Registry No. 1, 72-43-5; 2, 7388-31-0; 3, 2132-70-9; 4, 28463-03-8; 5, 79648-83-2; 6, 75938-34-0; 7, 61002-54-8; 9, 2971-36-0; 10, 13005-40-8; 11, 14868-03-2; 12, 90047-64-6; 13, 79639-27-3; 14, 90047-65-7; 15, 79639-28-4; 16, 79639-30-8; 17, 79639-32-0; 18, 79639-31-9; 19, 79639-33-1; 20 isomer I, 90047-66-8; 20 isomer II, 90047-67-9; 26, 611-99-4; 27, 79639-29-5; 2-[4-(benzyloxy)-3-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene, 90047-68-0; 4-(benzyloxy)-3-methoxybenzaldehyde, 2426-87-1; (trichloromethyl)lithium, 2146-66-9; 1-[4-(benzyloxy)-3-methoxyphenyl]-2,2,2-trichloroethanol, 90047-69-1; anisole, 100-66-3; 2-[4-(benzyloxy)-3-methyoxyphenyl]-1,1,1-trichloro-2-(4-methoxyphenyl)ethane, 90047-70-4; 2-[3-(benzyloxy)-4-methoxyphenyl]-1,1-dichloro-2-(4-methoxyphenyl)ethene, 90047-71-5; 3-(benzyloxy)-4-methoxybenzaldehyde, 6346-05-0; 1-[3-(benzyloxy)-4-methoxyphenyl]-2,2,2-trichloroethanol, 90047-72-6; 2-[3-(benzyloxy)-4-methoxyphenyl]-1,1,1-trichloro-2-(4-methoxyphenyl)ethane, 90047-73-7.

Supplementary Material Available: A list of the solvents used and 6 pages of diagrams showing in detail the isolation of the metabolites from feces, urine, and bile (7 pages). Ordering information is given on any current masthead page.

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A Rapid Automated Procedure for the Determination of Trypsin Inhibitor Activity in Soy Products and Common Foodstuffs

Bonnie A. Charpentier* and Dale E. Lemmel

An automated method for the determination of trypsin inhibitor activity has been developed based on an improved procedure using the synthetic substrate benzoylarginine-*p*-nitroanilide. The method has been found to be rapid and reproducible and has been applied to a variety of foodstuffs including eggs, cheese, beef, several vegetables, and soy products. Results show that trypsin inhibitor activity decreases dramatically during the processing of soy flour to food products. Activities found in the foods analyzed have been compared on fresh weight, dry weight, and protein content bases. The advantages and limitations of the method are discussed.

Trypsin inhibitors are widely distributed in nature, occurring in a large variety of both plant and animal components of the human diet (Liener and Kakade, 1980; Belitz et al., 1982; Doell et al., 1981). Much work has been done to characterize various trypsin inhibitors and to study their physiological effects in animals and humans. Interest has centered in particular upon trypsin inhibitors present in soybeans, a valuable source of protein for both animals and humans. Soy protease inhibitors have been of interest because of their antinutritional effects and interference with protein digestion (Hill et al., 1982; Krogdahl and Holm, 1981; Liener, 1981; Satterlee et al., 1979). They have also been shown to cause pancreatic enlargement in chicks (Chernick et al., 1948) and in mice and rats (Rackis, 1965, 1974; Yamatori and Fujita, 1976; Kakade et al., 1973). In other studies, however, using animals such as dogs, calves, pigs, and monkeys (Kakade et al., 1975; Yen et al., 1974; Struthers et al., 1983), such pancreatic effects were not produced. Heating of soy products has been shown to destroy both antinutritional and pancreatic effects (Liener, 1975, 1981; Struthers et al., 1983). Reviews by Liener and Kakade (1980) and by Rackis and Gumbmann (1981) summarize the properties and nutritional significance of known protease inhibitors.

The Procter & Gamble Company, Winton Hill Technical Center, Cincinnati, Ohio 45224.

In view of the interests noted above, the need for an accurate and widely applicable analysis for trypsin inhibitors is obvious. Various column chromatographic methods (Whitaker and Sgarbieri, 1981), affinity chromatography (Gomes et al., 1979; Chan and de Lunen, 1982; Lin et al., 1980), and electrophoresis (Lewosz et al., 1981; Sgarbieri and Witaker, 1981) have proved valuable for the isolation and characterization of diverse trypsin inhibitors; these methods, however, are not suitable for rapid quantitation of trypsin inhibitor activity. The use of a pH stat to measure tryptic proteolysis and trypsin inhibitor content of soy flours has proved difficult with some samples and has shown no advantage as yet over conventional methods (Stinson and Snyder, 1980; Hill et al., 1982).

The most widely used method for measuring trypsin inhibitor activity evolved from a procedure developed by Kunitz (1947) in which the hydrolysis of casein by trypsin was measured spectrophotometrically in the presence and absence of inhibitors. Erlanger et al. (1961) introduced the use of a synthetic substrate, benzoylarginine-*p*-nitroanilide (BAPNA), in place of casein; subsequent work by Kakade et al. (1969) showed the use of BAPNA to be preferable because of simplicity and accuracy. Kakade's work, using the definition of 1 trypsin unit (TU) to be an

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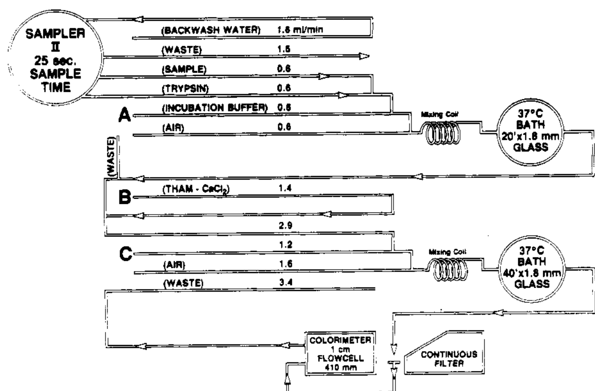

Figure 1. Schematic of automated trypsin inhibitor method.

increase in absorbance of 0.01 units, established 0.019 absorbance unit as equivalent to 1 μg of pure tyrpsin. A collaborative study by a Committee on Soybean Trypsin Inhibitor Analysis (Kakade et al., 1974; Rackis et al., 1974) resulted in several modifications of the procedure designed to improve both accuracy and reproducibility. A semiautomated procedure (Egbert et al., 1975) based on this improved method was developed. Later work (Hamerstrand et al., 1981) introduced further modifications designed to overcome difficulties encountered in extrapolating trypsin inhibitor activity to zero using the four specified sample dilutions of the standard method. This work showed improvement in reproducibility by using a single dilution of a sample to produce a level of trypsin inhibition in the 40–60% range. Additional improvements in the trypsin inhibitor method were recently introduced by Lehnhardt and Dills (1982). These workers have shown the modified analysis to be applicable in the range of 10–80% inhibition under the conditions of the assay. They have also shown that 1 μg of pure trypsin produced 2.16 TU under the conditions of the modified assay, and this factor was substituted for the 1.9 TU factor used in the Kakade et al. (1969) procedure.

This paper reports the development of an automated procedure based on the modified method of Lehnhardt. The procedure offers advantages in speed of analysis and reproducibility not attainable by a manual method. The accuracy and precision of the automated method and problems encountered in extraction of trypsin inhibitors are discussed. The method has been satisfactorily applied to a variety of soy protein products and common foodstuffs.

MATERIALS AND METHODS

Apparatus. The automated method utilized a Technicon AutoAnalyzer proportioning pump and autosampler equipped with a magnetic mixer and a continuous filter. The flow diagram for the analysis is shown in Figure 1. The sample wheel was modified to accommodate two sample cups in each sample position. The detector used was a Beckman Model 25 spectrophotometer with a 1-cm flow cell. The spectrophotometer was interfaced to a Hewlett-Packard 9825 desk-top computer.

Reagents. Trypsin Type III (2× crystallized from bovine pancreas), soybean trypsin inhibitor Type 1-S, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) hydrochloride, and tris(hydroxymethyl)aminomethane (Tris) (reagent grade) were obtained from Sigma Chemical Co., St. Louis, MO. Stock solutions were prepared as follows. Concentrated Tris buffer was prepared by dissolving 60.5 g of Tris in 900 mL of deionized water, adjusting to pH 8.2 with HCl, and diluting to 1 L with deionized water; this solution is very stable when stored at 4 °C. Concentrated $CaCl_2$ solution was prepared by dissolving 29.5 g of $CaCl_2$ in 900 mL of deionized water and diluting to 1 L; this solution is stable at room temperature. Concentrated BAPNA solution was prepared by dissolving 4.0 g of BAPNA in 100 mL of dimethyl sulfoxide. This solution is stable at room temperature for a minimum of 6 months. These stock solutions were used to prepare the following solutions fresh for each day's analyses: solution A (incubation buffer), 20 mL of concentrated Tris and 100 mL of concentrated $CaCl_2$ made to 200 mL with distilled water; solution B, 40 mL of concentrated Tris and 40 mL of concentrated $CaCl_2$ made to 250 mL with distilled water; solution C, 6 mL of concentrated BAPNA and 25 mL of dimethyl sulfoxide made to 250 mL with distilled water.

Other solutions for the analysis were prepared as follows. Standard trypsin solution was prepared by dissolving 40 mg of trypsin in 1 mM HCl to a total volume of 1 L; this solution is very stable when stored at 4 °C. Sodium hydroxide solution (for sample extraction) was prepared by dissolving 0.4 g of NaOH in distilled water to a total volume of 1 L.

Sample Preparation and Extraction. Dry samples (soy flour, concentrate, isolate, instant potatoes) were ground to pass through a 100-mesh screen. Those samples for which it was desirable to remove potentially interfering fats (Smith et al., 1980; Wang et al., 1975) were extracted with petroleum ether or hexane. Wet samples (cabbage, green beans, beef, eggs, potatoes, soy products) were either finely macerated or freeze-dried, ground, and hexane extracted. For spiking and recovery studies, trypsin inhibitor standard was added to the sample prior to hexane extraction.

One to five grams of sample was suspended in 50 mL of 0.01 M NaOH, adjusted to a constant pH of 8.2, and stored overnight at 4 °C. This suspension was diluted to 100 mL with distilled water. Some samples required further dilution to fall within the 10–80% inhibition range.

Automated Procedure. The automated procedure was set up to reproduce as closely as possible the manual method of Lehnhardt. The flow scheme for the addition of solutions A, B, and C is shown in Figure 1. Two cups were sampled simultaneously at each sample position. For trypsin standards, one cup contained tryspin standard solution and the other 1 mM HCl. For trypsin inhibitor measurements the cup containing 1 mM HCl was replaced with sample extract. For sample blanks, one cup contained sample extract and the other 1 mM HCl. Ten minute incubation and hydrolysis steps were provided by glass coils inside a 37 °C oil bath. Samples were filtered through No. 410 filter paper with a Technicon continuous filter running at 2 in./min before entering the spectrophotometer. Absorbance was read at 410 nm. Since sample filtrates were read at precisely timed intervals, the reaction termination step involving addition of acetic acid was eliminated. Calculations of milligrams of trypsin inhibited per gram of sample were done with the desk-top computer using the factor 2.16 for trypsin units inhibited determined by Lehnhardt. The determination of this factor was necessary since assay conditions, in particular the level of calcium present in the assay mixture, affect trypsin activity. The purity of the trypsin was determined by active site titration by the method of Chase and Shaw (1970).

The calculations are

$$\frac{\text{mg of trypsin inhibited}}{\text{g of sample}} = \frac{(A)(\text{sample dilution factor})}{(0.0216)(1000\ \mu\text{g/mg})(\text{sample wt, g})}$$

Table I. Comparison of Manual and Automated Methods

sample	mg of trypsin inhibited/g of sample	
	manual method[a]	automated method
soy flour	25.33	22.93 (12)[b] SD = 0.43 rel SD = 1.86%
soy isolate	9.41	9.68 (9)[b] SD = 0.20 rel SD = 2.05%

[a] Values from W. F. Lehnhardt (personal communication). Values given are the average of three dilutions of extract. [b] Number of aliquots of extract run.

where A = (absorbance of trypsin standard) − (absorbance of sample) − (absorbance of sample blank). Several replicates were done for each sample.

Other Analyses. Sample moisture, unless otherwise noted, was determined by weight before and after drying in a vacuum oven overnight at 70 °C. Percent nitrogen was determined by the Kjeldahl procedure (AOAC, 1980). Protein content was estimated by multiplying percent nitrogen by the factor 6.25.

RESULTS AND CONCLUSIONS

Validation of Automated Method. Several experiments were carried out to determine the accuracy and precision of the automated procedure. Linearity of response was checked initially by preparing a calibration curve with soybean trypsin inhibitor standard (Type 1-S, Sigma) at 5-, 10-, 15-, 20-, and 25-μg levels. A least-squares fit to a plot of absorbance vs. concentration of inhibitor produced a correlation coefficient of 0.9998.

Samples of a soy flour and a soy isolate for which TI levels had been determined at A. E. Staley Manufacturing Co. by the manual method (Lehnhardt and Dills, 1982) were run on the automated method for comparison. Several determinations from single extracts of each sample were made to establish the precision of the method. Results are shown in Table I. In order to include the extraction procedure in the precision measurement, the same soy flour and isolate samples were extracted on different days, independently, by two analysts. For the soy flour, the average for 29 determinations was 22.97 mg of TI/g of sample, with a relative standard deviation of 3.50%. Eighteen determinations of the isolate produced an average of 9.67 mg of TI/g of sample with a relative standard deviation of 3.25%.

Extraction and Spiking Studies. Efficiencies of the extraction procedures were determined by spiking samples with known amounts of soybean trypsin inhibitor and measuring recoveries of spikes with a standard curve. Recoveries were good for samples such as green beans, which did not require removal of fats; green beans spiked at levels of 4 and 6 μg/mL in the extract gave recoveries of 90 and 95%, respectively.

For samples requiring fat extraction, both extraction of dry samples and extraction of aqueous suspensions (Smith et al., 1980) were tried. All attempts at extraction of aqueous suspensions (including centrifugation and washing with organic solvent in a separatory funnel) produced losses of trypsin inhibitor activity. The most satisfactory method for extracting these samples was to freeze-dry, grind, and extract the samples with organic solvent before suspending in NaOH solution. Hexane and petroleum ether worked equally well for fat extraction.

Determination of Trypsin Inhibitor Activity in Soy Products and Common Foods. Values obtained for trypsin inhibitor activity in several soy protein products and common foodstuffs are shown in Table II. On a dry weight and protein basis, raw whole egg contained the highest TI activity of all samples run. The values shown here for raw egg are very similar to those found by Doell et al. (1981).

Soy flour contained the second highest TI activity on a dry weight and protein basis. Results from other soy products show that TI activity dropped markedly with further processing. Several soy concentrates had similar values approximately an order of magnitude below that of soy flour. Values for further processed soy products prepared from these concentrates were among the lowest obtained. these findings are expected since heating and other processing conditions are known to destroy trypsin inhibitor activity.

Other foods were run for which TI activity values are not shown in Table II. For these samples high backgrounds and low inhibition readings prevented precise determinations of TI values. These samples included cream cheese, cottage cheese, yogurt, and apples.

The method was found to be also applicable to rat diets prepared from soy isolate. A diet calculated by composition to contain 2.0 mg of TI/g of diet produced a value of 2.1 mg of TI/g by the automated method. The same diet after pelleting had a value of 1.6 mg/g, apparently due to loss of TI activity produced by the heating in the pelleting process.

Table II. Trypsin Inhibitor Activities of Soy Products and Common Foods

sample	mg of TI/g of sample	% moisture	% N (Kjeldahl)	% protein	mg of TI/ g dry wt	mf of TI/ g of protein
whole egg (raw)	7.2 ± 0.3 (3)[a]	73.7[b]	2.10	13.13	27.4	54.8
egg white	8.6 ± 0.8 (4)	87.6[b]	1.99	12.44	69.4	69.1
egg yolk	2.2 ± 0.1 (4)	51.1[b]	2.70	16.88	4.5	13.0
cheese (mozzarella)	0.1 (1)	50.8	4.12	25.75	0.2	0.4
beef (raw)	0.1 ± 0.01 (5)	57.7	2.79	17.44	0.2	0.6
cabbage (raw)	0.1 (2)	92.2	0.39	2.44	1.3	4.1
whole green beans	0.3 ± 0.06 (3)	86.3[c]	0.76[3]	4.75	2.2	6.4
white potato tubers						
raw	0.7 ± 0.05 (5)	83.2	0.46	2.88	4.2	24.3
boiled 20 min	0.2 ± 0.01 (5)	74.0	0.50	3.13	0.8	6.4
instant potato flakes	0.1 (2)	5.0	1.29	8.06	0.1	1.2
soy flour	23.0 ± 0.8 (29)	5.3	8.46	52.9	24.2	43.3
soy protein isolate	9.7 ± 0.3 (18)	1.9	14.82	92.6	9.8	21.3
soy concentrate	3.1 ± 0.2 (38)	6.3	10.61	66.30	3.2	4.6
further processed soy products from conc.	0.1 ± 0.03 (13)	39.3	5.84	36.78	0.2	0.3

[a] Values given averages ± SD; number of determinations shown in parentheses. [b] Values from Composition of Foods, Agriculture Handbook No. 8, USDA. [c] Average of beans and pods determined separately.

Conclusions. An automated procedure, based on an improved method for analyzing trypsin inhibitor activity, has been developed. The method has advantages in speed and in reproducibility, eliminating much of the tedium and resulting potential for error of manual methods. It is applicable to a large variety of foods and soy products.

The results of this work demonstrate that processing of soy flour and soy isolates produces products with very low TI activity, especially when compared to the TI activity of other commonly consumed foods.

Registry No. BAPNA, 911-76-2; trypsin inhibitor, 9035-81-8.

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Varietal and Environmental Differences in Soybean Glycinin and β-Conglycinin Content

Patricia A. Murphy* and Adoracion P. Resurreccion

The amounts of glycinin and β-conglycinin were measured in 12 soybean varieties by using rocket immunoelectrophoresis. These two proteins constitute 55–75% of the soluble protein in the soybean seed. When 10 varieties (Corsoy, Hodgson, Kitamusume, Tokachi-nagaha, Toyosuzu, Vinton, Wase-kogane, Weber, and Yuuzuru) were grown in a uniform environment in 1980 and 1981, the glycinin content (as seed protein) was 46.9–54.4% and 46.7–57.2%, respectively. The average glycinin content was 51.0%. β-Conglycinin content for the 2 years examined averaged 18.5%, with a range of 16.8–20.1% and 16.5–20.9%, respectively. Vinton and Weber soybeans from several growing seasons and different environments had glycinin contents with a range of 11.8% and 14.5%, respectively. β-Conglycinin content of these soybeans varied by 5.0%. There seemed to be no relationship between glycinin and β-conglycinin content in these soybeans. Environmental influences seem to have a much greater impact on glycinin concentration in soybeans than on β-conglycinin content. Genetics also has an influence on the expression of these two proteins but to a lesser extent than environment.

Glycinin and β-conglycinin are the two major protein fractions in the soybean seed. Their physical, chemical, and functional properties are an area of considerable research interest. These two proteins are reported to produce different properties in a number of food products (Saio et al., 1973, 1974). The heat stabilities of the two proteins are quite different (Saio et al., 1975; German et al., 1982; Damodaran and Kinsella, 1982). The reactivity of the two proteins to metal ions, and in particular Ca^{2+}, is different (Saio et al., 1973; Briggs and Wolf, 1957). Damodaran and Kinsella (1981) have reported a difference in binding constants with the two proteins for a number

Department of Food Technology, Iowa State University, Ames, Iowa 50011.

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