Thermal Inactivation of Trypsin Inhibitors in Aqueous Extracts of Soybeans. Studies on Substances That Accelerate Inactivation

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ABSTRACT

Substances separated from soybean extracts by gel filtration on Sephadex G-75 accelerated thermal inactivation of trypsin inhibitory activity of soybean extracts and purified inhibitors. Aqueous extracts of soybean showed faster antitryptic activity destruction in the presence of these factors at all inactivation times. The effect was appreciable after a few minutes of treatment at 96° C, and after 15 min the residual activity was considerably smaller in the presence of these accelerators. Samples of trypsin inhibitors, purified by gel filtration on Sephadex G-75 and subjected to heat treatment at 96° C for 15 min, showed increases in inactivation practically up to completion with increasing concentration of accelerators. The accelerating effect was observed with all fractions of trypsin inhibitors purified by Sephadex G-75 and diethylaminoethyl cellulose chromatography, but the extent of the effect was not the same for all inhibitors. Carboxymethylation with sodium iodoacetate of the Sephadex G-75 fraction containing the accelerators led to a loss of the accelerating effect at the same time as the carboxymethylation of their free mercapto groups took place.

Substances other than the trypsin inhibitors (TI) themselves may participate in the thermal inactivation of TI activity in aqueous extracts of soybeans and other seeds. Results of gel filtration on Sephadex G-75 columns support this conclusion because purified inhibitors, obtained in the fraction of intermediate molecular weight, have greater thermal stability than do inhibitors in extracts. Moreover, in the presence of the highest molecular weight fraction of the same gel filtration, the thermal inactivation of these inhibitors is accelerated (Ellenrieder et al 1980).

This article reports the results of experiments made to obtain more information about the process of thermal inactivation of TI activity.

MATERIALS AND METHODS

Materials

Benzoyl-DL-arginine-*p*-nitroanilide (BAPA), bovine trypsin (twice crystallized), sodium iodoacetate, and 5,5′ dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma, St. Louis, MO; Sephadex G-75 from Pharmacia, Upsala, Sweden; and diethylaminoethyl (DEAE) cellulose DE 32 from Whatman, Maidstone, Kent, England. Soybean (*Glycine max*) was of the cultivar Cerrillos W 65, crop year 1978.

Defatted soy flour was prepared according to the method described by Ellenrieder et al (1980).

Preparation of Accelerator and TI Fractions

FI, the fraction of highest molecular weight obtained by gel filtration on Sephadex G-75, containing the substances that accelerate the thermal inactiviation of soybean TI, and FII, the fraction of intermediate molecular weight containing all of the TI activity in soy extracts, were produced according to the following procedure. Defatted soybean flour (10 g) in 100 ml of distilled water was stirred for 1 hr. The suspension was centrifuged for 10 min at 20,000 × *g*. The pH of the supernatant was adjusted to 6.0 with 1*N* HCl, after which it was centrifuged again. The supernatant was dialyzed against water at pH 7.0 and 4° C and was then lyophilized. The gel filtration on Sephadex G-75 was performed according to the method described previously (Ellenrieder et al 1980). The chromatographic fractions of interest were dialyzed and then lyophilized.

TI Activity

TI activity, determined by the method of Kakade et al (1974) with BAPA as the substrate, was expressed in terms of trypsin units inhibited (TUI), as defined by Kakade et al (1969). The original activity of untreated extracts was 4,050 TIU per milliliter. Inactivation of the heat-treated samples was expressed as a percentage of the TUI of untreated samples.

Measurement of Effects of FI on Termal Inactivation of TI

TI in Aqueous Extracts of Soybeans. Aqueous extracts were prepared by stirring 5 g of defatted soy flour in 100 ml of distilled water for 1 hr then centrifuging for 10 min at 20,000 × *g*. FI (40 mg/ml) was dissolved into a portion of this aqueous extract. The original pH of the extract was 6.5; after the addition of FI it was 6.05. It was adjusted again to pH 6.5 with 1*N* NaOH and centrifuged as before. Samples with and without FI were treated at 96° C during the times indicated, and then the inactivation was determined.

Purified TI. FII was dissolved in 10m*M* Na-K phosphate buffer, pH 6.5, to give a final concentration of 3.75 mg/ml in the presence of variable amount of FI. The combined solution was centrifuged and then heated for 15 min at 96° C. Residual TI activity was determined.

TI Purified by DEAE-Cellulose Chromatography. The TI of FII were further fractionated by chromatography on DEAE cellulose, using the method described by Obara et al (1970). A column of 1.6 × 50 cm was used. About 50 mg of FII dissolved in 1 ml of 10m*M* Na-K phosphate buffer, pH 7.6, was eluted by a linear gradient of NaCl fed by two 250-ml flasks, one with buffer and the other with buffer containing 0.3*M* NaCl. Fractions of 2.5 ml were collected and TUI was determined in the tubes. The eluates, which were pooled into five fractions (FD1-FD5) according to the peaks of TI activity, corresponded to fractions I-V of Obara et al (1970). Reproducibility was tested by three repetitions of the chromatographic run. The freeze-dried products were reconstituted in 5 ml of 10m*M* Na-K phosphate buffer, pH 6.5, and were heat-inactivated for 15 min at 96° C with and without FI.

Carboxymethylation of FI

Samples (1 ml) of the supernatant of a centrifuged solution of FI (20 mg/ml) in Na-K phosphate buffer, pH 7.6, were treated with different amounts of sodium iodoacetate for 1 hr at 30° C in the dark. They were dialyzed against 10-m*M* Na-K phosphate buffer, pH 6.5, and adjusted to a volume of 1.5 ml. The mercapto group concentration of these samples was determined using DTNB (Habeeb 1972); 0.1 ml of 5m*M* DTNB was added to 0.9 ml of carboxymethylated sample, and the absorbance increase was observed at 412 nm. A value of 14,150 for the molar absorptivity was used (Riddler et al 1979). The accelerating effect of these carboxymethylated samples was determined by mixing 0.2 ml of the same with 0.2 ml of FII dissolved in Na-K phosphate buffer, pH 6.5, and heating 15 min at 96° C. The TI activity of these treated samples was determined as described.

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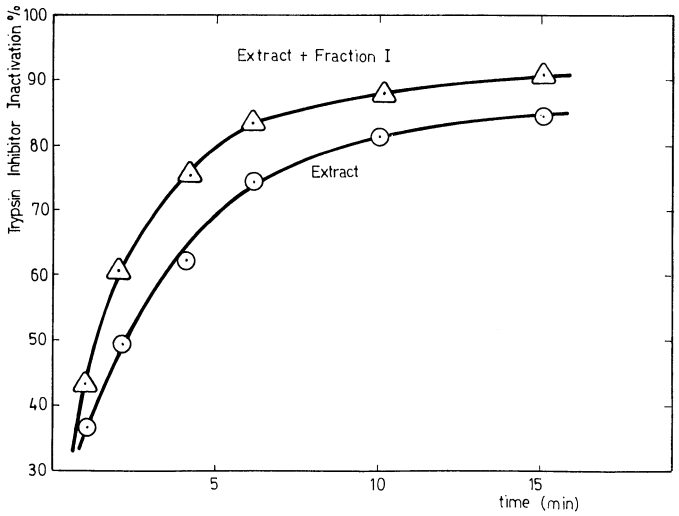

Fig. 1. Effect of accelerator (fraction I of Sephadex G-75 chromatography) on the thermal inactivation (96° C) of trypsin inhibitory activity in soybean aqueous extracts.

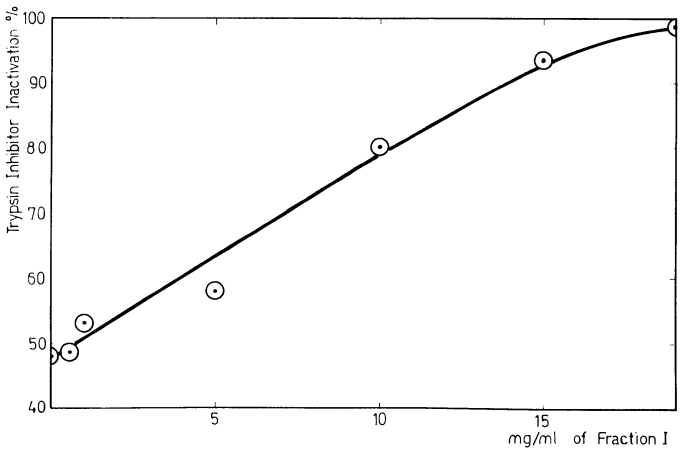

Fig. 2. Effect of accelerator (fractions I of Sephadex G-75 chromatography) on the thermal inactivation (96° C) of trypsin inhibitor purified by Sephadex G-75 chromatography.

RESULTS AND DISCUSSION

Effects of Accelerator on Thermal Inactivation of TI

Figure 1 illustrates the effect of FI on TI activity in soy extracts heated at 96° C for different times. The inactivation was always greater in the presence of FI. With the accelerator, most of the TI are inactivated within 5 min. After 15 min the residual activity is appreciably smaller in the presence of accelerator.

Figure 2 plots the thermal inactivation of the TI in FII against the concentration of added FI. The loss of antitryptic activity is almost total at the highest FI concentration. Because of the heterogeneity of the inhibitors in FII (Obara et al 1970), one can infer from these results that the accelerators of FI have an effect on all of the inhibitors in FII. To confirm this assumption, a chromatographic separation of the TI on DEAE cellulose was performed according to the method of Obara et al (1970). Activity peaks similar in number to those reported by Obara et al were obtained, but their relative size was different. The percentages of eluted TI activity were: FD1, 5.1; FD2, 8.3; FD3, 6.1; FD4, 48.2; and FD5, 32.3. A further difference from the results of Obara et al (1970) was that FD5 appeared as a double peak, but this could have been caused by differences in plant variety. Kamoi et al (1977) found that the F5 fraction from similar DEAE cellulose chromatography of TI of soybean cultivar Onihedakazaki No. 1 consisted of two subfractions, shown by isoelectric focusing chromatography. The fraction FV was identified by Obara et al (1970) as the Kunitz soybean TI. That this protein is absent in a soybean variety is

TABLE 1
Effect of Accelerator Fraction (FI) on the Thermal Inactivation of Trypsin Inhibitors (TI) Purified by DEAE-Cellulose Chromatography

Chromatographic TI Fraction[a]	Percentage Inactivation[b]	
	Without FI	With FI (10 mg/ml)
FD1	25.2	36.4
FD2	1.7	12.3
FD3	6.0	12.5
FD4	0.0	38.1
FD5	40.2	71.0

[a] Fractions correspond to the TI activity peaks of the DEAE-cellulose chromatography.
[b] Samples were inactivated by heating 15 and at 96° C.

TABLE II
Effect of Carboxymethylation of FI[a] Solutions on the FI Acceleration of Thermal Inactivation of Trypsin Inhibitory Activity

Concentration of Na Iodoacetate in the Carboxymethylation Reaction (m*M*)	Free Mercapto Groups in the Inactivation Experiment (sulfhydryl moles per liter $\times 10^5$)	Precent Inactivation of FII[b] (15 min at 96° C)
0.0	2.69	65.5
1.0	0.83	61.9
10.0	0.52	47.0
100.0	0.32	44.7
Inactivation without FI	···	43.5

[a] Fraction (by G-75 Sephadex chromatography) containing accelerators.
[b] Fraction (by G-75 Sephadex chromatography) containing trypsin inhibitors.

further evidence of plant varietal differences (Orf and Hymowitz 1979). The accelerators of FI effected all the inhibitor fractions (Table I). However FD1–FD3 showed more resistance to the effect of FI than did FD4 and FD5. An interesting difference between these two groups of TI is in their cystine content (Obara et al 1970). FD1–FD3 have a low cystine content, whereas FD4 has a high cystine content and FD5 is intermediate. Nordal and Fosum (1974) proposed that the accelerating effect of meat protein on the thermal inactiviation of soybean TI was caused by an interaction between the mercapto groups of meat and the disulfide bonds of the inhibitors. If this is also the case for the interaction of the accelerators of FI with the soybean TI, the difference in resistance of the TI fractions may depend upon the number of disulfide bonds in their molecules.

Carboxymethylation of FI

Carboxymethylation experiments were conducted to determine the role of the mercapto groups in the accelerated inactivation of TI. Results are given in Table II. The analysis of the mercapto groups indicates that, even with the highest iodoacetate concentration, the DTNB reaction (Habeeb 1972) was not completely blocked. Carboxymethylation of FI eliminated its accelerant effect (Table II). No linear relationship was found between the concentration of free mercapto groups and the "accelerant effect," but many types of sulfhydryl groups with different reactivities probably exist. Iodoacetic acid can also carboxymethylate imidazole and amino groups (Gurd 1972), but the conditions used were not adequate for these reactions. The results of these experiments could be considered evidence that the sulfhydryl groups are responsible for acceleration of the inactivation of TI by fraction FI.

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