THE PROTEASES OF THE SOYBEAN[1]

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ABSTRACT

Column chromatography with gradient elution on diethylaminoethyl cellulose resulted in separation of the proteolytic activity of the soybean into six distinct fractions. An inhibitor was separated from the active fractions. The properties of the crude extract and four purified fractions were determined. Although the crude extract was inhibited by iodoacetate, the activity of the pure fractions was not associated with sulfhydryl groups.

Although the proteolytic activity of the soybean has been described in the literature, the work was either a report of the presence of proteolytic activity in the beans, or an investigation of the possible effects on baking properties. Ofelt, Smith, and Mills (1) describe the proteolytic activities of the soybean with special reference to their possible relation to baking. This paper continues the above work, with emphasis on the purification and properties of the enzymes.

Materials and Methods

Enzyme Extraction. Soybeans of the Ogden variety were reduced to a powder in a Waring Blendor with pauses every 2 min. to prevent overheating. Soy flour (100 g.) was treated with 1 liter of distilled water in the blender. This volume filled the container to capacity and minimized foam production. The suspension was blended for 6 min. with pauses at 2-min. intervals to prevent overheating. For the same reason, the container was cooled to 2°C. in a refrigerator before use. The resulting suspension was centrifuged at 15,000 r.p.m. for 10 min. A thin, white, oily layer was skimmed off. Determinations by the methods described below showed a specific activity of 53 proteolytic units per mg. protein in the supernatant from 10 g. soy flour and 6.9 proteolytic units per mg. protein in the residue from the same quantity of soy flour. Since the supernatant contained most of the proteolytic activity (88.3%), this supernatant was used in subsequent work.

A 0.98% extract was also prepared in a pestle homogenizer, care being taken to prevent overheating by cooling and by grinding slowly. Here also the activity was mainly in the supernatant containing 81.6 proteolytic units per mg. protein from 10 g. soy flour, with only negli-

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gible amounts remaining in the precipitate (3%). The pH values were 6.2–6.3 and 6.0 for the Waring Blendor extract and the homogenizer extract respectively.

An acetone powder of the soy flour was prepared by extraction twice with five volumes of cold diethyl ether (0°C.) and then twice with five volumes of cold acetone (0°C.), with subsequent decantation and vacuum evaporation of the solvent. The enzyme was extracted from this acetone powder by both methods described above.

A 9.2% extract had 64 units per mg. protein from 10 g. soy flour, which is an increase of 20.7% in activity for the acetone-treated preparation. The 0.98% extract showed an increase of 30% under the same conditions.

Proteolytic Activity Determination. The absorbance of the trichloroacetic acid(TCA)-soluble extract at 280 mμ (Beckman DU spectrophotometer) of an incubated mixture served as a measure of proteolysis. This was compared with the analysis of the TCA extract by the method of Lowry for tyrosine (2), and by micro-Kjeldahl determination. Results were identical.

In subsequent work, therefore, the absorbance at 280 mμ of the TCA supernatant was taken as a measure of proteolysis. At first, the protein was precipitated with 2 ml. of a TCA solution containing 1.43 g. per ml. (14 ml. incubation mixture described in the next paragraph). At times, however, the supernatant was cloudy and the TCA concentration was reduced to 0.85 g. per ml. The absorbance of the TCA-soluble extract, obtained when 0.85 g. TCA per ml. was used, was identical with that obtained when 1.43 g. TCA per ml. was used.

The soy extract was first incubated at 40° and later on at 50°C. (the optimum) with buffer and casein suspension according to Ofelt *et al.* (1). When proteolytic activities were determined on suspensions incubated in a 100-ml. Erlenmeyer flask, the absorbance of the TCA-soluble extract at 280 mμ was 0.95, whereas it was 0.79 when a test tube was used under the same conditions. Subsequent incubations were therefore done in 100-ml. Erlenmeyer flasks. Incubation of the mixture in a rotary shaker (100 r.p.m.) resulted in TCA-soluble extract with an absorbance of 1.10 (280 mμ), whereas the incubation of the same quantities without shaking gave an absorbance of 1.14 (280 mμ) for the TCA-soluble extract. The subsequent incubations were therefore done without shaking.

The quantities used by Ofelt *et al.* (1) were later reduced to 4 ml. soy extract, 4 ml. casein suspension, and 6 ml. buffer, these being in the same proportions as the larger volumes used above. For determination of the proteolytic activity of chromatographed samples, half

the above-mentioned quantities were used. The casein was vitamin-free assay grade (Nutritional Biochemicals Corp., Cleveland, Ohio). The buffer was 0.05*M* citrate or acetate.

As described in the same paper (1) the assay of the TCA-soluble extract was a measure of the proteolytic activity at the end of the incubation period (originally 5 hr. but changed to 2.5 hr. as a result of preliminary experiments).

Units. For convenience, the following units of proteolytic activity were used. For the 9.2% extract a change of 0.1 in absorbance at 280 mμ of the TCA-soluble extract was considered as one unit of activity. Similarly for the 0.98% extract a change in absorbance at 280 mμ of 0.01 was considered as one unit.

Soy Self-Digestion. Originally, the blank was made up of all the ingredients used in the incubation mixture to which TCA was added at the outset. When proteolytic activity was determined at various time intervals, the TCA-soluble extract increased linearly up to 4 hr., then decreased, and again increased to its previous level at 6 hr. (Fig. 1). Constant repetition gave the same results.

This unusual behavior led to incubation of the proteolytic extract with buffer but without substrate. Every hour, TCA was added to a pair of Erlenmeyer flasks and the absorbance (280 mμ) of the TCA-soluble extract was determined. Figure 2 gives the results obtained. The increase in TCA-soluble extract up to 5 hr. of incubation is quite marked.

To correct for the apparent self-digestion described above, a double blank was used. One contained the enzyme extract and buffer and the other the casein solution. Both were incubated for the same length of time as the complete incubation mixture and then both blanks were mixed and TCA was added as with the incubation mixture. When this

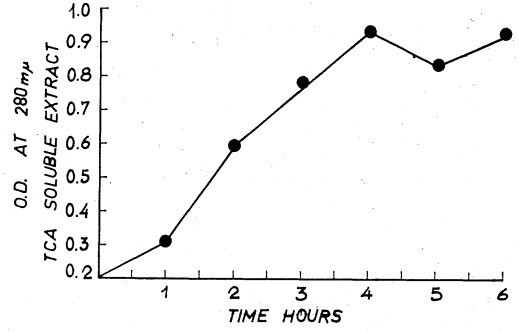

Fig. 1. Change in TCA-soluble extract with time on incubation of soybean protease with casein.

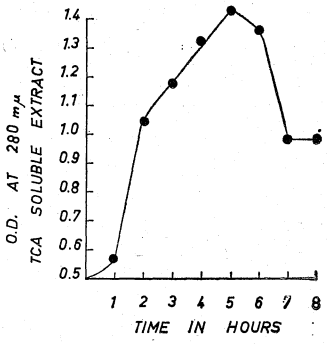

Fig. 2. Change in TCA-soluble extract with time of soybean protease incubated in buffer at pH 5.5.

double blank was used, the anomalous dip in the digestion at 5 hr. disappeared, and the TCA-soluble extract (absorbance at 280 mμ) was linear with time up to 3 hr. and then was constant. A 2.5-hr. incubation period was used in subsequent work. For a 2.5-hr. incubation period, both the apparent self-digestion and the increase in TCA-soluble extract on a casein substrate, when the one blank was used, are linear and we therefore assume that the self-digestion with or without casein was the same.

Results

pH Activity Curve. The pH activity curve of Ofelt *et al.* (1) was confirmed. It was extended to pH 7.0 on the one hand and to pH 3.0 on the other. Figure 3 gives the results obtained. The optimal pH was 5.5.

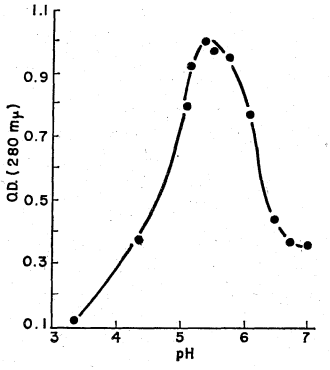

Fig. 3. Effect of pH on crude soybean protease activity. The absorption of the TCA-soluble extract at 280 mμ of the protease incubated with casein for 2.5 hr. at 50°C. is given.

Effect of Temperature. The absorbance of the TCA supernatant after incubation of the soy extract with casein and buffer at pH 5.5 for 2.5 hr. at the various temperatures is given in the table below. The optimum is 50°C. Proteolytic activity was determined from the absorbance of the TCA-soluble extract after incubation.

Temperature °C.	*Absorbance at 280 mμ*
20	0.14
30	0.60
40	0.98
50	1.13
60	0.92

In another experiment, the stability of the enzyme at various temperatures was determined. For this purpose the enzyme was held at the temperature investigated for 30 min. and then its proteolytic activity was determined by the normal procedure. Table I gives the results obtained. The 30-min. preincubation period at temperatures up to 40°C. had no deleterious effect on the proteolytic activity. At 50°C., on the other hand, the 30-min. preincubation reduced the enzyme activity to 27% of the activity of an enzyme preparation preincubated at 40°C. for 30 min. Preincubation at higher temperatures was more destructive.

TABLE I

EFFECT OF STORAGE TEMPERATURE ON STABILITY OF THE PROTEOLYTIC ACTIVITY OF SOYBEAN EXTRACT[a]

TEMPERATURE	T.C.A.-SOLUBLE EXTRACT[b]	TEMPERATURE	T.C.A.-SOLUBLE EXTRACT[b]
5	0.73	40	0.74
10	0.73	50	0.20
20	0.82	60	0.19
30	0.80	100	0.00

[a] The enzyme was held at the temperature indicated for 0.5 hr. at pH 5.5 and then its proteolytic activity was determined on a casein substance pH 5.5 (2.5 hr. of incubation).
[b] Absorbance 280 mμ.

Physical and Chemical Properties. On dialysis of 9.2% soy protein suspension against cold distilled water, the proteolytic activity remained in the undialyzable fraction.

Table II gives the results obtained when various inhibitors were used. It can be seen that the only substance found with inhibitory properties was iodoacetic acid. Further determinations with fractions purified by column chromatography (described in the next section), however, revealed no effect at all of iodoacetate on the proteolytic

activity. This was verified by using *p*-chloromercuribenzoate (PCMB) by the method described by Boyer and Segal (3). Neither varying the enzyme concentration nor varying the PCMB caused any change from the control.

TABLE II

EFFECT OF VARIOUS REAGENTS ON THE PROTEOLYTIC ACTIVITY OF SOYBEAN PROTEASE[a]

REAGENT ADDED	ABSORBANCE 280 mμ	REAGENT ADDED	ABSORBANCE 280 mμ
None	0.135	$NaNH_3$	0.099
Iodoacetic acid (neutralized)	0.022	NaCN	0.11
$MgCl_2$	0.125	EDTA (neutralized)	0.14
NaF	0.130	Cysteine (neutralized)	0.175
$CaCl_2$	0.081		

[a] The proteolytic activity was determined from the absorbance of the TCA-soluble extract after 2.5 hr. of incubation of 0.98% extract in homogenizer.

TCA at 5% concentration precipitated 79% of the proteolytic activity. The TCA concentration could be reduced still further to 0.034%, where 74% of the activity precipitated. The resuspended precipitate was actively proteolytic. Seventy-six percent of the activity precipitated at pH 4.5, the point of maximum precipitation.

Purification. The TCA precipitate was placed on a diethylaminoethyl (DEAE) cellulose column 1.5 cm. in diameter and 10 cm. high, which was mounted on a Buchler refrigerated fraction collector. Acetate buffer (50 ml.), pH 4.8, was allowed to percolate through the column. This treatment solubilized part of the precipitate. Eighteen percent of the original activity was recovered in the first 15 ml. The specific activity of this fraction was increased thirtyfold. The rest of the TCA precipitate remained on the column.

The crude enzyme preparation (prepared in a homogenizer) was dialyzed against distilled water which was changed until the dialysate showed no precipitate with silver nitrate. Five milliliters, containing 5.58 mg. protein (4.17 proteolytic units), was placed on a DEAE cellulose column as described above and was eluted with a gradient of acetate buffer 0.05*M* to 1*M*, pH 5.6, the Buchler apparatus being used for maintaining the gradient. Ten-milliliter fractions were collected.

Recovery of protein and proteolytic activity are given in Fig. 4.

The presence of six fractions is quite evident. The recovery here was 4.37 mg. protein or 78.3% and 398.6 activity units, an increase more than ninety-five-fold.

The Michaelis constants of four of the six proteolytic fractions (I, II, III, VI, Fig. 4) were determined on a casein substrate. The calcu-

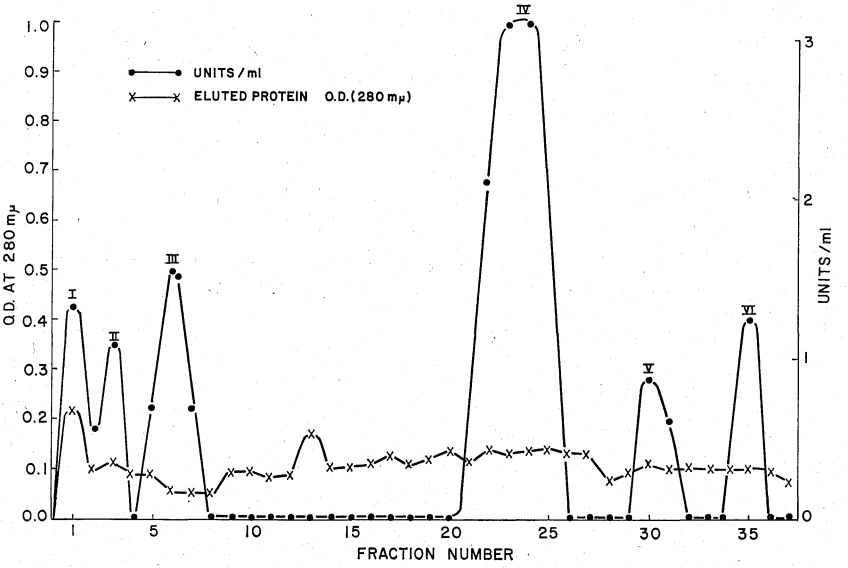

Fig. 4. Column chromatography on DEAE-cellulose of soybean protease. The eluant was a gradient of acetate buffer, pH 5.6, from 0.05*M* to 1*M*. The absorbance at 280 mμ of the TCA-soluble extract after incubation of each fraction with casein and buffer as described is the basis for the calculation of proteolytic activity.

lations were on a weight basis. They are listed in the table below.

Fraction	*Km* %
I	0.46
II	0.50
III	0.81
VI	1.12

Fractions I and II appear to be similar. Fractions III and VI, however, have constants quite different. This fact leads us to conclude that these fractions, at least, are different from the others.

Paper electrophoresis of the crude enzyme in equal volumes of 2.5% formic acid and 7.5% acetic acid, pH 2.3, gave five distinct bands when stained with amido black. Fraction I, under the same conditions, gave one band only. The other fractions are under investigation.

Discussion

The quantities of pure chromatographed protease were insufficient to complete the necessary determinations. In this respect the apparent self-digestion of the original crude extract is of interest. Work is in progress at present to prepare quantities of each chromatographically

pure protease, to determine whether self-digestion is actually observed with these fractions or whether one fraction hydrolyzes another. The amount of self-digestion in the crude extract is very important and when not taken into account can cause considerable error. For example, the maximum occurred after 5 hr. with the single blank and after 3 hr. with the double blank.

Figure 2 shows a drop in TCA-soluble extract after 5 hr., and leveling off at 8 hr. We have no explanation for this phenomenon.

Although the inhibitory effect of iodoacetate and the stimulatory effect of cysteine on the crude enzyme preparation suggested the involvement of sulfhydryl groups, no inhibitory effect of iodoacetate or of PCMB was observed on any of the six active proteolytic fractions separated by column chromatography.

Cyanide had very little effect, showing that the enzyme did not require cyanide activation, as would be expected from a papain-type protease.

The precipitate after TCA treatment remained actively proteolytic. The active centers were therefore not affected by this precipitation.

Although the protease was divided into six active fractions, by column chromatography the greatest quantity of enzyme was always eluted in the first fraction. The other fractions together made up less than 20% of the active enzyme.

The remarkable enrichment of the proteolytic activity after chromatography strongly suggests the removal of protease inhibitors.

The higher specific activity of the acetone powder is most likely due to removal of lipids.

Literature Cited

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