Chem. Pharm. Bull. 33(2) 746—752 (1985)

Studies on the Quality of Commercially Available Semi-alkaline Proteinase Preparations Using High-Performance Liquid Chromatography

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(Received February 27, 1984)

The quality of commercially available semi-alkaline proteinase (SAP) preparations was studied by using high-performance liquid chromatography (HPLC) on a TSK G-3000 SW column, with 0.2 M phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS) as a mobile phase. We could quantitatively separate SAP from enteric coating, excipients and degradation products of SAP in pharmaceuticals of various dosage forms, and follow the changes in their activities during storage. When the content of SAP in pharmaceuticals was determined by this HPLC method, commercially available SAP preparations were found to contain 60—78% of the labeled amount of SAP. HPLC chromatograms obtained in the heat stability test demonstrated that SAP both in the liquid and the solid state is degraded into small fragments in the course of inactivation. Inactivation of powdery SAP by ultraviolet irradiation and by compression force in tabletting could also be followed by using HPLC.

This simple and precise HPLC method was proved to be useful for assessing the stability of SAP preparations and for investigating the mechanism of inactivation of this enzyme.

Keywords——semi-alkaline proteinase; *Aspergillus melleus*; HPLC; stability; antiphlogistic enzyme

Many proteases, including trypsin, α-chymotrypsin, papain, bromelain, serratia protease (*Serratia* sp. E-15) and semi-alkaline proteinase (*Aspergillus melleus*), are now widely used as anti-inflammatory drugs, though their action mechanisms have not yet been clarified. As these enzyme preparations are generally unstable proteins and their catalytic activities are readily affected by factors such as temperature, humidity and pH, special care must be taken to maintain the enzyme activities during the process of pharmaceutical manufacturing or storage.[1–4] These enzyme preparations are mostly for oral administration. Generally, only the potencies of the enzyme preparations are expressed on the labels, and little attention has been paid to the purities of these preparations except in the case of injectable formulations.

Recently high-performance liquid chromatography (HPLC) has been extensively used for the separation or characterization of proteins or peptides.[5–7] Thus, the aim of the present work was to study the quality of commercially available semi-alkaline proteinase (SAP) preparations by using HPLC with a TSK-GEL G-3000 SW column.

Materials and Methods

Reagents——Casein and sodium dodecyl sulfate (SDS, specially prepared reagent) were obtained from Wako

Pure Chemical Ind., Co. and Nakarai Chemical Co., respectively. Commercially available semi-alkaline proteinase preparations were purchased from three pharmaceutical companies. All other reagents were of reagent grade.

Purification of Semi-alkaline Proteinase——Semi-alkaline proteinase was extracted from the commercial tablets with 10 mM acetate buffer (pH 5.0). The extracted solution was applied to an SP-Sephadex C-50 column previously equilibrated with 10 mM acetate buffer (pH 5.0) containing 1 mM $CaCl_2$, and then eluted with a linear gradient of NaCl (0—0.2 M) in the same buffer. The purified SAP gave a single protein band on disc electrophoresis (pH 9.4). The concentration of active site of the purified SAP was determined by using *N*-acetylcinnamoylimidazole as a titrant.[8,9] The result of the active site titration demonstrated that the purity of the standard SAP was 100%.

Assay of Enzyme Activity——The caseinolytic activity was measured by the method of Hagiwara *et al.*[10] with a minor modification. 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 the filtrate at 280 nm was measured with a Hitachi 200-10 spectrophotometer. One unit of proteolytic activity was defined as the amount of the enzyme that liberated trichloroacetic acid-soluble hydrolysate whose absorbance at 280 nm is 1.0 under the above conditions.

Determination of Protein——The concentration of protein was determined by the method of Lowry *et al.*[11] using bovine serum albumin as a standard.

HPLC Instrumentation——HPLC was performed on a Hitachi 638-80 liquid chromatograph equipped with a Hitachi 635M multi-wavelength ultraviolet monitor. A TSK-GEL G-3000 SW column was obtained from Toyo Soda Co. The flow rate was 0.5 ml/min and the effluent from the column was monitored at 220 nm. Peak area was determined by weighing the paper cut from underneath the peak on the chromatogram.

Preparation of the Sample for HPLC——Semi-alkaline proteinase was extracted from the commercial SAP preparations with 0.1 M phosphate buffer (pH 7.8) and then the extracted solution was centrifuged for 10 min at 3000 rpm. The supernatant was filtered through a 0.4 μm microfilter (Fuji Photo Film Co., Ltd.). The filtrate was diluted with 0.1 M phosphate buffer (pH 7.8) to make a solution containing 1 mg of SAP (labeled amount) per ml. Five μl of the sample was injected into the HPLC column.

Results and Discussion

Determination of Semi-alkaline Proteinase by High-Performance Liquid Chromatography

Initially, the separation and activity recovery of SAP from the column were investigated by using four buffer systems as a mobile phase. When 0.2 M Tris–HCl buffer (pH 7.0) was used as the elution buffer, the recovery of SAP from the HPLC column was 85%. In the presence of 0.1% SDS, the recovery was slightly higher (87%). When 0.2 M phosphate buffer (pH 7.0) was used for elution, the recovery of SAP from the column was 94%. As a mobile phase, 0.2 M phosphate buffer (pH 7.0) containing 0.1% SDS was found to be most suitable for the present work, because the activity and protein of SAP were completely recovered (100%). Thus, 0.2 M phosphate buffer (pH 7.0) containing 0.1% SDS was employed as the elution buffer. Standard SAP purified from a tablet was eluted as a single peak on the chromatogram using this buffer system and the elution volume of SAP was 22.3 ml.

The amounts of SAP in standard solutions were plotted against the peak areas on the chromatograms (Fig. 1). The calibration curve obtained was linear and passed through the origin.

The reproducibility of the present method was tested by injecting 0.48 μg of SAP seven times and measuring the peak areas. The mean value of the weight of the peak area cut from the chart and the coefficient of variation were 55.5 ± 1.1 mg and 2.0%, respectively.

Quantitative Determination of Semi-alkaline Proteinase in the Commercial Preparations

The recovery of SAP from pharmaceuticals was investigated. Standard SAP (5.0 mg) was added to 0.1 M phosphate buffer (pH 7.8) containing 10.0 mg of tablet, capsule or granule, then extraction of SAP was performed, and 5 μl of the extracted solution was injected into the HPLC instrument. The recoveries of SAP from the pharmaceuticals were over 99.0% (unpublished data). Thus, we applied the HPLC method to the quantitative determination of SAP in pharmaceutical materials.

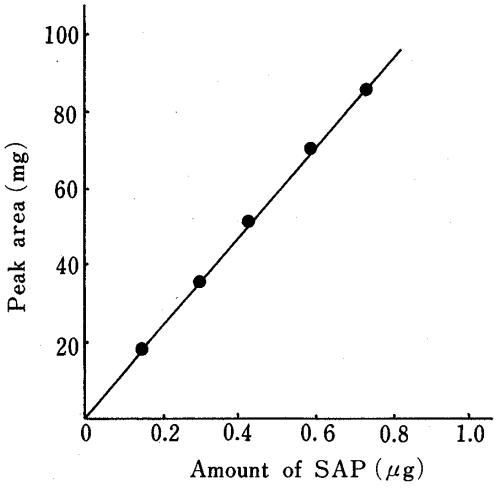

Fig. 1. Calibration Curves for SAP

Each point is the mean value of five experiments.

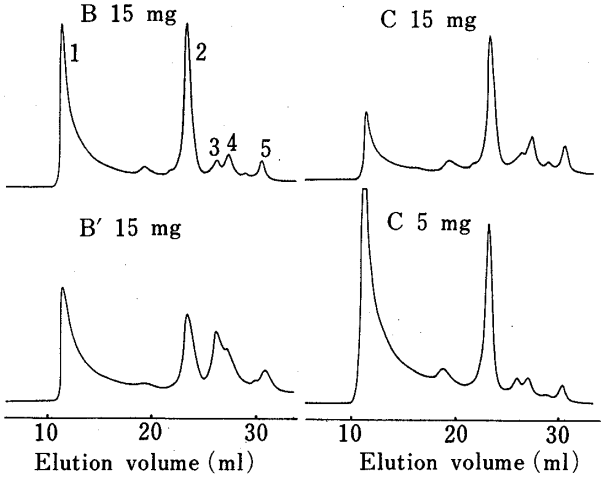

Fig. 2. Chromatograms of Commercially Available SAP Tablets

Five μl of SAP solution (1 mg/ml) was injected into the HPLC column.

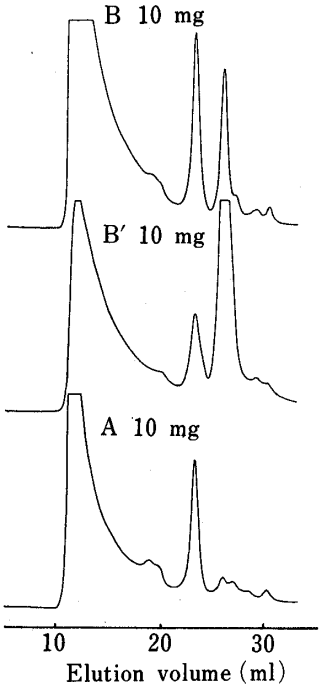

Fig. 3. Chromatograms of SAP Capsules

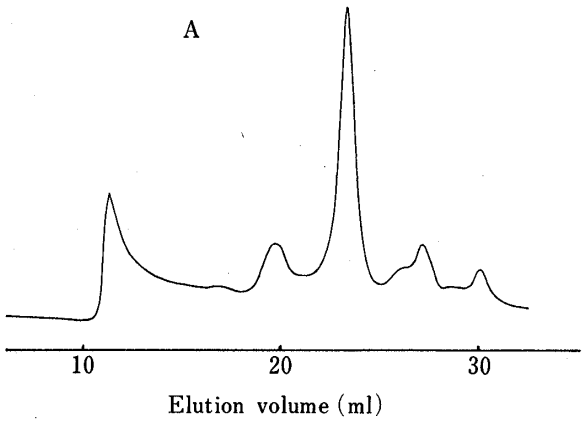

Fig. 4. Chromatogram of SAP Granules

Figure 2 shows chromatograms of the commercial SAP tablets. Peak 1 was considered to be components of the enteric coating, because the peak area of this fraction decreased when acetate buffer (pH 5.0) was used as an extracting buffer. Peak 2 coincided with that of SAP and peaks 3, 4 and 5 were thought to be the excipients or the decomposition products of SAP. In the chromatogram of sample (B′), a decrease in the peak area of SAP and an increase in the decompositon products were observed as compared to that of sample (B), which indicated the deterioration of the enzyme preparation during storage. SAP was clearly separated from all other compoennts of the tablets.

Figure 3 shows the chromatograms of the commercial SAP capsules. A large amount of high molecular weight component was observed. The chromatogram of the capsule (B′) also indicated deterioration of the enzyme preparation during storage. Figure 4 shows the

TABLE I. Total Protein, Total Activity and Content of Semi-alkaline Proteinase in Commercially Available Preparations

SAP Preparation[a)]	Total activity (U)	Total protein[b)] (mg)	Total amount of SAP[c)] (mg)
Tablet			
A 15 mg	250.4±4.6	11.6±0.49	9.9±0.18
B 15 mg	242.7±4.2	11.7±0.41	9.7±0.16
B′ 15 mg[d)]	105.4±6.7	11.2±0.47	4.1±0.12
C 15 mg	224.0±3.8	11.9±0.53	9.5±0.13
C 10 mg	165.8±1.2	8.3±0.34	6.7±0.09
A 5 mg	80.0±2.4	4.2±0.33	3.2±0.09
C 5 mg	86.1±0.8	4.1±0.22	3.5±0.07
Capsule			
A 10 mg	169.9±2.8	8.9±0.40	6.8±0.11
B 10 mg	188.4±4.0	9.3±0.29	7.6±0.19
B′ 10 mg[d)]	76.4±4.8	8.2±0.24	3.1±0.19
Granules			
A 1 g	184.2±1.3	9.0±0.21	7.3±0.13

Experiments were independently performed 3 times. Each value represents the mean value±S.D. of 3 determinations.
a) SAP preparations were obtained from 3 pharmaceutical companies (A, B, C).
b) Content of protein was determined by the method of Lowry *et al.*[11]
c) Amount of SAP was determined by the HPLC method.
d) B′ was a sample whose effective date had already expired. The sample was stored at room temperature until one year had passed beyond the effective date.

chromatogram of the commercial SAP granules. SAP was eluted separately from other components in the granules.

The contents of semi-alkaline proteinase in pharmaceutical materials was thus quantified by the HPLC method and the results are summarized in Table I. Total proteinase activity and total protein in each pharmaceutical are also given in Table I. When the content of SAP in pharmaceuticals was compared with the total proteinase activity, a good correlation ($r=0.999$, $n=11$) was obtained. This result suggests that the peak area of SAP is directly proportional to the amount of SAP and also parallels the enzyme activity in these pharmaceutical preparations. The content of SAP in pharmaceuticals determined by HPLC was compared with the labeled amount for 11 individual enzyme preparations. It was found that the SAP contents of the test samples were 60—78% of the labeled amount.

This HPLC method is useful for the simultaneous separation and determination of SAP, excipients and decomposition products of SAP in pharmaceutical materials of different lots or various dosage forms.

Time Course of Degradation of Semi-alkaline Proteinase Preparation in the Liquid and Solid States

The solid-state stability of enzyme preparations during storage is usually affected by many factors such as temperature, humidity, light and bacterial contamination. The compression force may also contribute to enzyme inactivation when an enzyme powder is compressed into a tablet. Thus, the HPLC method was applied to follow the inactivation of SAP preparations under accelerated conditions. The effect of heat on the stability of SAP preparations in the liquid and solid states was investigated.

Figure 5(a) shows the time course of inactivation of a 0.1% solution of SAP (A, SAP tablet 15 mg) in 0.1 M phosphate buffer (pH 7.8) during heat treatment at 50 °C. Peak I of SAP on the HPLC chromatograms decreased with time. On the other hand, peaks II, III and IV

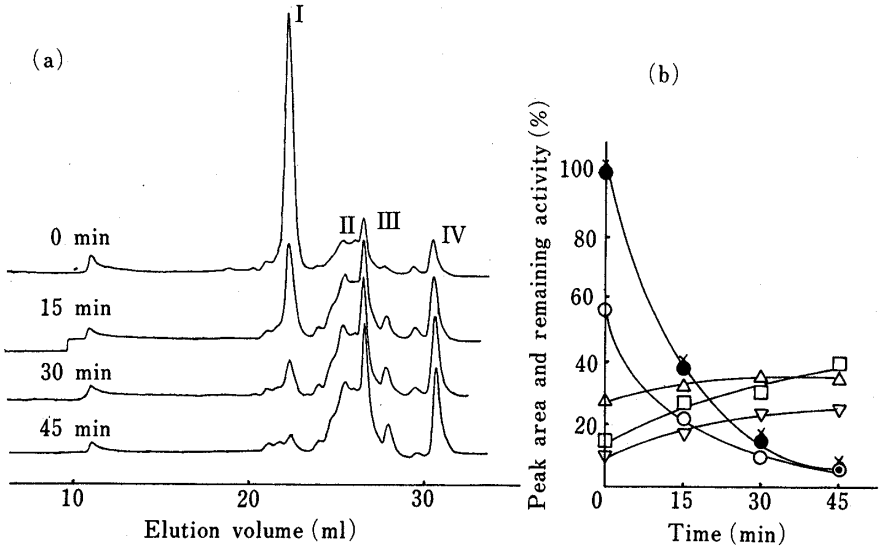

Fig. 5. Time Course of Inactivation of SAP Solution during Heat Treatment at 50 °C

A SAP tablet (A, 15 mg) was dissolved in 15 ml of 0.1 M phosphate buffer (pH 7.8). This solution was incubated at 50 °C, and 5 μl of the sample was injected into the HPLC column at the indicated times. The peak area of each fraction is expressed relative to the total peak area (open symbols). ○, I; △, II; ▽, III; □, IV.

●, the ratio of the peak area I after incubation to the peak area I at 0 min; ×, remaining proteinase activity.

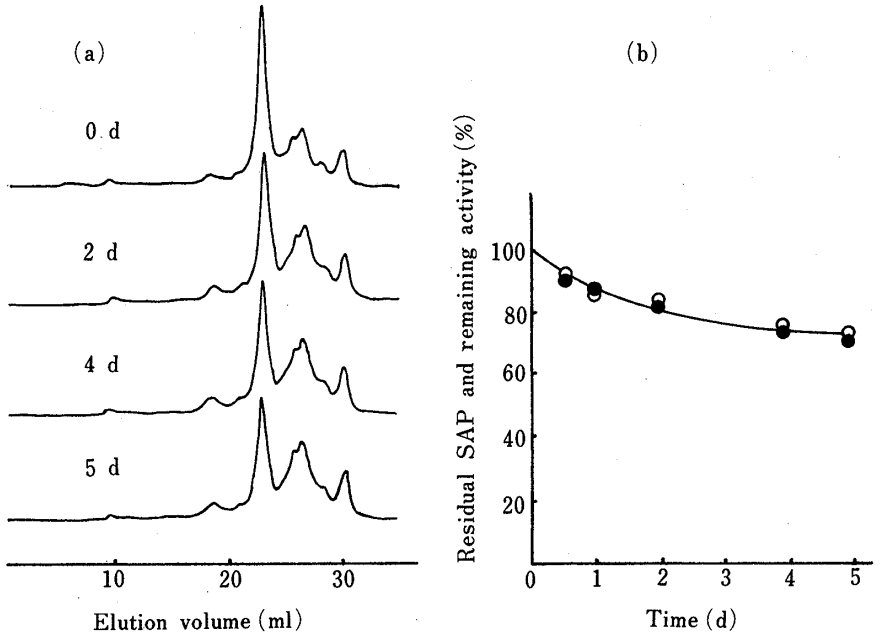

Fig. 6. Time Course of the Inactivation of SAP Powder during Heat Treatment at 90 °C

The enzyme powder (20 mg) was kept in a vial (4 ml) at 90 °C for the indicated time, then the sample was dissolved in 0.2 M phosphate buffer (pH 7.8) to make a 0.1% solution of SAP. Five μl of the sample solution was injected into the HPLC column.

○, remaining activity; ●, residual peak area of SAP.

increased. The ratio of the peak area of each fraction to the total peak area and the remaining peak area of SAP after the heat treatment were plotted as a function of time (Fig. 5(b)). The decrease in the peak area of SAP was directly proportional to the loss of enzyme activity.

Figure 6 showed the time course of the heat-inactivation of SAP powder. The peak area

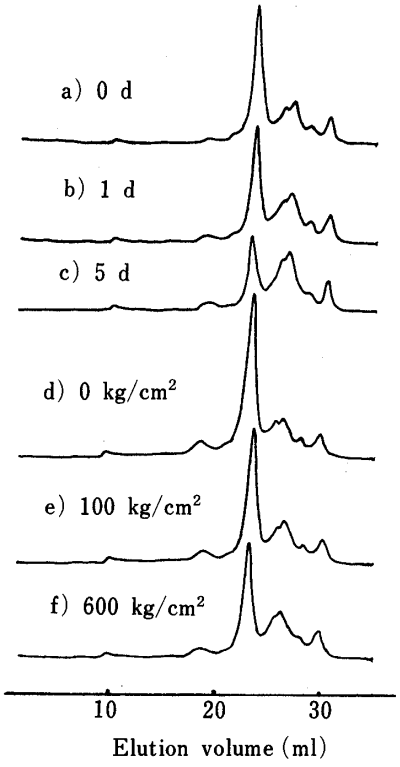

Fig. 7. HPLC Chromatograms of Inactivation of SAP Powder by Ultraviolet Irradiation and Compression Force during Tabletting

SAP powder (20 mg) was irradiated at a distance of 20 cm from the ultraviolet lamp at 25 °C for the indicated time (a—c). SAP powder (50 mg) was poured into a single set of flat-faced punches and a die, and compressed by means of an oil press (Rikenseiki, P-16B) to give a punch pressure of 100 and 600 kg/cm² (d—f). Samples were injected into the HPLC instrument according to the method described in Fig. 6.

of SAP decreased and those of its degradation products increased as time passed. As shown in Fig. 6(b), the residual peak area of SAP was proportional to the remaining activity. When the inactivation behavior in the solid state was compared with that in solution, no significant difference was seen.

Figure 7 showed the time course of inactivation of SAP powder by ultraviolet irradiation and compression force during tabletting. After ultraviolet irradiation for 1 and 5 d, the enzyme activity of SAP powder decreased to 79 and 52% of the original activity, respectively. When SAP powder was compressed under pressures of 100 and 600 kg/cm², the enzyme activity reduced to 84 and 72% of the original activity, respectively. In both cases, it was found that the residual peak area of SAP directly corresponded to the remaining activity.

These results suggest that during the course of inactivation, the peak area of SAP in the HPLC chromatogram is proportional to the enzyme activity, and this HPLC method is useful for assessing the stability of SAP preparations in both the solid and liquid states. The action mechanism of the antiphlogistic enzymes is still obscure. Our results indicated that inactivation of semi-alkaline proteinase preparations resulted in an increase in the decomposition products. As SAP preparations are usually administered orally, the decomposed products may be absorbed into the body. Therefore, it is important to define the quality of the enzyme preparations.

This simple and precise method should be useful for the routine quality control of SAP preparations and seems to be applicable for testing the potencies of enzyme preparations during storage or in the course of pharmaceutical manufacturing.

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