PARTIAL PURIFICATION AND PROPERTIES OF PROTEASES FROM DEFATTED SOYBEAN FLOUR

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Summary Four chromatographically different proteases were partially purified from defatted soybean flour, and their pH optima were around 5.0 to 5.6 using casein as the substrate. These soybean proteases were designated S1, S2, S3 and S4 according to their order of elution from a DEAE-cellulose column. Each gave a single peak of caseinolytic activity on a Sephadex G-200 column chromatogram, and corresponded to the molecular weights of about 50,000(S1), 35,000(S2), 60,000(S3) and 200,000(S4). The proteases could hydrolyze casein and poly-Glu. α-Casein was more rapidly hydrolyzed than β-casein, but the esters or dipeptide could not be hydrolyzed. Aliquots of $10^{-3}$ M Hg$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ inhibited the caseinolytic activities by 70% to 90%, while other cations, Mn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$ and Ni$^{2+}$, at the same concentration did not. SPI ($10^{-5}$ M) inhibited 80–90% of their activities, and EPNP ($10^{-5}$ M) inhibited their activities 30–60%, but DFP ($10^{-3}$ M), SSI ($10^{-3}$ M), PCMB ($10^{-4}$ M), NEM ($10^{-3}$ M) and EDTA ($10^{-3}$ M) were not inhibitory. The above results indicate that proteases S1, S2, S3 and S4 from defatted soybean flour can be classified as acid proteases.

Keywords proteases, soybean, defatted soybean flour, purification, protease inhibitor, acid proteases

Since LAUFER et al. (1) reported proteolytic activity in soybean seeds, investigators have described the purification and the properties of the enzyme in the seed. OFELT et al. (2) reported the presence of a protease most active at pH 5.5 in soybean flour. The partial purification and some properties have been

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Abbreviations: DFP, diisopropylfluorophosphat; PCMB, p-chloromercuribenzoate; NEM, N-ethylmaleimide; EPNP, 1,2-epoxy-3(p-nitrophenoxy) propane; SSI, subtilisin inhibitor; SPI, pepsin inhibitor; ATEE, acetyltyrosineethyl; BAEE, benzoylarginineethyllest; TAME, tosylarginine-methylleste; poly-Glu, poly-glutamic acid.
reported by Weil et al. (3), who separated six active proteolytic fractions from dormant soybean cotyledons. They showed that these enzymes had optimal pH values between pH 5.0 and 5.4 by using casein as the substrate, and different Michaelis constants on poly-Glu. Fukazawa et al. investigated the proteases in germinating and dormant soybean seeds (4) and found four protease fractions in the dormant seeds on DEAE-sephadex column, although the activities were weak.

In this paper, the authors present the results of the partial purification of the proteases from defatted soybean flour and their enzymatic properties.

MATERIALS AND METHODS

Materials. Defatted soybean flour was a generous gift from Yoshiwara Seiyu Co., Ltd., Kobe. Z-Phe-Tyr, SSI and SPI were gifts from Prof. S. Murao of the Department of Agriculture, University of Osaka Prefecture. DFP was obtained from Sigma Co., Ltd. BAEE, ATEE and poly-Glu were obtained from the Protein Research Foundation (Osaka, Japan). All other chemicals were obtained from commercial sources of the guaranteed grade of purity. α-Casein and β-casein were purified by the method of Warner (5). Casein of Hammarsten grade, Wako Pure Chemical Ltd., Japan, was purified by dissolving in alkaline pH, precipitating at acidic pH, and washing with aq. methanol (6).

Enzyme assay. The purified casein was dissolved in alkaline pH at a concentration of 1%. The reaction mixture, consisting of 0.5 ml of the casein solution, 1.0 ml of enzyme solution and 0.5 ml of 0.5 M acetate buffer (pH 5.5), was incubated at 40°C for 120 min. The reaction was terminated by the addition of 0.5 ml of 40% trichloroacetic acid. The solutions were kept at room temperature for one hour, and the precipitates were removed by centrifugation; the concentration of the splits in the supernatant was determined by absorbancy at 280 nm. The blank was performed by adding the substrate solution to the mixture of the enzyme and buffer solution after the introduction of trichloroacetic acid (7). One unit was defined as the amount of the enzyme which produced an increase in the absorbancy of 0.01 during 120 min at 40°C.

For the assay of proteolytic activity in the sample of polyacrylamide gel, the amount of splits was determined by using either ninhydrin (8) or phenol reagent (9) instead of measuring the absorbancy at 280 nm.

Assays with ATEE and BAEE were performed by a spectrophotometric method (10). Assays with Z-Phe-Tyr and poly-Glu were performed by the ninhidrin method of Yenn and Cocking (8).

Polyacrylamide gel electrophoresis. Disc electrophoresis with 7.5% polyacrylamide gels was conducted according to the procedure of Davis (11). Tris-glycine buffer, pH 8.3 served as the electrode tray buffer and bromphenol blue as the tracking dye. A current of 2 mA per gel tube was applied for 30 min. Two gel tubes were prepared for a sample. One was stained for protein bands with
Amide Black 10B, while the other was cut into 3-mm lengths, and each chip was homogenized with 0.5 M acetate buffer (pH 5.5) in a small glass homogenizer. The proteolytic activity from the disc gel homogenate was assayed by the method described above.

**Molecular weight determination by gel filtration.** Molecular weights of the proteases were determined by a gel filtration technique according to ANDREW (12). Sephadex G-200 equilibrated with 50 mM phosphate buffer (pH 7.0) was packed into a glass column of 1.0 × 60 cm. As standard proteins for molecular weight determination, horse cytochrome c (12,500), bovin serum albumin (67,000), egg albumin (45,000), rabbit muscle aldolase (158,000) and beef liver catalase (240,000) were used.

**Protein concentration.** Protein concentration was determined by the method of LOWRY et al. (13), using crystalline bovine serum albumin as a standard.

## RESULTS

**Purification of proteases**

**Step 1.** Preparation of crude extract: The defatted flour was suspended in 10 volumes of extraction medium and stirred for 15 min in an ice cold bath. The suspension was centrifuged at 15,000 rpm for 30 min. The effect of medium on extraction is shown in Table 1. As the best result was obtained with regard to both total activity and specific activity with 50 mM phosphate buffer it was used in the following purification. For the purification of proteases, 200 g of the soybean defatted flour was suspended in 2,000 ml of medium and stirred for 15 min. A clear supernatant was obtained after centrifugation of the suspension at 15,000 rpm for 30 min.

| Extracting media                  | Activity (units) | Protein (mg) | Specific activity (units/mg) | Relative specific activity |
|-----------------------------------|------------------|--------------|-----------------------------|----------------------------|
| H₂O                               | 8.9              | 20.0         | 0.45                        | 1.0                        |
| 1 M NaCl                           | 8.6              | 22.4         | 0.38                        | 0.7                        |
| 50 mM phosphate buffer pH 7.0      | 12.5             | 12.0         | 1.04                        | 2.3                        |
| 50 mM phosphate buffer pH 7.0 with 1 M NaCl | 9.1              | 18.4         | 0.49                        | 1.1                        |

**Step 2.** Preparation of the pH 5.5 soluble protein fraction: As a preliminary experiment, a portion of the clear supernatant (step 1) was divided into several fractions and in each the pH was adjusted to between 7.0 and 3.3 by acetic acid. The precipitates were collected by centrifugation and dissolved in
medium. An aliquot of the solution was used for proteolytic activity and protein content. At pH 5.5, the ratio of non-protease protein to proteolytic activity was greatest in the precipitate. Thus, supernatant at pH 5.5 was gathered by centrifugation at 10,000 rpm for 15 min. In this step, protease was purified about 2.4-fold (Table 2).

**Step 3.** Ammonium sulfate fractionation: To the pH 5.5 soluble fraction, solid (NH₄)₂SO₄ was added gradually to 65% saturation, then the suspension was kept for over one hour in an ice bath. The precipitate was collected by centrifugation at 10,000 rpm for 15 min, dissolved in a minimum volume of medium and stored frozen state until use. Before using, it was dialyzed against medium.

| Step                  | (a)  | (b)  | (a)  | (b)  |
|-----------------------|------|------|------|------|
| Crude extract         |      |      |      |      |
| pH 5.5 sup            | 8,000| 1.6  | 11,310| 4.6 |
| peak 2                |      |      |      |      |
| Sephadex G-150        | 2,500| 20.0 | 2,100| 15.0 |
| S1                    |      |      |      |      |
| S2                    |      |      |      |      |
| S3                    |      |      |      |      |
| S4                    |      |      |      |      |
| DEAE-cellulose 1      | 1,400| 42.9 | 588  | 70   |
| DEAE-cellulose 2      | 784  | 160  | 980  | 46.6 |

(a) Total activity (units).
(b) Specific activity (units/mg protein).

Fig. 1. Chromatogram of the supernatant fraction of a crude extract acidified to pH 5.5 on Sephadex G-150 column. Proteolytic activity (-----), protein (-----).
Step 4. Sephadex G-150 column chromatography: The preparation from Step 3 was subjected to Sephadex G-150 column, equilibrated with medium, and two peaks of proteolytic activity were obtained (Fig. 1). The fractions, 25–35, and 51–60, were combined respectively, concentrated with ammonium sulfate (60%), and kept frozen until the next step.

Step 5. DEAE-cellulose column chromatography: The concentrated fraction (fraction numbers 25–35) in Step 4 were dissolved in medium and dialyzed.
against the same buffer. This dialyze was applied to a DEAE-cellulose column (2.0 × 65 cm), previously equilibrated with the same buffer. Then, the column was washed with 400 ml of the same buffer and eluted with medium containing NaCl from 0 M to 0.5 M in a linear gradient. Rechromatography of the active fraction was performed on a DEAE-cellulose column prepared as described above. The elution pattern of the rechromatography is shown in Fig. 2, and the fractions (numbers 62–73) were pooled and kept frozen (soybean protease S4).

The concentrated sample consisting of fraction number 51–60 from Step 4 was dialyzed and loaded on a DEAE-cellulose column. The same condition for chromatography as described above revealed a typical elution pattern as shown in Fig 3. The proteolytic activity was separated into three fractions, and they were designated as soybean protease S1 (first fraction) and soybean protease S2 (second fraction). The third fraction (numbers 52–62) was rechromatographed on a DEAE-cellulose column under the conditions described above. The active fraction was designated soybean protease S3 (Fig.3). The soybean proteases S1, S2 and S3 were concentrated with ammonium sulfate, dissolved in medium and stored frozen at −20°C. A typical purification process is outlined in Table 2.

Homogeneity and determination of the apparent molecular weight

Soybean proteases S1, S2, S3 and S4 gave a single peak of proteolytic activity on Sephadex G-200 column. Using standard proteins, their molecular weights were determined as about 50,000 for S1, 35,000 for S2, 60,000 for S3 and 200,000 for S4, as shown in Fig. 4. Disc electrophoresis on polyacrylamide gel of S2 revealed a single protein band, but S1, S3 and S4 gave several minor protein bands. The protein band and proteolytic activity for S2 coincided well (Fig. 5).

![Fig. 4. Determination of the molecular weights of proteases S1, S2, S3 and S4 by gel filtration on a Sephadex G-200 column.](image)
Fig. 5. Electrophoresis of proteolytic activity and protein of protease S2. Proteolytic activity was assayed by A) the ninhydrin method and B) the Lowry method.

Fig. 6. Effect of pH on activities of proteases. Conditions and components were the same as in the standard assay method, except that each buffer was used at the indicated pH. 0.1 M acetate buffer (○—○), 0.1 M phosphate buffer (●—●).
Effects of pH on proteolytic activity

The effects of pH on proteolytic activity are shown in Fig. 6. Maximum activity was obtained at pH 5.5 (S1), 5.0 (S2), 5.0 (S3) and 5.5 (S4).

Effects of inhibitors

Several types of inhibitors on the proteolytic activities were assayed for S1, S2, S3 and S4. The enzyme activities were not inhibited by serine protease inhibitors, DFP (10^{-3}M), SSI (10^{-3}M), thiol protease inhibitors, PCMB (10^{-4}M), NEM (10^{-3}M) or EDTA (10^{-2}M). Acid protease inhibitor SPI (10^{-5}M) inhibited the activity to varying extents from 80% to 90% and EPNP (10^{-5}M) inhibited by 30% to 60%, as shown in Table 3.

Effects of metal ions

The requirement of metal ions for the proteolytic activities of S1, S2, S3 and S4 was assayed in the presence of 10^{-3}M metal ions. Hg^{2+}, Cu^{2+} and Zn^{2+} inhibited the enzyme activities by 70% to 90%, whereas Mn^{2+}, Mg^{2+}, Ca^{2+} and Ni^{2+} had very little effect on the enzyme activities (Table 4). The enzyme activities, which had been inhibited by heavy metal ions (10^{-3}M) were restored by more than 100% by the addition of SH reagent, mercaptoethanol or dithiothreitol, at a concentration of 10^{-3}M.

Specificities of the proteases

The proteolytic activities of the soybean proteases were examined. These proteases hydrolyzed polymer substrates, α-casein or poly-Glu, while their actions on β-casein were weak and BAEE, ATEE, TAME and Z-Phe-Tyr were not hydrolyzed under these conditions.

Table 3. Effects of inhibitors on the proteolytic activity.
Proteolytic activity was assayed at pH 5.5 after the addition of inhibitor at the indicated concentration.

| Inhibitor | Final concentration | S1 (Relative activity, %) | S2 (Relative activity, %) | S3 (Relative activity, %) | S4 (Relative activity, %) |
|-----------|---------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| None      |                     | 100                       | 100                       | 100                       | 100                       |
| DFP       | 1 mm                | 80                        | 90                        | 90                        | 95                        |
| SSI       | 1 mm                | 80                        | 87                        | 91                        | 86                        |
| PCMB      | 0.1 mm              | 91                        | 100                       | 100                       | 100                       |
| NEM       | 1 mm                | 100                       | 100                       | 100                       | 100                       |
| EDTA      | 1 mm                | 100                       | 100                       | 100                       | 100                       |
| SPI       | 0.01 mm             | 22                        | 24                        | 13                        | 17                        |
| EPNP      | 0.01 mm             | 73                        | 68                        | 48                        | 63                        |
Table 4. Effects of metal ions on the proteolytic activity.
Proteolytic activity was assayed at pH 5.5 after addition of metal ions (1 mM).

| Metal salt | S1  | S2  | S3  | S4  |
|------------|-----|-----|-----|-----|
| None       | 100 | 100 | 100 | 100 |
| MnCl₂      | 71  | 83  | 100 | 91  |
| MgCl₂      | 93  | 92  | 100 | 80  |
| CaCl₂      | 93  | 100 | 100 | 93  |
| NiSO₄      | 94  | 75  | 100 | 91  |
| HgCl₂      | 13  | 2   | 11  | 8   |
| ZnCl₂      | 58  | 35  | 24  | 20  |
| CuCl₂      | 29  | 12  | 16  | 16  |

DISCUSSION

In the present experiment, the four proteases that we separated from defatted soybean flour had pH optima between 5.0–5.5. In other dormant seeds, cotton (14), barley (15) and hempseed (16), the presence of acid proteases having pH optima of 2–4 have been described, but proteases in sunflower seeds had pH optima at 5.2 and were classified as acid proteases (17). The application of specific protease inhibitors of microbial origin have facilitated the characterization of proteases in biological materials. Thus, we were able to demonstrate that the soybean proteases S1, S2, S3 and S4 are acid proteases from the effects of inhibitors, SSI or SPI (Table 3).

Although we studied the enzymatic properties of these proteases, there was little difference in the optimum pH, effects of inhibitors, effects of metal ions or specificities for substrates; however, the molecular weights differed markedly. Furthermore, the enzyme activities, which had been inhibited by heavy metal ions (10⁻³M), were restored by more than 100% by the addition of SH reagents. However, the enzymes are not inhibited by SH-blocking reagents such as PCMB or NEM. Thus, it is suggested that free SH groups on the enzymes are participating in the reactivation, but do not contribute to the active sites. Detailed characterization of the proteases in soybean seeds must await further investigation.

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