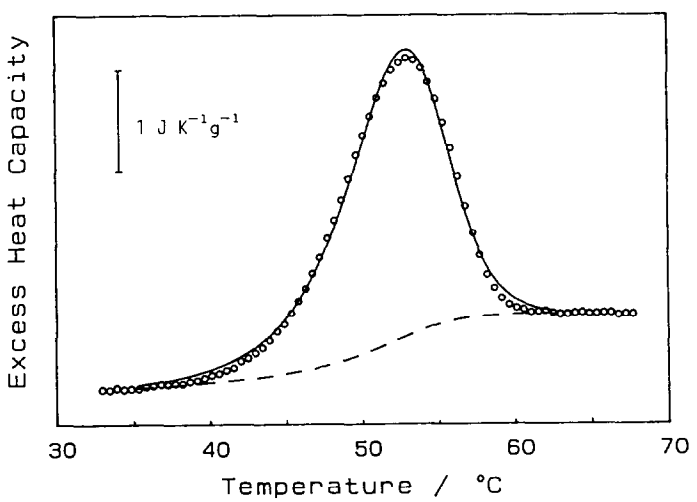

Fig. 4. Curve fitting of the excess heat capacity of acid proteinase A at pH 5.6 based on the two-state transition with the dissociation to light and heavy chains. O, observed data; ——, calculated curve; ——, calculated base line.

more reversible than at pH 5–6. Especially the second peak was found to be fully reproducible. A gel filtration experiment showed that at neutral pH the enzyme is dissociated into two peptide chains at 37°C [6]. This fact obviously indicates that the second peak reflects the transition of a structure remaining after the dissociation of the heavy and light chains, presumably the structural change in the heavy chain.

Privalov et al. [4], reported in their DSC study on pepsin denaturation at neutral pH that its unfolding shows an excess heat capacity curve with two peaks, only the second peak being reversible. On the basis of the curve deconvolution technique they concluded that each of the two peaks observed for the unfolding of pepsin is deconvoluted into two components; thus the enzyme is composed of four domains. This situation is quite different from our result obtained here with acid proteinase A. It is undoubtedly true that this difference stems from the structural difference in both enzymes.

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