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# Autolysis of Semi-alkaline Proteinase from *Aspergillus melleus*

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The autolysis of semi-alkaline proteinase (SAP) purified from *Aspergillus melleus* was studied from the viewpoint of enzyme stability. We also prepared inactive phenylmethylsulfonyl semi-alkaline proteinase (PMS–SAP) in order to avoid the influence of autolysis and used it to study the denaturation profile or structural change in urea solution. Experiments with native SAP and PMS–SAP were performed in parallel under the same conditions. It was found that the rate of inactivation of this enzyme, as determined from the decrease in enzyme activity, followed first-order kinetics and that there was a good relationship between the degree of inactivation and the amount of autolyzed products during urea treatment. The rate of urea denaturation of PMS–SAP was followed by high-performance liquid chromatography (HPLC) and circular dichroism (CD) spectral measurement; the denatured enzyme could be completely separated from the intact PMS–SAP by HPLC. The results suggested that the inactivation of the enzyme was a result of the denaturation, which was accompanied by conformational change. Thus, it seems likely that the cause of the inactivation of SAP is denaturation rather than autolysis, because during autolysis, SAP was proteolyzed through the denatured form produced in the process of inactivation.

**Keywords**——semi-alkaline proteinase; *Aspergillus melleus*; autolysis; high-performance liquid chromatography; stability

Semi-alkaline proteinase (SAP) purified from *Aspergillus melleus* is now widely used as an anti-inflammatory drug. In general, the effectiveness of enzyme preparations largely depends on their enzyme activities, and stability of enzyme activity is considered to be an important parameter of drug quality.

It is well known that the catalytic activities of many proteolytic enzymes such as trypsin and chymotrypsin are affected by temperature, pH and high concentrations of urea and guanidine salts. The kinetics of inactivation of these enzymes have been extensively studied. Some workers reported that the inactivation of trypsin is a first-order reaction,[1] but others have proposed a second-order reaction.[2,3] It is also uncertain whether the inactivation of chymotrypsin follows first-order kinetics or not.[4,5]

However, little work has been done on the mechanism of the autolysis of proteolytic enzymes.[4,6,7] It is still unclear whether the autolysis of proteolytic enzymes during purification or pharmaceutical manufacturing processes affects the enzyme stability or not. It is difficult to study the conformational change or the denaturation process of these enzymes during autolysis, because inactivation and autolysis of the enzyme occur simultaneously, and interpretation of the experimental results is difficult.

In this paper, we prepared phenylmethylsulfonyl semi-alkaline proteinase (PMS–SAP), having no proteolytic activity, to avoid the influence of autolysis and this modified enzyme was used to examine the denaturation process. As we found that denatured PMS–SAP could be separated from intact PMS–SAP by high-performance liquid chromatography (HPLC) on

a TSK-GEL G-3000 SW column, we applied this method to follow the autolysis process of semi-alkaline proteinase during urea treatment by performing parallel experiments with PMS–SAP and native SAP.

## Materials and Methods

**Materials**——Urea (specially prepared reagent) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Nakarai Chemical Co. Fluorescamine was obtained from Sigma Chemical Co. DEAE-Biogel A was a product of BIO-RAD Laboratories. Sephadex G-25 was obtained from Pharmacia Fine Chemicals Co. All other reagents were of reagent grade.

**Enzyme**——SAP was purified from *Aspergillus melleus* according to the method of Ito and Sugiura[8] with a minor modification. Before use, the enzyme solution was applied to DEAE-Biogel A column (3 × 30 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and eluted with a linear gradient of NaCl (0—0.2 M) in the same buffer. The active fraction was concentrated by ammonium sulfate precipitation. PMS–SAP was prepared as follows. PMSF was added to afford 10 times the molar concentration of SAP. The reaction mixture was allowed to stand at room temperature for 30 min, then unreacted reagent was removed by passage through a Sephadex G-25 column (1.5 × 10 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$. This modified enzyme gave a single protein band on disc electrophoresis (pH 9.4). No significant change in amino acid composition was observed after chemical modification.

**Assay of Enzyme Activity**——The caseinolytic activity of native SAP was measured. Hammarsten casein (1.2%) dissolved in 0.1 M phosphate buffer (pH 7.0) was used as a substrate. One-half ml of enzyme solution was mixed with 2.5 ml of substrate and incubated for 10 min at 37 °C. The reaction was stopped by addition of a solution (2.5 ml) containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid. The mixture was left for 20 min at 37 °C and then filtered through No. 131 filter paper (Toyo Roshi). The absorbance of filtrate at 280 nm was measured with a Hitachi 200-10 spectrophotometer.

**Measurement of the Amount of N-Terminal Residues**——The degree of autolysis of native SAP was followed by fluorometric assay of decomposed products. Ten $\mu$l of the test sample was added to 1.5 ml of 0.2 M phosphate buffer (pH 6.1) and then 0.5 ml of fluorescamine (0.3 mg/ml acetone) was added with vigorous stirring. The fluorescence intensity at 475 nm was measured with a Hitachi 650-30 fluorescence spectrophotometer excitation at 390 nm.

**Instrumental Analysis**——HPLC was performed on a Hitachi 638-80 liquid chromatograph equipped with a Hitachi 635M multi-wavelength ultraviolet (UV) monitor. A TSK-GEL G-3000 SW column (7.5 × 600 mm) was obtained from Toyo Soda Co. As a mobile phase, 0.2 M phosphate buffer (pH 7.0) containing 0.1% SDS was used at a flow rate of 0.5 ml/min and the effluent was monitored at 220 nm. Circular dichroism (CD) spectral measurement was carried out with a Jasco J-40C automatic recording spectropolarimeter at a protein concentration of 0.035 mg/ml in a cell of 10 mm light path at 20 °C.

## Results

### Effect of Urea on the Activity of SAP

The inactivation (loss of enzyme activity) of SAP in the presence of urea proceeded with first-order kinetics (Fig. 1). The rate of enzyme inactivation increased as the concentration of urea was increased. As SAP was decomposed into small peptides and amino acids during the autolysis, the degree of autolysis was followed by fluorometric assay of decomposed products. It was found that the increase in the amount of autolyzed products paralleled the loss of the enzyme activity.

### Urea Denaturation of SAP

As the autolysis of SAP interfered with the studies of the denaturation profile during urea treatment, we prepared inactive PMS–SAP. This modified enzyme did not show any detectable change in the CD spectrum as compared with that of native SAP. Furthermore, in Ouchterlony's double immunodiffusion test, PMS–SAP and native SAP gave a single precipitation line against antibody to the native SAP and their precipitation lines fused completely.

Figure 2 shows an Arrhenius plot of the thermal stability data for PMS–SAP and native SAP. After heat treatment at various temperatures, the content of the native enzyme

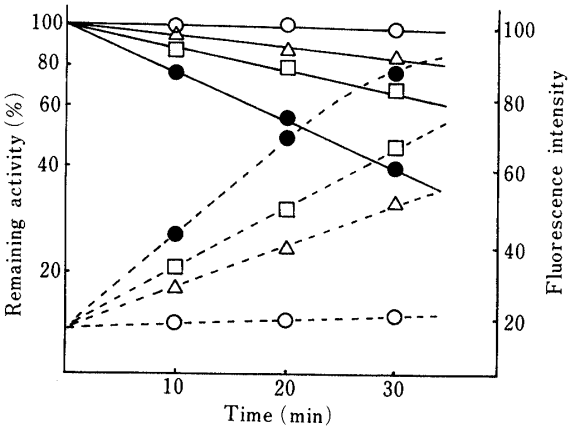


Fig. 1. Effect of Urea on the Stability of SAP

The enzyme ($2.9 \times 10^{-5}$ M) was incubated at 37 °C in urea solution containing 10 mM Tris-HCl buffer (pH 8.0), and the remaining activity (——) was determined. The degree of autolysis (---) was measured by fluorometric assay of autolyzed products. Fluorescence intensity of the sample was expressed relative to that of leucine (13.1 $\mu$g) taken as 100.0%. Urea concentrations were; ○, none; △, 4 M; □, 6 M; ●, 8 M.

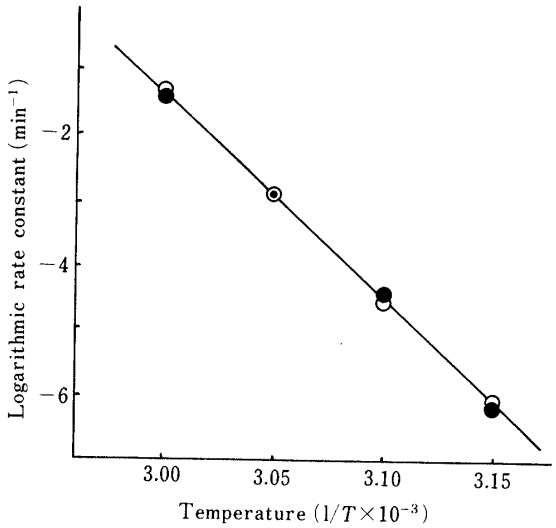


Fig. 2. Arrhenius Plot of Thermal Stability Data for PMS–SAP and Native SAP

The content of native enzyme was measured by HPLC. ○, PMS–SAP; ●, native SAP.

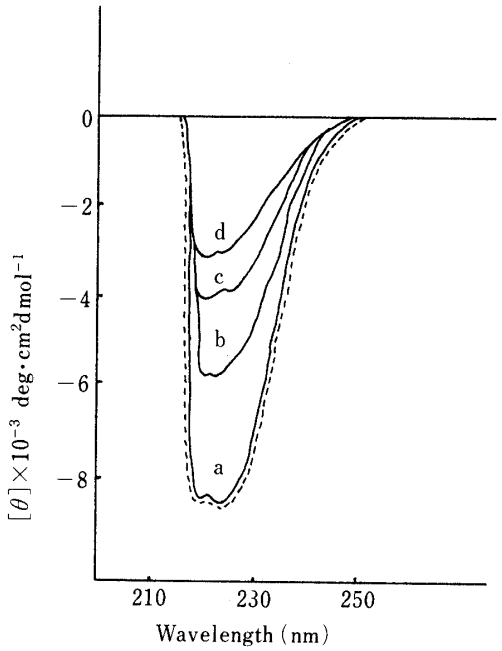


Fig. 3. CD Spectra of PMS–SAP in the Presence of 8 M Urea

The temperature of the sample cell was maintained at 37 °C by using circulating water. The broken line shows the CD spectrum of native SAP.

Data are expressed in terms of mean residue ellipticity [$\theta$]. a, 0 min; b, 10 min; c, 20 min; d, 30 min.

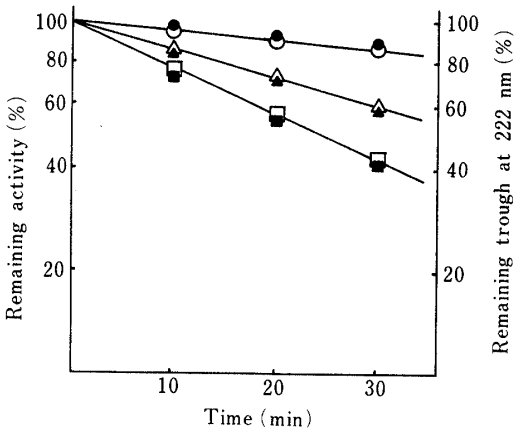


Fig. 4. Inactivation and Conformational Change of SAP in the Presence of Urea

PMS–SAP ($2.9 \times 10^{-5}$ M) was incubated at 37 °C in various concentrations of urea and the CD spectra of the urea-treated samples were measured at the indicated times. The trough at 222 nm was plotted (closed symbols). Native SAP was treated under the same conditions as PMS–SAP and the remaining activity was assayed (open symbols). Urea concentrations were; ○●, 4 M; △▲, 6 M; □■, 8 M.

(undenatured enzyme) was determined by HPLC. There is no difference in thermal stability between PMS–SAP and SAP. These results demonstrate that no appreciable changes in the tertiary structure of this enzyme are caused by the chemical modification. Therefore, we used inactive PMS–SAP in place of native SAP to study the denaturation process.

Figure 3 shows the CD spectra of PMS–SAP in 8 M urea with respect to time. The trough in the 222 nm region gradually decreased with time. Native SAP showed the same time course of conformational change in terms of the CD spectra during urea treatment. The rate of decrease in the trough at 222 nm followed first-order kinetics at any concentration of urea tested, and the rate of spectral change is closely related to the decrease in enzyme activity (Fig. 4).

## HPLC of SAP

The denaturation process of SAP was followed by HPLC. The HPLC was performed in the presence of 0.1% SDS to reduce the strong adsorption of SAP on the column; SAP was completely recovered from the column, and no decrease in the enzyme activity was observed in the effluent. Figures 5(A) and (B) show the time courses of denaturation of native SAP and inactive PMS–SAP in 8 M urea solution, respectively. The elution volume of native SAP was found to be 22.3 ml (Fig. 5(A)). The peak area of the native enzyme decreased as the enzyme activity decreased.

As completely denatured PMS–SAP (identified on the basis of its CD spectrum) was eluted at 15.2 ml, the former of the two peaks shown in Fig. 5(B) was assigned to denatured PMS–SAP. The denatured enzyme increased with time, whereas the original PMS–SAP peak decreased. The peak of the denatured enzyme was not observed in the chromatograms of native SAP, because the denatured enzyme produced during urea treatment is proteolyzed by the remaining active proteinase. It seems that this process corresponds to the apparent autolysis of SAP.

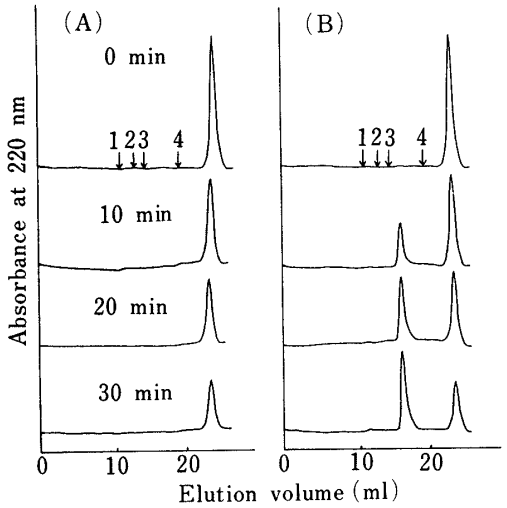


Fig. 5. Chromatograms Showing the Time Courses of Denaturation of Native SAP (A) and PMS–SAP (B) in the Presence of 8 M Urea

Native SAP and PMS–SAP (both $2.9 \times 10^{-5}$ M) were incubated in urea solution containing 10 mM Tris-HCl buffer (pH 8.0) at 37 °C, and samples (2 μl) were periodically subjected to HPLC. The arrows showed the elution volumes of standard proteins. 1, γ-globulin (160000); 2, bovine serum albumin (68000); 3, egg albumin (43500); 4, cytochrome c (13500).

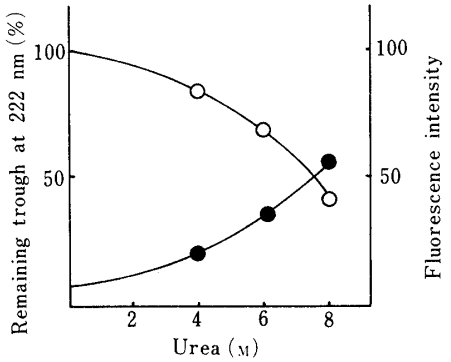


Fig. 6. Relationship between Urea Denaturation and Susceptibility to Proteolysis by Semi-alkaline Proteinase

PMS–SAP was incubated at 37 °C for 30 min in urea solution and the denaturated enzyme was digested with immobilized SAP at 37 °C for 10 min. The degree of digestion was measured by fluorometric assay of proteolyzed products (closed symbols). The extent of denaturation of PMS–SAP was followed by CD spectral measurement (open symbols).

The remaining activity was found to be directly proportional to the peak area of the native enzyme. The specific activity was constant in any concentration of urea tested at all reaction times, which indicates that no intermediate state is formed during the urea denaturation.

The arrows in Fig. 5 show the elution volumes of standard proteins. The molecular weight of SAP is 34000, and it consists of a single polypeptide chain with no disulfide bond.[9] Based on the elution volumes of the standard proteins, the denatured PMS–SAP was eluted at the position corresponding to the above molecular weight. On the other hand, a remarkable delay was observed in the case of the native enzyme. It was reported that some gel permeation chromatography (GPC) columns exhibit a considerable degree of retention of solutes as a result of the interaction with silanol groups on the stationary phase.[10-12] TSK-GEL G-3000 SW column is a silica based aqueous GPC support.[13] Thus, this column may have some silanol groups on the silica surface. Differences, in the degree of interaction with the accessible silanol groups are probably responsible for the separation of the two components (native and denatured SAP).

During the autolysis, the denatured enzyme is considered to act as a substrate for the remaining active proteinase. To confirm this, the susceptibility of the urea-denatured enzyme to proteolysis, was investigated. For the digestion of urea-denatured PMS–SAP, semi-alkaline proteinase immobilized on Sepharose 4B was used in order to avoid the possible autolysis of active enzyme. The denaturation of PMS–SAP was followed by CD spectral measurement and the degree of digestion of the denatured enzyme was determined by fluorometric assay of the proteolyzed product.

As shown in Fig. 6, the digestibility by the active SAP paralleled the extent of the denaturation. This result demonstrated that the increase in the susceptibility to proteolysis could be attributed to the denaturation of SAP, and that during the autolysis, SAP was digested through the intervention of the denatured form by the remaining active enzyme.

## Discussion

We prepared inactive PMSF-modified semi-alkaline proteinase (PMS–SAP) to avoid the influence of autolysis during studies on the denaturation process during urea treatment. The results obtained by HPLC and CD spectral measurement were essentially the same for native and PMS–SAP. The inactivation of this enzyme, which followed first-order kinetics, closely paralleled the decrease in the content of the intact enzyme measured by HPLC and the rate of the conformational change determined by CD spectral measurement. As CD spectral changes of PMS–SAP in urea solution are similar to those which occur during urea treatment of native SAP, the conformational change reflected in the CD spectra is considered to be the denaturation of the enzyme rather than the hydrolysis of peptide bonds due to autolysis. It seems that the inactivation is a result of denaturation. The denatured enzyme can not return to the native state after the removal of the urea by dialysis (unpublished data). These results suggest that the denaturation of SAP involves an irreversible two-state transition (native→denatured form) without a stable intermediate.

The process of the autolysis of SAP may thus be as follows. The first step is the denaturation of enzyme, followed by proteolysis of the denatured form by remaining active enzyme. During the autolysis, the denaturation step must be the rate-limiting process, not proteolysis. This process of autolysis differs from the mechanism of autolysis of chymotrypsin proposed by Hein *et al.*[6] They proposed that the inactivation of chymotrypsin is a second-order reaction and during the autolysis, the rate-limiting step is initial attack of the enzyme molecule on another enzyme molecule. Bender *et al.*[7] also reported that the inactivation of chymotrypsin follows second-order kinetics at neutral pH. They considered the loss of

catalytic activity to be due to autolysis. However, our results showed that the inactivation of SAP is a first-order reaction, and autolysis occurs as a result of denaturation. Hofstee[4] found from the kinetic studies that denaturation was a rate-limiting factor in the autolytic process of chymotrypsin. Our experimental data support this idea.

Protease digestion has been successfully used to detect the native-denaturation transition or thermally induced conformational change of lysozyme or ribonuclease A.[14-18] These experiments are based on the hypothesis that the native protein is resistant to proteolysis and only the denatured protein is digested by proteases. This hypothesis is consistent with the process of autolysis of SAP proposed by us.

In summary, the autolysis of SAP does not affect the inactivation kinetics, and the autolysis of SAP occurs by proteolysis of the denatured form.

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