The Role of Calcium in Semi-alkaline Proteinase from *Aspergillus melleus*

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The role of calcium in the thermal stability of semi-alkaline proteinase (SAP) from *Aspergillus melleus* was investigated. As removal of calcium from active SAP induced an autolytic degradation, phenylmethylsulfonyl fluoride-modified SAP (PMS-SAP), having no proteolytic activity, was used. The calcium binding isotherm obtained by equilibrium gel chromatography indicated that PMS-SAP bound a maximal amount of 2 mol of calcium per mol of enzyme. The results of high-performance liquid chromatography and differential scanning calorimetry of the EDTA-treated PMS-SAP suggested that removal of calcium lowered the transition temperature of the thermal denaturation, accompanied with a decrease in the enthalpy change (ΔH) and in the entropy change (ΔS) for the denaturation. No significant change was observed in the circular dichroism spectrum or immunological properties when one of the two calciums was removed from PMS-SAP. The other one was not removed by dialysis against various concentrations of EDTA or EGTA at pH 8.0, but was removed when PMS-SAP was irreversibly denatured under acidic conditions (pH 2.0). It seems likely that the calcium atom which is removed by dialysis is present on the surface of the enzyme molecule and stabilizes the enzyme against thermal denaturation, whereas the remaining calcium atom seems to be firmly bound to the enzyme or buried in the molecular interior.

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

Semi-alkaline proteinase (SAP) from *Aspergillus melleus* is now commercially available as an anti-inflammatory drug. Care is necessary in the pharmaceutical processing and storage of enzyme preparations due to the relative instability of proteins as compared with synthetic drugs. Thus, investigation of the factors which affect the enzyme stability is necessary both to manufacture enzyme preparations of good quality and to prevent degradation during storage.

It is well known that calcium protects a number of proteases from autolytic degradation and stabilizes them against thermal or chemical denaturation.[1−6] SAP was also found to be protected from thermal inactivation by calcium.[7] Thermodynamic and kinetic analyses of the stabilization of proteases by bound calcium could be performed if proteases with different calcium content could be prepared. However, one of the problems in studying the binding of calcium to proteases is that the rate of autolysis increases rapidly as the amount of bound calcium decreases. This makes it difficult to obtain low-calcium proteases. Therefore, inactive phenylmethylsulfonyl fluoride-modified semi-alkaline proteinase (PMS-SAP) was prepared to avoid the problem of autolysis and was used for experiments on the thermal stability and the structure of low-calcium PMS-SAP.

The object of the present work was to investigate the role of calcium in the thermal stability of SAP in more detail by using inactive PMS-SAP.

Materials and Methods

Reagents——Ethylenediamine-N, N, N', N'-tetraacetic acid (EDTA), ethyleneglycol bis(2-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA) and phenylmethylsulfonyl fluoride were purchased from Nakarai Chemical Co. Sephadex G-25 was obtained from Pharmacia Fine Chemical Co. DEAE-Biogel A was a product of BIO-RAD Laboratories. All other reagents were of reagent grade.

Enzyme——Semi-alkaline proteinase 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 a 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. Phenylmethylsulfonyl semi-alkaline proteinase (PMS-SAP) was prepared as follows; phenylmethylsulfonyl fluoride was added to afford 10 times the molar concentration of SAP dissolved in 10 mM Tris–HCl buffer (pH 8.0). The mixture was allowed to stand at room temperature for 30 min, then unreacted PMSF 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 the chemical modification. Circular dichroism (CD) and ultraviolet (UV) spectra of PMS-SAP were the same as those of the native SAP. No difference in thermal stability between SAP and PMS-SAP was observed when the thermal stability was checked by using the high-performance liquid chromatography (HPLC) method.[9]

Instrumental Analysis——HPLC was performed on a Hitachi 638-80 liquid chromatograph equipped with a Hitachi 635M multi-wavelength 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% sodium dodecyl sulfate (SDS) was used at a flow rate of 0.5 ml/min and the effluent was monitored at 220 nm. The CD spectrum was measured with a Jasco J-40C automatic recording spectropolarimeter at a protein concentration of 0.035 mg/ml in 5 mM Tris–HCl buffer (pH 8.0) in a cell of 10 mm light path at 20 °C. Calorimetric measurements were performed with a Rigaku Denki differential scanning calorimeter (DSC) at a heating rate of 5 °C/min and a protein concentration of 100—120 mg/ml in 10 mM Tris–HCl buffer (pH 8.0). Calcium concentration was determined with a Hitachi 170-50 atomic absorption spectrophotometer. Calcium measurement was performed with a Ca–Mg hollow cathode lamp at 422.8 nm.

Calcium-Binding Isotherm——The amount of bound calcium per mol of PMS-SAP was obtained by equilibrated gel chromatography according to the method of Voordouw and Roche.[2] A sample of 8 mg of protein (1 ml) was applied to a Sephadex G-25 column (1.5 × 50 cm) equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing a given concentration of $CaCl_2$. Fractions of 3 ml were collected and analyzed for calcium and protein. The protein concentration was determined according to the method of Lowry *et al.*[10]

Preparation of Semi-alkaline Proteinase Antibody——Purified SAP (10 mg in 1 ml of saline) was emulsified in the same volume of Freund's complete adjuvant and subcutaneously injected into rabbits. The rabbits received four further injections (10 mg of SAP) at one-week intervals. One week after the final injection, blood was withdrawn and serum was separated. The immunoglobulin G (IgG) fraction was prepared from the rabbit antisera by fractionation with 33% ammonium sulfate, followed by passage through a DEAE-cellulose column equilibrated with 10 mM Tris–HCl buffer (pH 8.0). Then, the IgG was eluted with a linear gradient of NaCl (0—0.2 M) in the same buffer.

Results and Discussion

Effect of Calcium on the Thermal Stability of SAP

Figure 1 shows the effect of calcium on the thermal stability of SAP. Low-calcium SAP was prepared by dialysis against 0.5 mM EDTA in 10 mM Tris–HCl buffer (pH 8.0), followed by passage through a Sephadex G-25 column equilibrated with 10 mM Tris–HCl buffer (pH 8.0). EDTA-treated SAP (1.04 mol of calcium per mol of the enzyme) was incubated in 10 mM Tris–HCl buffer (pH 8.0) with various concentrations of $CaCl_2$ at 37 °C for 10 min, and then the remaining activity was assayed according to the method previously reported.[9] Greater thermal stability was obtained as the calcium concentration was increased. This result suggests that calcium significantly contributes to the thermal stability of SAP. Autolysis makes it difficult to investigate the quantitative relationship between the amount of bound calcium and the stability of SAP, because the rate of inactivation rapidly increased with decrease in bound calcium. PMS-SAP, which has no proteolytic activity, was therefore prepared to avoid the effect of autolysis and used for the following studies on the effect of the removal of bound calcium on the thermal stability and the structure of PMS-SAP.
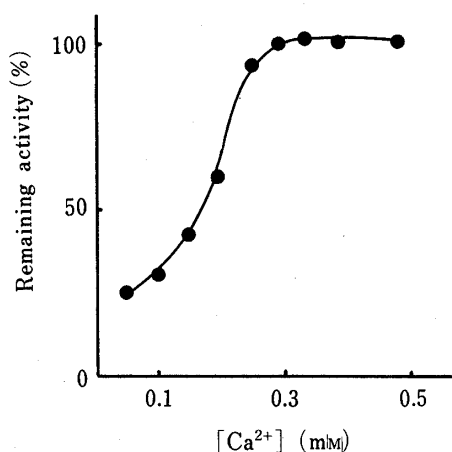
Fig. 1. Effect of Calcium on the Thermal Stability of SAP

Low-calcium SAP $(2.9 \times 10^{-5} M)$ was added to various concentrations of $CaCl_2$ in 10 mM Tris–HCl buffer (pH 8.0). After incubation at 37 °C for 10 min, the remaining activity was assayed.
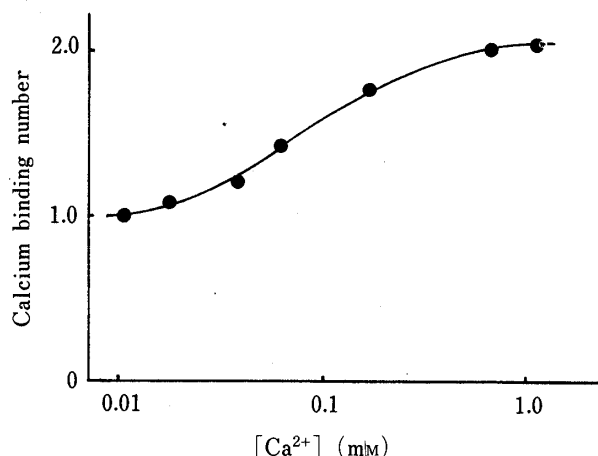
Fig. 2. Calcium Binding Isotherm of PMS-SAP in 10 mM Tris–HCl Buffer (pH 8.0)

Calcium Binding Isotherm

The amount of calcium bound to PMS-SAP was determined by the equilibrium gel filtration method, and the concentration of calcium in each fraction was measured by using an atomic absorption spectrophotometer. Figure 2 shows the calcium binding number (mol per mol of enzyme) as a function of the concentration of free calcium. PMS-SAP binds a maximum of 2.0 mol of calcium per mol of the enzyme. A Scatchard plot of the calcium binding data indicated that the association constant for the binding of the EDTA-removable calcium to PMS-SAP was $1.4 \times 10^4 M^{-1}$.

To investigate the relationship between stability and calcium content, we obtained preparations containing 1.5 mol and 1.0 mol of calcium per mol of PMS-SAP by dialysis against 10 mM Tris–HCl buffer (pH 8.0) containing 0.5 mM EDTA. Excess EDTA and calcium were removed from PMS-SAP by gel filtration on Sephadex G-25. These fractions are referred to as 1.5-calcium and 1-calcium PMS-SAP, respectively. However, complete removal of the remaining calcium from 1-calcium PMS-SAP by dialysis against various concentrations (0.5, 5 and 10 mM) of EDTA or EGTA at pH 8.0 was unsuccessful.

Stability of EDTA-Treated PMS-SAP

HPLC was used to determine the stabilizing effect of bound calcium on the thermal denaturation of PMS-SAP. We previously showed that this HPLC method is useful for the simultaneous determination of the denatured form and the native form of the original PMS-SAP; the elution volumes of the denatured form and the native form of PMS-SAP were 15.2 and 22.3 ml, respectively.[9] Two peaks were observed in the chromatograms of the heat-treated PMS-SAP (Fig. 3), at 15.2 ml and at 22.3 ml elution volume. The chromatograms obtained showed that the thermal stability of PMS-SAP decreased as the amount of the bound calcium decreased.

The kinetics of thermal denaturation of PMS-SAP with various calcium contents was investigated by using HPLC. PMS-SAP $(2.9 \times 10^{-5} M)$ was incubated at an appropriate temperature in 10 mM Tris–HCl buffer (pH 8.0) and 2 μl aliquots were withdrawn at 5 min intervals up to 30 min, then subjected to HPLC. When the logarithm of the remaining SAP protein (percent) determined by HPLC was plotted as a function of time, the thermal denaturation of PMS-SAP was found to follow first-order kinetics. The rate constants of the
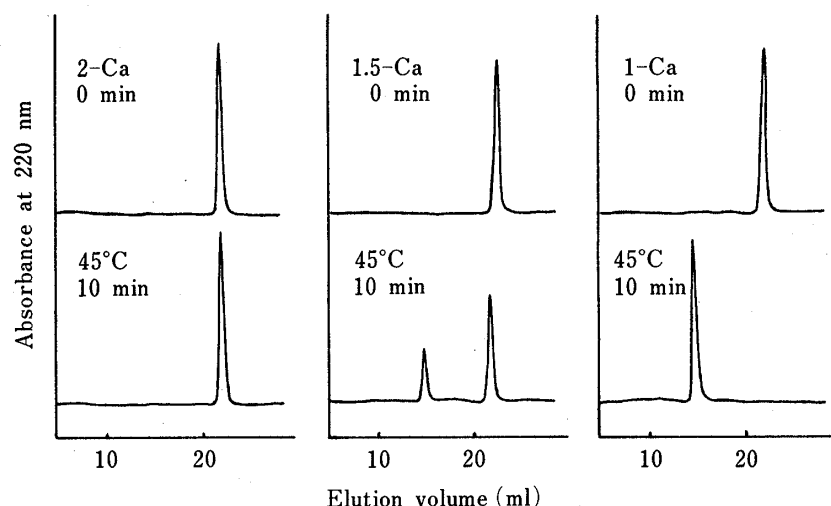
Fig. 3. Thermal Stability of 1-Ca, 1.5-Ca and 2-Ca PMS-SAP

PMS-SAP (2.9×10^{-5} M) was incubated at 45 °C for 10 min, and then the sample (2 μl) was injected to the HPLC instrument.
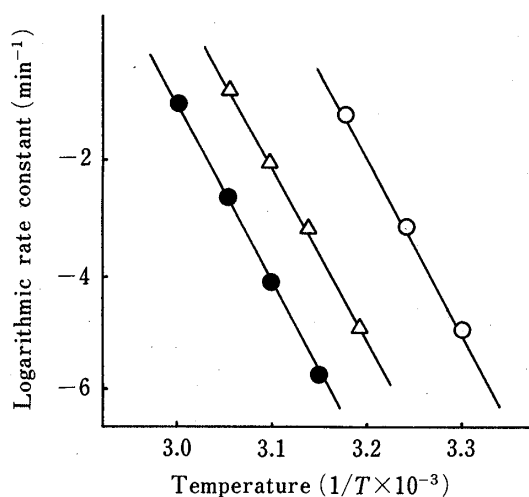

Fig. 4. Arrhenius Plots of the Thermal Dena-
turation of 1-Ca, 1.5-Ca and 2-Ca PMS-SAP

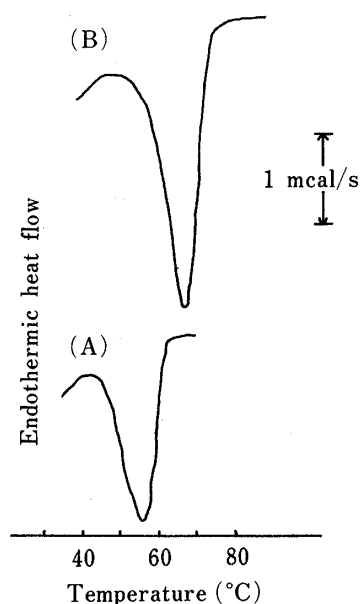●, 2-Ca PMS-SAP; △, 1.5-Ca PMS-SAP; ○, 1-Ca
PMS-SAP.

Fig. 5. DSC Thermogram of 1-Ca (A) and
2-Ca (B) PMS-SAP

thermal denaturation of 1-calcium, 1.5-calcium and 2-calcium PMS-SAP were measured at various temperatures by the HPLC method and Arrhenius plots of the data are presented in Fig. 4. It is evident that PMS-SAP was effectively stabilized against thermal denaturation by bound calcium. The activation energy for the thermal denaturation was not influenced by the amount of bound calcium and its value was around 70 kcal/mol. These results suggest that the denaturation of 2-calcium, 1.5-calcium and 1-calcium PMS-SAP goes through the pathway whose transition-state energy is not affected by the amount of bound calcium and that calcium is necessary for SAP to maintain a structure which is resistant to heat denaturation.

Differential Scanning Calorimetry

Differential scanning calorimetry was used for the investigation of the thermal stability

of 2-calcium and 1-calcium PMS-SAP (Fig. 5). An endothermic peak was observed and the enthalpy change for the denaturation (ΔH) was calculated from the peak area in the thermograms. The temperature of the thermal denaturation (T_d) shifted to higher temperature as the amount of bound calcium increased. This increase from 56 to 67 °C was also accompanied with an increase in the enthalpy from 350 to 460 kcal/mol. The entropy change for the thermal denaturation (ΔS) was calculated from the relation $\Delta S = \Delta H/T_d$. Values of 1064 and 1352 cal·mol^{-1}·degree^{-1} were obtained for ΔS for 1-calcium and 2-calcium PMS-SAP, respectively. These results indicate that removal of calcium decreased the thermal stability of SAP and caused a decrease in ΔH and ΔS for the thermal denaturation. Therefore, we propose that there are two reasons why calcium thermodynamically stabilizes SAP. First, stabilization of SAP by calcium is due to the increase of enthalpy needed for the denaturation. Secondly, calcium stabilizes SAP by increasing the difference in free energy between the native and the denatured state.

CD Spectrum and Immunological Properties of EDTA-Treated PMS-SAP

The effect of removal of calcium on the structure of PMS-SAP was investigated by CD spectral measurement. Figure 6 shows the CD spectra of 1-calcium and 2-calcium PMS-SAP. The CD spectrum of 1-calcium PMS-SAP is identical with that of 2-calcium in the region from 200 to 250 nm. The broken line showed the spectrum of the enzyme after incubation at 37 °C for 10 min. No significant change was observed in the CD spectrum after heat-treatment of 2-calcium PMS-SAP. In the case of 1-calcium PMS-SAP, a large structural change was observed after treatment at 37 °C. These results suggested that calcium is needed for the maintenance of the structural integrity of SAP.

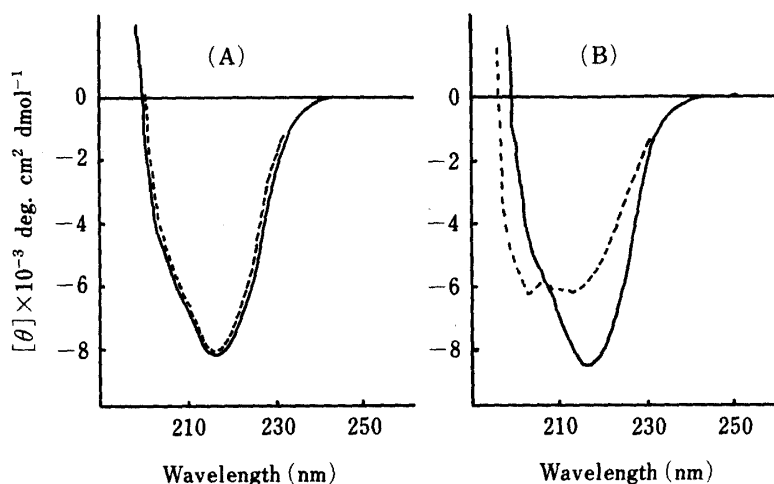Immunological studies of 2-calcium, 1.5-calcium and 1-calcium were performed by

Fig. 6. CD Spectra of 2-Ca (A) and 1-Ca (B) PMS-SAP
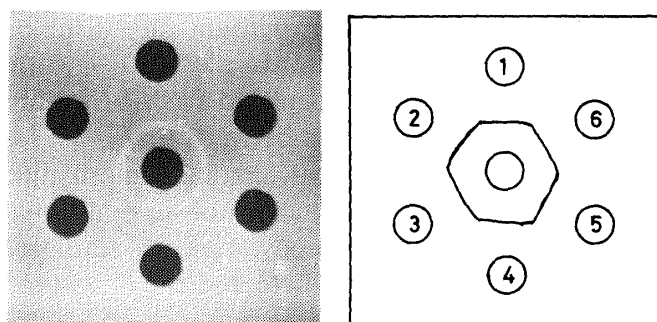
————, after treatment at 37 °C for 10 min.

Fig. 7. Ouchterlony Immunodiffusion of 1-Ca, 1.5-Ca and 2-Ca PMS-SAP against Native SAP Antibody

Well 1, native SAP; well 2, 2-Ca PMS-SAP; wells 3, 5, 1.5-Ca PMS-SAP; wells 4, 6, 1-Ca PMS-SAP.
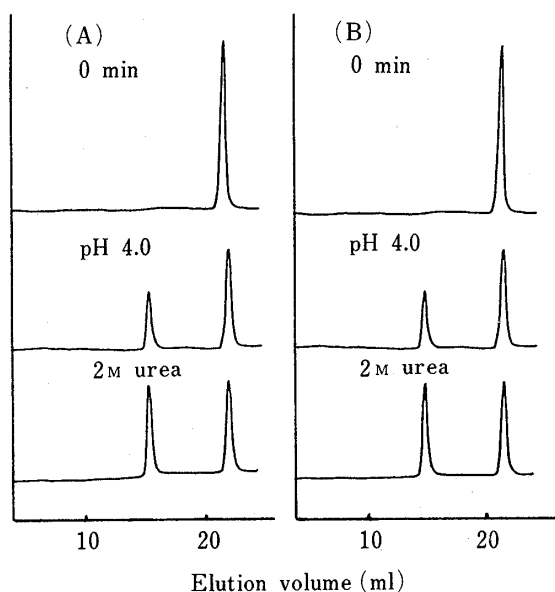
Fig. 8. Stability of 1-Ca PMS-SAP in the Absence (A) and Presence (B) of 1 mM EDTA

After 1-Ca PMS-SAP (2.9 × 10^{-5} M) had been treated at 37 °C for 10 min under the indicated conditions, a sample (2 μl) was subjected to HPLC.

Ouchterlony's method.[11] Each enzyme showed a single precipitation line against antibody to the native enzyme and the precipitation lines fused completely (Fig. 7). This result indicated that SAP still maintains the antigenic structure after loss of one calcium.

Effect of EDTA on the Stability of 1-Calcium PMS-SAP

To study the role of the remaining one calcium, the stability of 1-calcium PMS-SAP was investigated in the presence and absence of 1 mM EDTA by using HPLC (Fig. 8). For the treatment of 1-calcium PMS-SAP, we chose relatively severe conditions. The chromatogram showed that 1-calcium PMS-SAP in the absence of 1 mM EDTA was 20 and 43% denatured after a 10 min incubation at 37 °C in 10 mM Tris–HCl buffer (pH 8.0) and 10 mM acetate buffer (pH 4.0), respectively. In 10 mM Tris–HCl buffer (pH 8.0) containing 2 M urea, PMS-SAP was 48% denatured. The addition of 1 mM EDTA did not affect the rate of denaturation of 1-calcium PMS-SAP under denaturing conditions. When 1-calcium PMS-SAP was irreversibly denatured with 0.01 N HCl and dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA for 24 h, the acid-treated enzyme lost the last calcium. We concluded from these results that one calcium may be firmly bound to the enzyme or is buried inside the enzyme molecule.

Weaver et al.[12] reported that one of four bound calcium ions of thermolysin was not removed by EDTA and suggested that the role of the firmly bound clacium is to link together the two molecular lobes of thermolysin. Semi-alkaline proteinase requires at least 2 mol of calcium per mol of the enzyme for maintenance of the thermal stability, and one of the two bound calciums is easily removed by the chelating agents while the other is not. Hisue et al.[13] suggested that calcium in α-amylase can replace disulfide bridges in effect and that calcium linkages maintain the structural rigidity of the active center.

Semi-alkaline proteinase consists of a single polypeptide chain without any disulfide bonds and the enzyme is not a metal protease but a serine protease.[14] CD spectral measurement demonstrated that 2-calcium PMS-SAP and calcium-depleted PMS-SAP have the same structure, but differences in thermal stability were observed between them.

It is supposed from these results that the easily removable calcium in semi-alkaline proteinase probably maintains the structural integrity by the introduction of an additional bond as in the case of α-amylase,[13] and the additional bond thermodynamically stabilizes SAP by increasing the difference in free energy between the native state and the denatured state, and by increasing the enthalpy of the denaturation.

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