Thermal Inactivation of Trypsin Inhibitors in Aqueous Extracts of Soybeans, Peanuts, and Kidney Beans: Presence of Substances That Accelerate Inactivation

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ABSTRACT

Thermal inactivation of trypsin inhibitors in aqueous suspensions and centrifuged water extracts of dehulled and defatted soy and peanut flours and of flours prepared from whole kidney beans depended on concentration. Thermal stability (after heating at 96°C for 15 min) of trypsin inhibitor activity was greatly increased when these aqueous suspensions and extract were diluted. High molecular weight components, separated from soybean extracts by gel filtration on Sephadex G-75, accelerated thermal destruction of trypsin inhibitor activity in purified inhibitor preparations.

Trypsin inhibitor (TI) of the soybean are undesirable antinutritional factors (Rackis 1974) that are readily inactivated by moist heat treatment (Baker and Mustakas 1973, Liener and Kakade 1969). Thermal inactivation of antitryptic factors in soybeans, (Albrecht et al 1966, Baker and Mustakas 1973) and in purified preparations (Birk 1961, Obara and Watanabe 1971) has been investigated extensively. Results show that the extent of destruction of TI activity in beans, grits, and flours and in purified preparations differs widely.

This article reports the effect of concentration on TI thermal stability in aqueous extracts and suspensions of soy, peanut, and kidney bean flours and on the isolation of high molecular weight substances that enhance the thermal inactivation of purified soybean TI preparations.

MATERIAL AND METHODS

Materials

Benzoyl-DL-arginine-*p*-nitroanilide (BAPA) was obtained from Nutritional Biochemical Corp., Cleveland, OH; casein (Hammersten) from BDH, Poole, England; bovine trypsin (twice crystallized) from Sigma, St. Louis, MO, and Sephadex G-75 from Pharmacia, Upsala, Sweden. The seeds were: soybean (*Glycine max*) variety Cerrillos W 65, kidney bean (*Phaseolus vulgaris*) variety Alubia, and peanut (*Arachis hypogaea*) variety Manfredi.

Flour Suspensions and Extacts

Dehulled legume seeds were ground in a modified coffee grinder mill, defatted by two extractions with 10 volumes of hexane, and then ground again to −80 mesh.

Aqueous suspensions were prepared by stirring flour in distilled water for 1 hr at room temperature.

Extracts of soybean, peanut, and kidney bean flours were obtained by centrifuging 10% (w/v) suspensions for 20 min at about 1700 × *g*. The supernatants were diluted to the concentrations used in the thermal inactivation experiments.

Trypsin Inhibitory Activity

Trypsin inhibitory activity was determined by the method of Kakade et al (1969, 1974), with BAPA as substrate. In some analyses, casein substrate was also used (Kakade et al 1969). TI activity was expressed in terms of trypsin units inhibited (TUI), as defined by Kakade et al (1969). Inactivation of heat-treated samples was expressed as percent destruction of TUI of untreated samples.

Sephadex G-75 Chromatography

Chromatography was done according to the method of Obara and Kimura (1967) and Obara et al (1970) on a 2.5 × 100-cm column. The elution buffer was 0.01*M* phosphate, pH 7.6; 0.4*M* NaCl; and 0.01*M* 2-mercaptoethanol. (In some runs a buffer without 2-mercaptoethanol was also used). Elution rate was 60 ml/hr. Samples for chromatography were prepared by stirring 10 g of soy flour in 100 ml of distilled water for 1 hr. The suspensions were centrifuged, dialyzed against distilled water at 4°C, and lyophylized. About 200 mg of this extract was dissolved in 2 ml of elution buffer and applied to the column. Effluent fractions of 5 ml were collected. The absorbance at 280 nm and the TUI of each tube were determined. The tubes were pooled to obtain four fractions: fraction I, tubes 28–42, contained the highest molecular weight substances; fraction II, tubes 43–73, accounted for all of the TI activity; fraction III, tubes 74–112, and fraction IV, tubes 113–145, contained the lowest molecular weight substances. The four fractions were dialyzed, lyophylized, and then dissolved in 5 ml of 0.01 *M* phosphate buffer, pH 7.6.

RESULTS AND DISCUSSION

Thermal Inactivation of TI Activity in Aqueous Extractions

The thermal inactivation of TI activity in aqueous suspensions and extracts of soybeans at different concentrations was investigated by heating samples at 96°C for 15 min. During the experiments, the suspensions were continuously stirred. Results are given in Fig. 1. Results show that thermal inactivation was greatly decreased when both suspensions and extracts were diluted. At intermediate concentrations, however, heat inactivation of TI activity was much lower in suspensions than in centrifuged extracts. These data indicated that substances removed by centrifugation partially protect the trypsin inhibitors from thermal destruction.

As shown in Fig. 2, thermal stability of TI activity in extracts of soy, peanut, and kidney bean flours is greater at low concentration. This behavior suggests that the process of TI inactivation could be the same for the three legumes. TI thermal stability is greater in peanut extracts than in those of the other two seeds. The rate of inactivation of inhibitors in aqueous extracts depends on concentration; the process is therefore not unimolecular, and participation of substances other than the inhibitors is very probable.

Thermal Stability of Purified Inhibitors

A simple method for separating TI from higher molecular weight constituents in soybean extracts by gel filtration in Sephadex columns has been reported (Obara and Kimura 1967, Obara et al 1970). Sephadex G-75 was used to fractionate soy extracts, according to the method of Obara et al (1970), and results were similar to those they reported. The major portion of the UV-absorbing substances representing the highest molecular weight material was eluted in the first peak. All of the TI activity was eluted in the next two partly separated peaks, followed by other minor absorbance peaks containing lower molecular weight substances. All these peaks were pooled as described.

TI thermal stability in fraction II obtained by gel filtration was greater than that of the original extract (Table I). When samples of

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TABLE I
Thermal Inactiviation of Soybean Trypsin Inhibitors Partially Purified by Sephadex G-75 Chromatography

Sample[a]	Percent Inactivation after Heating 15 min at 96°C
Extract before chromatography	86.4
0.3 ml Fraction II	39.2
0.3 ml Fraction II	
+0.3 ml fraction I	55.2
+0.7 ml fraction I	85.7
+0.7 ml fraction III	37.3
+0.7 ml fraction IV	39.3

[a]All samples were diluted to 1,200 trypsin inhibitor units per milliliter before heat inactivation.

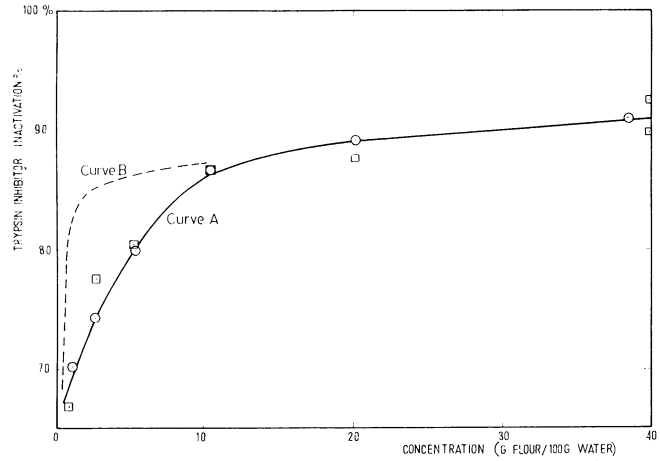

Fig. 1. Thermal inactivation of trypsin inhibitors (TI) in aqueous suspensions and extracts of defatted soy flour at different concentrations. Heating: 15 min at 96°C. Curve A, aqueous suspensions. ⊙ = experimental points obtained with casein as the substrate; ⊡ = experimental points obtained with benzoyl-DL-arginine-*p*-nitroanilide as the substrate; Curve B, aqueous extracts. This is the same as curve B, Fig. 2, but data points are omitted for better clarity. This curve is equivalent to that of TI inactivation of supernatants obtained from centrifugated suspensions of the indicated concentrations.

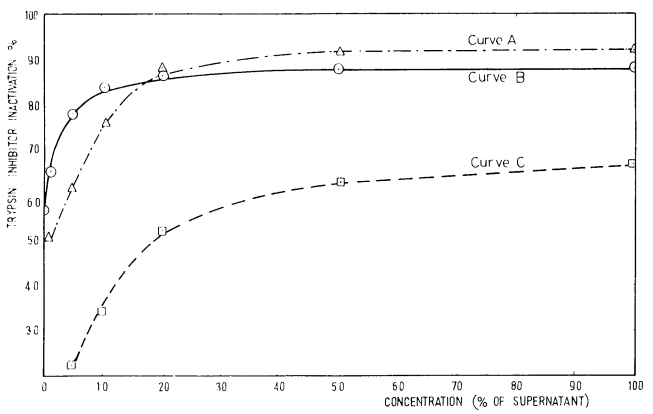

Fig. 2. Thermal inactivation of trypsin inhibitors in aqueous extracts of legume flours at different concentrations. Curve A, kidney beans; curve B, soybeans; curve C, peanuts. Concentration is expressed as percent of the supernatants from centrifugated suspensions of 100 g/L. Heating: 15 min at 96°C. Original activity with benzoyl-DL-arginine-*p*-nitroanilide as substrate: kidney bean 35.2, soybean 83.7, and peanut 22.5 trypsin inhibitor units per milligram, respectively.

Fraction II were diluted with aliquots of Fraction I, however, the thermal stability of TI was appreciably reduced. As shown in Table I, fractions III and IV had no effect on the TI thermal stability of fraction II. To avoid differences due to concentration dependence of TI thermal stability, the determinations in Table I were made on samples of the same initial TI concentration (ie, the same TUI/ml). In these experiments the chromatographic fractions were carefully dialyzed to remove 2-mercaptoethanol, which reduces the stability of TI (Kassell, 1970). By avoiding the use of this reagent in duplicate chromatographic runs, the same reduction of TI stability was observed as when fractions I and II were combined. Fraction I alone showed no activity when tested toward BAPA.

Thermal stability in purified soybean TI preparations is greater than that in soybean seeds, grits, or flours; residual TI activity in heat-treated soy flour (steamed at 100°C for 15 min) is approximately 10% of its original value in raw flour (Kakade et al 1974, Rackis 1974). A purified Kunitz soybean TI solution, however, loses only 50% of its activity after treatment at 90°C for 30 min (Kunitz 1947). Bowman Birk inhibitor solutions do not lose any of their activity after heating for 30 min at 100°C (Birk 1961). Other purified inhibitors show similar thermal resistance to inactivation (Obara and Watanabe 1971). The increased stability of TI activity in purified preparations may result from removal of certain factors during purification. These factors, when present in crude soybean extracts, accelerate TI inactivation. The high molecular weight components of fraction I, which accelerated inactivation of TI activity of fraction II, probably are the same factors that reduce stability of TI in whole soybean preparations. The concentration dependency of the thermal stability of TI activity in aqueous extractions may result from an interaction of TI with these macromolecular "thermal inactivators."

The data in Table I suggest that the destabilizing factors are high molecular weight substances, most likely proteins. As shown by Obara and Kimura (1967) and Obara et al (1970), however, nonprotein constituents are also present in fraction I obtained by Sephadex column chromatography.

Substances accelerating thermal inactivation of soybean TI were previously detected in other products. Nordal and Fossum (1974) found that soybean TI had lower thermal stability in soy-meat mixtures; high molecular weight substances were apparently responsible for this effect. A substance with similar properties is also present in cow milk whey and buttermilk (British Arkady Co. 1976). The mechanism by which these substances produce thermal inactivation of TI is now known. Nordal and Fossum (1974) suggest that interaction of sulfhydryl groups in meat proteins with disulfide bonds of the inhibitors can lead to inactivation. Such an assumption needs further support.

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