Partially Purified Proteolytic Enzymes from Wheat Flour and Their Effect on Elongational Viscosity of Cracker Sponges[1]

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ABSTRACT

Enzymes were extracted from wheat flour with ammonium sulfate and purified by gel-filtration chromatography. Two peaks of proteolytic activity were detected. Lubricated uniaxial compression was used to measure the changes in elongational viscosities of cracker sponges at pH 4 during fermentation. The elongational viscosities of the sponges decreased with fermentation time, indicating enzyme activity. The elongational viscosities of the sponges were not noticeably changed when the enzymes had been extracted from the flour. However, the elongational viscosities of the sponges again decreased with fermentation time when the extracted enzymes were added back to the flour. Only one of the two proteolytically active fractions eluted from Sephadex G-100 was responsible for the change in the elongational viscosity of the sponge during fermentation. Rechromatography was used to further purify the proteolytic enzyme and produce a single peak with high specific proteolytic activity. Since pepstatin inhibited most of the activity of the purified enzyme preparation, it contains an acid protease.

One of the purposes of the 18-hr fermentation process in making soda crackers is to obtain an optimum modification of gluten. In studying rheological changes of cracker sponges during fermentation, Pizzinatto and Hoseney (1980) reported that proteolytic enzymes native to flour appeared to be important in changing the rheological properties of the cracker sponge. The effects of proteolytic enzymes in altering rheological properties of cracker sponges were further investigated by Wu and Hoseney (1989). They found that resistance-to-extension (RTE) of the sponge did not change during fermentation when salt-extractable enzymes were extracted from the flour. However, a decrease in RTE, similar to that of the unextracted control flour, occurred when extracted enzymes were added back to the residue flour. The authors also showed that the decrease in RTE could be influenced by the amount of extracted enzymes added (Wu and Hoseney 1989).

Attempts have been made to reduce the long fermentation time required in cracker manufacturing. Proteolytic enzymes have a softening action on gluten and have been used successfully in many commercial products. However, commercial proteolytic enzymes have not been totally successful in reducing the fermentation time for cracker processing. Drapron and Godon (1987) reported that a considerable rheological change occurred when only a few peptide bonds of gluten were broken by proteolytic enzymes. Thus, the proteolytic enzymes in native wheat flour may act on specific peptide bonds of gluten during the sponge fermentation in cracker manufacturing. This proteolytic action causes the rheological changes and gives the unique texture to the final products.

In this study, proteolytic enzymes extracted from a commercial soft wheat cracker flour were purified by chromatography and characterized by measuring proteolytic activity and dough elasticity. The properties of the enzymes may help to explain how they affect the rheological changes in cracker sponges.

MATERIALS AND METHODS

The commercial soft wheat cracker flour (moisture 12.9%, protein 10.1%, and ash 0.45%) used in this study was supplied by Mennel Milling Co., Fostoria, OH. All chemicals used in this study were reagent grade.

Extraction of Proteolytic Enzymes from Wheat Flours

The isolation procedure was basically as described by Salgo (1981). Flour (20 g) was stirred with 120 ml of ammonium sulfate solution (18% saturation) at 4°C for 1.5 hr. The slurry was then centrifuged at 1,000 × *g* for 15 min. The supernatant was adjusted to 40% saturation by adding solid ammonium sulfate, allowed to set for 24 hr, and then centrifuged. The centrifugate was discarded. The supernatant adjusted to 80% saturation and allowed to set for another 24 hr, and then centrifuged at 1,000 × *g* for 15 min. The precipitate from 80% saturation was dialyzed against water and lyophilized. This was the crude-enzyme fraction.

Proteolytic Activity Assay

A Farrand model A4 fluorometer (Farrand Optical Co., New York) and a Pye-Unicam 1750B UV-VIS spectrophotometer (Pye Unican Ltd., Cambridge, England) were used to assay the proteolytic activity. The second largest of six aperture openings of the fluorometer was used. Round cuvettes (75 × 10 mm) were used for fluorometric readings; 1.0-cm square cuvettes were used for spectrophotometer (340 nm) readings.

Substrate solution was prepared by adding 0.5 g of bovine hemoglobin (Sigma Chemicals Co., St. Louis, MO) to 1 ml of 0.025*M* sodium acetate buffer, pH 4. Enzyme solutions (50 μl of 5 mg/ml) were added to 1 ml of substrate solution, mixed, and incubated at 40°C for different time intervals. After incubation, 30 μl of enzyme-substrate solution was removed and added to 200 μl of saturated borate buffer (pH 9.4) containing 1% sodium dodecyl sulfate (SDS) solution. Then, 25 μl of *o*-pthaldialdehyde reagent solution (2.0 ml of methanol, 220 μl of saturated borate buffer, 12.5 mg of OPA, and 22 μl of ethanethiol) was added to the tube. The combined solutions were mixed and allowed to react at room temperature for 15 min. Then 1.0 ml of methanol was added to the cuvette, the solution was mixed thoroughly, and fluorometer and spectrophotometer readings were taken. The method was basically as described by Mathewson et al (1988).

Gel-Filtration Chromatography

The crude-enzyme fraction (120 mg) extracted by ammonium sulfate was dissolved in 6 ml of 0.025*M* sodium acetate buffer (pH 4) containing 1 m*M* ethylenediaminetetraacetic acid (EDTA) and 2 m*M* mercaptoethanol. The suspension was centrifuged at 2,000 × *g* for 20 min. The supernatant was applied directly to the top of a column (2.6 × 90 cm, Pharmacia Fine Chemicals, Sweden), packed with Sephadex G-100, and equilibrated with 0.025*M* sodium acetate buffer (pH 4.0). The flow rate was 14 ml/hr. Fractions (10 ml) were collected by an LKB Ultrorac fraction collector. The void volume of the column was determined by the elution time for a solution containing 5 mg/ml of blue dextran in sodium acetate, pH 4. Fractions were analyzed by

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a spectrophotometer at 280 nm. The proteolytic activity of each fraction was measured by the fluorescence method described previously.

Rechromatography

Fractions 17–22, previously eluted from Sephadex G-100 and found to contain proteolytic activity, were pooled and concentrated from 70 ml to about 2.5 ml with a centriprep concentrator (MW cutoff: 8,000 Da) (Amicon Division, W. R. Grace & Co., MA). The concentrated (2.5 ml) enzyme extract (fraction 17–22) from the Sephadex G-100 purification was applied to the top of the 2.6 × 75-cm LKB column packed with Sephadex G-150 and previously equilibrated with 0.025*M* sodium acetate buffer (pH 4.0) and eluted with a flow rate of 14 ml/hr. Fractions (10 ml) were collected with an LKB automatic fraction collector.

Fractions 14–18 that eluted from the Sephadex G-150 column were found to contain proteolytic activity. They were pooled and concentrated with a centriprep as described above. This concentrated enzyme fraction was further applied to the top of a column packed with Sephadex G-50 (1.5 × 90 cm). Operating conditions were the same as those described above.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The SDS-PAGE procedure was essentially as reported in the Hoefer Scientific Instrument Catalog (1983). The enzyme solution was collected from each purification step and lyophilized. The Hoefer SE 600 system (160 × 180 × 1.5 mm) containing a linear gradient of 10–20% acrylamide was operated at 12°C for 5 hr at 30 mA constant current. Bio-Rad molecular weight markers (phosphorylase B, 97.4 kDa; bovine serum, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.5 kDa) were used. Coomassie blue stain in trichloroacetic acid (Lookhart et al 1982) was used for visualizing the proteins.

Flour Preparation for Reconstitution Experiment

Flour (625 g) was extracted with 4 L of ammonium sulfate solution (18% saturation), stirred for 1.5 hr at room temperature, and centrifuged at 1,000 × *g* for 15 min. The supernatant was adjusted to 40% saturation by adding solid ammonium sulfate, allowed to set for 24 hr, and then centrifuged. The centrifugate was discarded. The supernatant was adjusted to 80% saturation and allowed to set for another 24 hrs and then centrifuged at 1,000 × *g* for 15 min. The crude-enzyme fraction was obtained from the precipitate. The extracted flour residue was suspended in 4 L of water, stirred, and centrifuged at 1,000 × *g* for 15 min. The residue flour was separated again from the supernatant, and the water wash was repeated three times before the flour was lyophilized. The lyophilized flour was ground with a laboratory mill (model 7140, Dumore, Racine, WI) for the reconstitution experiment.

Lubricated Uniaxial Compression

To study the change in elongational viscosity of the cracker sponge during fermentation, lubricated uniaxial compression was performed with an Instron Universal Testing Machine (model 1130-C4) equipped with a 50-kg load cell. The metal platen surfaces were covered with adhesive-backed Teflon sheeting, and the sample-platen interfaces were lubricated with heavy mineral oil (Bagley and Christianson 1986, Bagley et al 1988).

Flour (10 g) with 56% water absorption was mixed in a mixograph for 1.5 min. The pH of the sponge was adjusted to 4.0 with lactic acid before fermentation, and it remained at that level. The fermentation was carried out in a fermentation cabinet with 85% humidity and 30°C for various time intervals. After fermentation, doughs were sheeted to 0.794-cm thickness in two passes through a sheeter (National Mfg. Co.). Three disk-shaped pieces were cut from each dough with a cookie cutter (diameter, 1.9 cm). The resulting test pieces were immediately immersed in heavy mineral oil (Dillon's, Hutchinson, KS) for 10 sec on each side. Before testing, the dough height was measured with calipers; that measurement was used to determine the time required to deform the sample to a strain level of 50% at a crosshead speed of 2.5 cm/min. The elongational viscosity is calculated by the following equation:

$$\text{Elongational Viscosity} = 2\,F\,h/R^2\,V_z$$

where F is the total force on the sample, h is the sample height (cm) after compression, R is the radius (cm) of the sample after compression, and V_z is crosshead speed (cm/min).

Proteolytic Enzyme Inhibitors Purified by Rechromatography

Five proteolytic enzyme inhibitors were used in this study: EDTA, pepstatin, and phenylmethylsulfonylfluoride (PMSF) from Sigma Chemicals Co.; iodoacetamide (IAA) from Fisher Scientific Co., Fair Lawn, NJ; and *N*-ethylmaleimide (NEMI) from Nutritional Biochemicals Corporation, Cleveland, OH. The concentration of inhibitors was 1 m*M* for pepstatin, PMSF, and NEMI; 2 m*M* for EDTA; and 0.01%, v/v, for IAA. Before the proteolytic activity assay, the purified material (0.5 ml of 40 ml total enzyme collection obtained by consecutive chromatographic separations via Sephadex G-100, G-150, and G-50) was mixed with the substrate-inhibitor solution at 40°C for 2 hr. The proteolytic activity of each enzyme-substrate-inhibitor mixture was measured by the fluorescence method as described previously.

RESULTS AND DISCUSSION

Purification of Proteolytic Enzymes by Gel-Filtration Chromatography

The flour extract separated by G-100 Sephadex chromatography is shown in Figure 1; the protein content and proteolytic activity of the enzyme fractions are labeled. One peak and a poorly resolved shoulder of proteolytic activity were found. The first peak (fraction 14–22) partially overlapped the void volume.

Changes in Elongational Viscosity

To study the possible effect of proteolytic enzymes on sponge rheology, the pH of the sponge was adjusted to the optimum for proteolytic action (Wu and Hoseney 1989). The elongational viscosity of the sponges decreased with fermentation time (Fig. 2). In a reconstitution study, sponges were made from flour residue with and without added enzymes. After 18 hr of fermentation at pH 4, insignificant changes were found in the elongational viscosity of sponges made from the flour residue (Fig. 3). This indicated that the enzymes extracted with ammonium sulfate were important in changing the rheological properties of sponges. However, the elongational viscosity of sponges decreased with fermentation time when the extracted enzymes were added back to the flour residue. These results show the effect of proteolytic enzymes on the rheology of sponge.

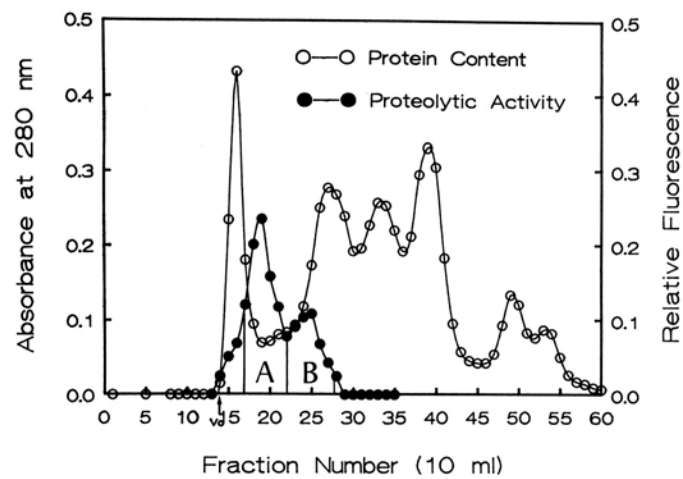

Fig. 1. Chromatogram of ammonium sulfate-extracted wheat enzymes on G-100 Sephadex. Peaks of proteolytic activity: **A,** 17–22, and **B,** 23–28. Arrow = column void volume.

Effect of Proteolytic Enzymes on Elongational Viscosity

Salgo (1981) reported that more than one proteolytic enzyme was found in wheat flour. In our work, two peaks of proteolytic activity were found after chromatography on Sephadex G-100 (Fig. 1). Therefore, we attempted to determine which peak of proteolytic activity was responsible for the rheological change in the sponge. The two peaks of proteolytic activity were labeled as A (17–22) and B (23–28) (Fig. 1). The two fractions were separately concentrated by ultrafiltration. In the reconstitution experiments, the proteolytically active enzyme solutions (portions A or B) were added back to flour residues individually to make sponges. The amount of enzymes in portion A added back to the flour residues is equivalent to that in the original flour. After fermentation for 18 hr at pH 4, a major decrease in elongational viscosity was found in the sponge made with portion A (Fig. 4). The enzymes in portion B did not change the elongational viscosity of the sponge. These results suggest that the enzymes in portion A were responsible for the rheological changes.

The enzymes in fraction A were active but not pure, as evidenced by partial overlapping with the void volume and fraction B (Fig 1). As shown in Figure 5, lane 3 contained multiple proteins. Therefore, further purification of the proteolytic enzymes was necessary.

Purification of Proteolytic Enzymes by Rechromatography

Fractions 17–22 (portion A) eluted from Sephadex G-100 were pooled and concentrated. The concentrated enzyme solution (2.5 ml contained 0.94 mg of protein) was applied to a Sephadex G-150 column. Two overlapping peaks were found in the chromatogram (Fig. 6). The peak at the void volume had no proteolytic activity, whereas the second peak (fractions 13–20) showed proteolytic activity. However, because the two peaks overlapped, further purification was necessary.

Fractions 14–18, eluted from the Sephadex G-150 column, were pooled and concentrated. This concentrated enzyme solution (2.5 ml containing 0.49 mg of protein) was loaded on a Sephadex G-50 column. About half of the protein (0.24 mg of protein) and all of the proteolytic activity eluted as a single peak (fractions 7–10) at the void volume (Fig. 7). The ratio of fluorescence to absorbance of the proteolytically active enzyme fraction purified by rechromatography was about 3.8, indicating that the purified material was an endoproteolytic enzyme, based on the observa-

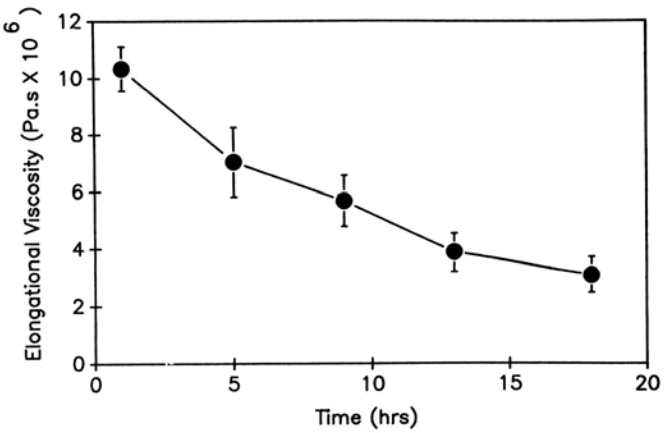

Fig. 2. Changes of elongational viscosity in sponges made from commercial cracker flour during 18-hr fermentation. Sponges set at pH 4, the optimum pH for proteolytic action.

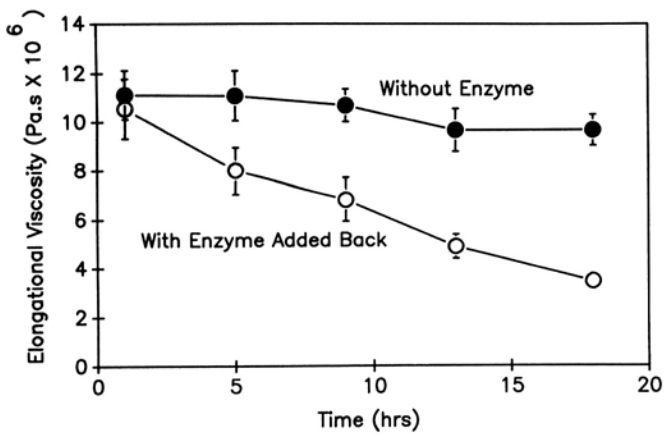

Fig. 3. Effect of extracted enzyme on elongational viscosity of sponges made from reconstituted flours, with and without enzymes, during 18-hr fermentation and reconstitution experiment. Sponges set at pH 4, the optimum pH for proteolytic action.

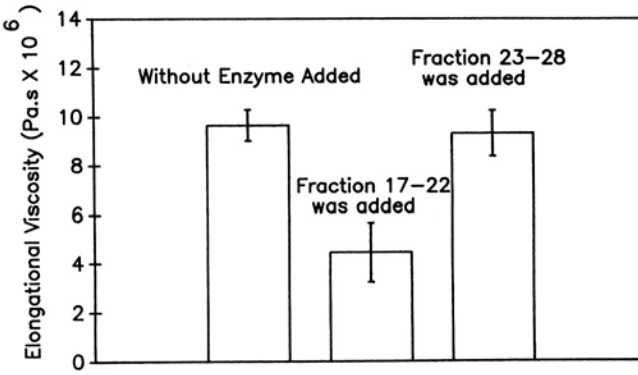

Fig. 4. Effect of proteolytically active fraction obtained from Sephadex G-100 on elongational viscosity of sponges set at pH 4.

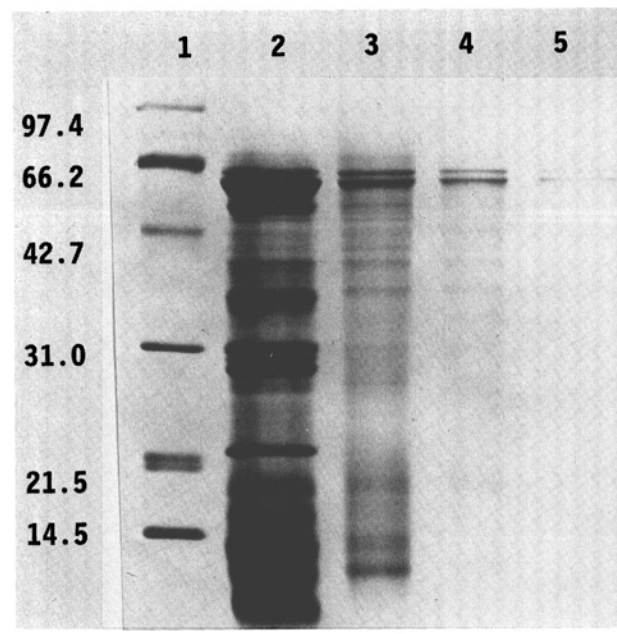

Fig. 5. Protein distribution of wheat extracts on each purified step examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis containing a linear gradient of acrylamide from 10 to 20%. Lane 1, Bio-Rad molecular weight markers. Lane 2, proteins purified by ammonium sulfate fractionation (20 μl of 5-mg crude enzyme/250 μl). Lane 3, 20 μl of a 250-μl solution containing 0.94 mg of protein purified by gel-filtration chromatography on G-100 Sephadex. Lane 4, 20 μl of a 250 μl solution (0.49 mg of protein) purified by rechromatography on G-100 and G-150 Sephadex. Lane 5, 20 μl of a 250-μl solution containing 0.24 mg of protein purified by rechromatography on G-100, G-150 and G-50 Sephadex. The solution in Lane 3 contained collected and pooled proteolytically active fractions 16–22 eluted from G-100 Sephadex. The solution was dialyzed against water and lyophilized. The lyophilized material was dissolved in a 250-μl sample-treatment solution of sodium dodecyl sulfate and 20 μl of the 250-μl sample was loaded. Solutions in Lanes 4 and 5 were obtained by the the same procedure on proteolytically active fractions 14–18 and 7–10 eluted from G-150 and G-50 Sephadex, respectively.

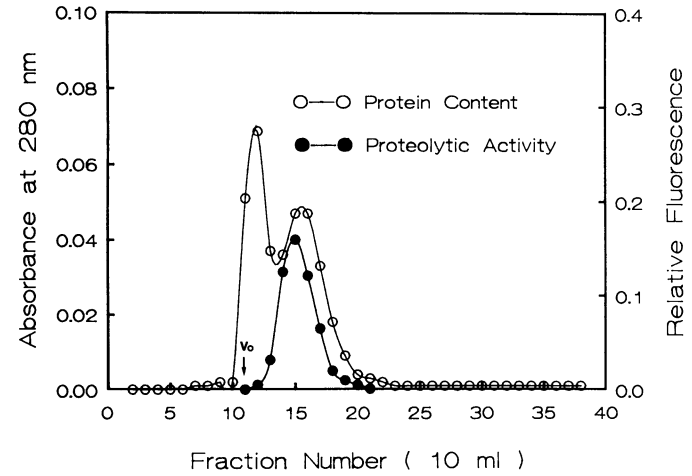

Fig. 6. Rechromatography of proteolytically active fractions from Sephadex G-100 on G-150. Arrow = column void volume.

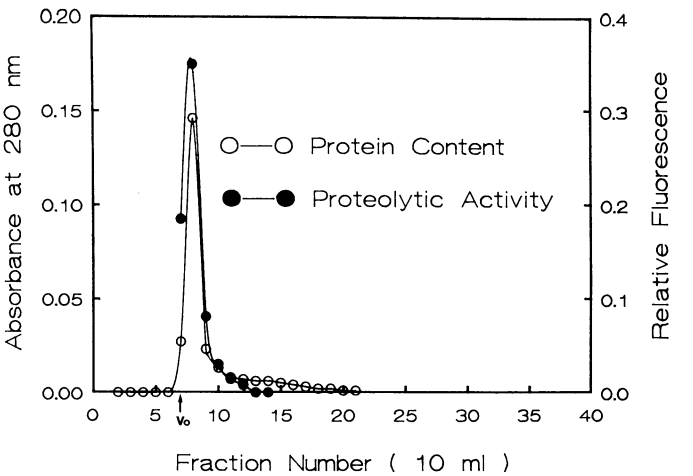

Fig. 7. Rechromatography of proteolytically active fraction from Sephadex G-150 on G-50. Arrow = column void volume.

tions by Mathewson and Seabourn (1988). Endo-proteolytic activity would be expected to produce more extensible doughs, such as those normally found in cracker sponge after fermentation. This was shown by adding the purified protease to the extracted flour and measuring the elongational viscosity. This fraction increased the elongational viscosity.

The protein distribution of the proteolytically active fraction from each chromatographic purification step was examined by SDS-PAGE (Fig. 5). A large amount of nonproteolytically active protein (Fig. 5, lanes 2 and 3) was removed by gel-filtration chromatography on Sephadex G-100. Further purification by rechromatography was effective in removing most of these nonactive (contaminating) proteins (Fig. 5, lanes 2 and 5). Table I shows the protein content and specific activity as a function of the purification step.

Effect of Inhibitors on Proteolytic Activity

Five inhibitors were used in this study to characterize the proteolytic enzyme purified by the multiple rechromatographic procedure. About 85% of the activity was inhibited by pepstatin, indicating that the enzyme is an acid protease. Hofmann (1974) reported that acid proteases have their main specificity toward the amino acids residue of tyrosine, phenylalanine, and tryptophan.

NEMI, IAA, PMSF, and EDTA had essentially no effect on the proteolytic activity; therefore no sulfhydryl protease, serine protease, or metalloprotease activity is involved in the system.

TABLE I
Protein Content and Specific Activity of Proteinase at Various Steps in Purification

Fraction	Fractions Collected	Protein Content/mg[a]	Specific Activity (relative fluorescence per mg of protein)
Ammonium sulfate		5.0	3.0
Sephadex G-100	17–22	0.94	16.4
Sephadex G-150	14–18	0.49	20.8
Sephadex G-50	7–10	0.24	67.2

[a] Determined after the indicated purification step.

These results are in agreement with those of Kawamura and Yonezawa (1982). They reported that pepstatin prevented the proteolytic action that decreases the viscosity of an enzyme-substrate solution. Because only 85% of the original activity was eliminated by pepstatin, more than one protease may be present in this purified material.

However, Salgo (1981) reported that wheat proteolytic enzymes were inhibited by PMSF and EDTA, suggesting that the enzymes were metalloproteases and serine proteases. Using the gluten-starch system and the same inhibitors as in Salgo's study, Wu (1987) investigated the effect of inhibitors on proteolytic activity as reflected by the change in RTE of sponges. None of the inhibitors in Wu's study stopped the proteolytic activity. However, pepstatin was not used in Wu's study, which may explain the lack of changes in the RTE of sponges. We would expect the addition of pepstatin to gluten-starch systems to inhibit the proteolytic activity and reduce the changes in the RTE of sponges.

CONCLUSION

Material extracted from soft wheat (cracker) flour with ammonium sulfate was purified by sequential chromatography through Sephadex G-100, then G-150, and finally G-50. The fractions from each gel-filtration column with proteolytic activity were collected, concentrated, and purified on successive columns. The homogeneity of the active fractions was examined by SDS-PAGE. Multiple electrophoretic bands were detected from the initial extract, but the number of bands decreased with rechromatography.

The purified enzyme preparation (from the G-50 column) exhibited endo-proteolytic acid-protease activity. This purified protease was effective in changing the elongational viscosity of sponges set at pH 4. When the cracker flour was extracted with ammonium sulfate (enzymes removed), essentially no changes in elongational viscosity were found. However, when the purified material was added back to the extracted flour, the changes in rheological properties of the sponges were similar to those of the original flour.

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