The Semiautomated Determination of Trypsin Inhibitors in Textured Soy Protein

A semiautomated method has been developed employing continuous flow methodology and the reaction of trypsin with benzoyl-DL-arginine-*p*-nitroanilide for the assay of trypsin inhibitors in textured soy protein (TSP). The specific inhibitor activity was defined by obtaining a best fit parabolic curve for the activity calculated from three dilutions in a defined trypsin inactivation range and solving the equation at 35% trypsin inactivation. Optimum inhibitor extraction conditions were ascertained and the method was applied to a broad range of TSP samples showing a pooled relative standard deviation of only 3.0%. The method showed acceptable recoveries (98–106%) when blended samples were analyzed.

With the increased role that soy protein plays in the world food supply, the nutritive quality related to trypsin inhibitor level is of major concern. The presence of trypsin inhibitors has been demonstrated in a wide variety of cereals and legumes and it has been reported that an increase in nutritive value of soy flour paralleled the destruction of trypsin inhibitor activity (Rackis, 1972). This increase in nutritive value with heating has been the subject of numerous studies which have been appropriately reviewed (Liener, 1969). Liener has suggested that trypsin inhibitors along with other growth inhibitors may account for the growth inhibition that exists in addition to that resulting from phytohemagglutinins in unheated soybeans (Liener, 1974).

Since it is well established that the nutritive quality of many legumes increases with heating concomitant with a decrease in phytohemagglutinin and trypsin inhibitor activity, the trypsin inhibitor assay has potential as a production control parameter in the manufacture of nutritious textured vegetable protein if the assay can be made reasonably convenient.

The benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA) substrate method, first introduced by Erlanger et al. (1961), has been used extensively for the analysis of trypsin and its inhibitors. The method has been automated using continuous flow methodology and employing the L-BAPNA enantiomer (Stewart, 1973). The automated method related the trypsin inhibitor level to standard soybean trypsin inhibitor. Kakade et al. (1969) demonstrated that trypsin inhibitor specific activity (micrograms of trypsin inhibitor per milliliter of extract) is a function of level and reported the activity by plotting level vs. specific activity and extrapolating to zero inhibitor level. This decrease of specific activity with level is presumably caused by the concentration dependent dissociation of the trypsin–trypsin inhibitor complex (Kakade et al., 1969).

Recently Kakade et al. (1974) have shown that the surface of insolubilized soy protein has trypsin inhibitor activity and they recommend equilibrating the uncentrifuged extract with trypsin to assess more accurately the inhibitor activity. It was the purpose of this study to obtain a semiautomated method employing DL-BAPNA for the determination of trypsin inhibitors which takes into account the recently reported surface activity and the variance of specific activity with level. Also, it was of interest to obtain reproducibility and recovery data.

EXPERIMENTAL SECTION

Apparatus. The flow scheme shown in Figure 1 was constructed from standard Technicon AutoAnalyzer II components. Calculations were made using a Hewlett Packard Model 10 programmable calculator.

Reagents. Tris buffer (0.1 *M*, pH 8.2) was prepared by dissolving 24.2 g of tris(hydroxymethyl)aminomethane (Sigma Chemical, St. Louis, Mo.) and 5.8 g of calcium chloride dihydrate in 1500 ml of distilled water. The pH was adjusted to 8.2 with 1.0 *N* hydrochloric acid and 40 ml of Triton X-100 (Rohm and Haas, Philadelphia, Pa., 0.05 g/ml of H_2O) reagent was added before the mixture was diluted to volume with distilled water. Tris buffer (pH 8.8), used for extraction of trypsin inhibitors, was prepared in the same manner; however, no Triton X-100 reagent was added.

The BAPNA reagent (0.45 mg/ml) was prepared by adding 90.0 mg of *N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (Nutritional Biochemical, Cleveland, Ohio) to a 250-ml beaker containing approximately 150 ml of distilled water. The BAPNA was dissolved by gentle heating on a steam bath (did not exceed 75°) and the solution was transferred to a 200-ml volumetric containing 4.0 ml of Triton X-100 reagent (0.05 g/ml of H_2O); the solution was diluted to volume after cooling to room temperature. The solution was stored in a refrigerator (5°) when not in use.

The 30% acetic acid used to quench the reaction contained 0.1% Triton X-100. The trypsin (Worthington Biochemical, Freehold, N.J., bovine pancreas, 2× salt free) was standardized using the method of Kakade et al. (1969) and found to be 60% active (1.0 μg of trypsin corresponds to 1.90 trypsin units as defined by Kakade). The trypsin stock solution (60 μg/ml) was prepared by dissolving 12.0 mg of trypsin (calculated from standardization) in 0.0025 *N* hydrochloric acid and diluting to 200 ml with the same solvent (prepared fresh weekly and stored at 5° when not used). The trypsin dilutions were prepared fresh each day using Tris buffer (pH 8.2 with Triton X-100). The trypsin inhibitor stock solution (200 μg/ml, Sigma Chemical, type 1-S) was prepared in 0.0025 *N* hydrochloric acid (prepared fresh weekly), and the inhibitor dilutions were prepared by diluting the appropriate aliquot of stock solution with Tris buffer (pH 8.2 with Triton X-100).

Procedure. The textured soy protein was ground in a Stein Mill to pass a 100 mesh screen. A portion of this material (100 mg uncooked, 300 mg cooked) was weighed into a 200-ml disposable polypropylene cup (Falcon Plastics, Oxnard, Calif.) and 50 ml of Tris buffer (pH 8.8) was transferred to the cup with a pipet. The wetted sample was allowed to set overnight in a refrigerator (5°) and then was shaken for 2 hr while attaining room temperature. Aliquots were taken from the stirred suspension (3 to 10 ml while maintaining uniform distribution of solid) and diluted to 25 ml with pH 8.2 Tris buffer. Three dilutions exhibiting a 15–60% decrease in trypsin activity were prepared for each sample. A 1-ml aliquot of the diluted material (uniform suspension) was transferred with an Eppendorf pipet to a test tube containing 4 ml of trypsin solution (9 μg/ml in pH 8.2 Tris buffer). A sample blank was obtained by adding another 1-ml aliquot of the most concentrated dilution to 4 ml of Tris (pH 8.2) buffer. The mixture was allowed to sit for 5 min at room temperature with occasional mixing (vortex mixer). A portion of mixture was placed in a sample cup, centrifuged for 5 min (3800 rpm), and then placed in the sampler where a portion of the supernatant was aspirated, mixed with BAPNA, and allowed to react 4 min at 38° before quench-

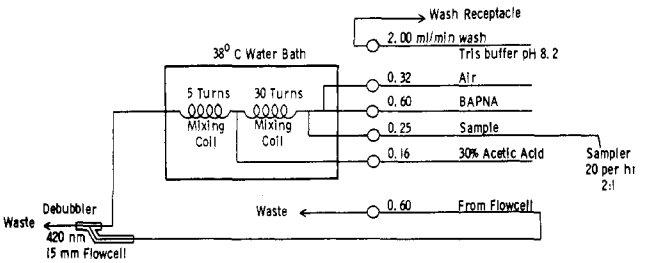

Figure 1. Flow scheme for the trypsin assay. The flowcell is mounted in a Technicon double beam colorimeter.

ing with 30% acetic acid as shown in Figure 1. The reaction coils were submerged in a constant temperature water bath. The *p*-itroaniline formed (absorbance measure continuously at 420 nm) by the trypsin-catalyzed hydrolysis of BAPNA was a measure of the active trypsin remaining.

The set of peaks corresponding to a sample consisted of the three dilutions and the sample blank followed by an additional sample cup containing wash. The trypsin level was assigned from a best fit curve (second-order polynomial, eq 1) of a set of trypsin standards as shown in Figure 2; the trypsin response decreased at higher concentrations.

After subtracting the portion of the blank corresponding to a particular dilution, the percent reduction of trypsin and the trypsin inhibited per gram of TSP extracted was calculated for each dilution (eq 2). From these data a best fit curve (eq 3) was obtained with a programmable calculator and the equation solved for 35% decrease in trypsin, thus determining the specific activity at a defined dilution.

Calculation. A programmable calculator was used to obtain the least-squares equations 1 and 3. A least-squares function (eq 1) was obtained for the set of trypsin standards to account for the nonlinearity of the trypsin response shown in Figure 2:

$$T = aX^2 + bX + c \qquad (1)$$

where T = trypsin concentration in micrograms/milliliter and X = absorbance at 420 nm in chart units. The trypsin concentration for each extract dilution was obtained from eq 1 and the results used to solve eq 2 for specific activity.

$$\frac{(7.2 - T)\ \mu g/ml \times 5\ ml}{\text{sample wt. (mg) added to trypsin}} = \frac{\text{mg of trypsin inhibited}}{\text{g of TSP}} = \text{specific inhibitor activity} \qquad (2)$$

Using percent inactivation of trypsin ($P = (7.2 - T)/7.2 \times 100$) and the corresponding specific activity calculated from eq 2 for the set of extract dilutions, eq 3 is obtained and solved for 35% inactivation. Thus, a specific activity independent of dilution is obtained.

$$\text{specific inhibitor activity} = a'P^2 + b'P + c' \qquad (3)$$

RESULTS AND DISCUSSION

Except for the pump tubes, the flow scheme shown in Figure 1 was completely constructed from glass; polyvinyl chloride (PVC) exhibited poor wash characteristics for this assay. When a PVC reaction coil was used, considerable adsorption and sample carry-over were observed. All pump tubes were PVC except the sample pump tube which was silicone rubber. The probe was constructed from glass reinforced Kel-F (0.7 mm i.d.) since it has been reported (Stewart, 1973) that stainless steel probes resulted in partial enzyme inactivation.

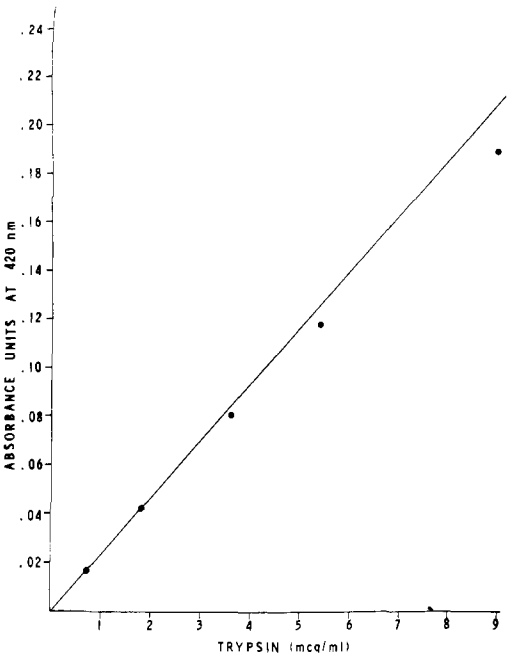

Figure 2. Set of trypsin standards demonstrates the decrease from linearity at higher levels.

The detergent, Triton X-100 (0.1% in all reagents except extraction buffer), improved the wash characteristics dramatically and allowed sampling at 20 per hour (sample to wash 2:1) with negligible sample carry-over and baseline drift. The most critical test of wash efficiency was with the high protein dilutions of TSP extract (extracts exhibited more carry-over problems than standards). However, with Triton X-100, a concentrated extract (2.4 mg of TSP extracted per ml) was carried through the procedure and sampled eight consecutive times with only 2.5% difference between the first and last sample. The method described herein calls for a blank and an extra sample cup containing wash after every set of three dilutions; hence, carry-over did not present a problem in this study. Increased levels over the 0.1% level of Triton X-100 did not improve the wash. Sodium dodecyl sulfate, frequently used to improve the wash in enzyme assays, caused precipitation in the lines even at 0.01% in wash solution and could not be used.

It was of concern to determine the effect of Triton X-100 on the trypsin-BAPNA reaction and on the trypsin-trypsin inhibitor complex formation. A set of trypsin standards (0.72 to 9.0 μg/ml) with Triton X-100 (0.1%) and another set without detergent were pumped through the flow scheme. There was no significant difference between the two sets indicating the detergent does not affect the enzyme-substrate reaction. An extract of TSP was used to prepare two sets of dilutions (0.24–2.4 mg of TSP extracted per ml). One set contained 0.1% Triton X-100 and was treated with trypsin containing detergent as described in the Experimental Section. The other set did not contain detergent and was treated with trypsin containing no detergent. When pumped through the system there was no significant difference between the two sets demonstrating negligible effect of the detergent on the enzyme-inhibitor complex.

That the specific activity varies with level is well known (Kakade, 1969) and is shown in Figure 3. This effect gave rise to differences in trypsin inhibitor specific activity values as large as 15% in the range 15–60% trypsin inactivation.

This problem had to be eliminated in order to properly assess comparative differences between samples extricated from the dilution effect. Previous workers have accounted

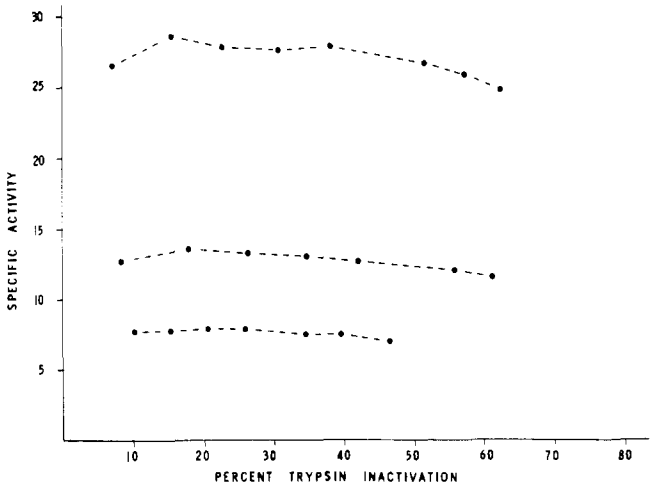

Figure 3. Specific activity (milligrams of trypsin inactivated per gram of TSP) as a function of the percent trypsin inactivated.

for this effect by extrapolating the curve shown in Figure 3 to zero trypsin reduction or have averaged the values obtained from a series of dilutions in a defined trypsin inactivation range (Kakade, 1974). For each sample, we have found it convenient to obtain a best fit parabolic curve for three dilutions in the range 15–60% trypsin inactivation and then arbitrarily assign the specific activity as that obtained at 35% trypsin inactivation. These calculations were easily made with a programmable calculator.

In an attempt to use crystalline trypsin inhibitor as a primary standard, a standard curve was obtained from a set of crystalline soybean trypsin inhibitor dilutions (3.4–30 μg/ml) and dilutions of the crude extract were assigned trypsin inhibitor activity in terms of an equivalent weight of the crystalline standard. There appeared to be no advantage to using this approach over the method adopted since the specific activity remained a function of the level. Presumably, this was caused by the presence of additional trypsin inhibitors in the crude extracts that were not present in the standard.

We obtained optimum extraction of the trypsin inhibitor by soaking a sample overnight in Tris buffer (pH 8.8) and then shaking the sample for 2 hr while attaining room temperature. This gave an average result on three samples comparable to that obtained by shaking the samples for 5 hr at room temperature in the same buffer.

The reproducibility of the enzme-substrate reaction was established by pumping 12 replicate samples of trypsin (7 μg/ml) through the flow scheme; a coefficient of variance of only 0.2% was observed. The reproducibility of the total trypsin inhibitor method was established by analyzing six samples representing different levels of trypsin inhibitor in triplicate. Each replicate was conducted on a different day over a 2-week period; the results are shown in Table I. A pooled relative standard deviation of 3.0% (18 degrees of freedom) was observed for the total method. This represents the total error in sampling, extraction, trypsin inhibition, and residual trypsin analysis.

In order to obtain some data on the accuracy of the method, 5-g portions of samples with a previously assigned inhibitor activity were blended to give a theoretical activity and then subjected to duplicate analyses. The determined values ranged from 98 to 106% of theoretical and are shown in Table I.

Table I. Reproducibility and Recovery Data for Trypsin Inhibitor Assay

Sample[a]	Act.[b] (mg of trypsin inactivated per g of TSP extracted ± std dev)	Rel std dev, %	Calcd act.	Recovery, %
1	14.3 ± 0.5	3.5		
2	34.5 ± 0.8	2.3		
3	11.5 ± 0.1	0.8		
4	6.2 ± 0.3	4.8		
5	8.7 ± 0.1	1.1		
6	17.3 ± 0.1	0.6		
Mixture	Act.[c]			
2 + 6	25.4 ± 1.2	4.7	25.9	98
1 + 6	15.6 ± 0.2	1.3	15.8	99
2 + 4	21.6 ± 0.5	2.3	20.4	106
5 + 4	7.6 ± 0.1	1.3	7.5	101

[a] The samples were subjected to different production conditions in order to obtain the wide range of inhibitor levels. Typical product levels are at the lower end of the range. [b] Average of three determinations. [c] Average of two determinations.

We found this automated method to be a reproducible and convenient way to deal with the many dilutions required for a trypsin inhibitor assay. Also, assigning the inhibitor activity at a specific inactivated trypsin level (35% decrease under conditions defined herein) proved to be a convenient way to define specific activity provided a programmable calculator is available.

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