An Improved Colorimetric Method for Determining Antitryptic Activity in Soybean Products

KESHUN LIU and PERICLES MARKAKIS[1]

ABSTRACT

The current colorimetric method for determining the antitryptic activity of soybean products has been modified as follows: 1) water rather than dilute alkali is used for extracting the inhibitors; 2) the aqueous extract is destabilized with Tris buffer and filtered before, rather than after, the reaction; 3) porcine rather than bovine trypsin is used; 4) the enzyme and not the substrate is added last to the reaction mixture; and 5) the volume of the reaction mixture is reduced from 10 to 4 ml. The proposed modification has a theoretical basis and was more sensitive than the current method. The relative standard deviation in 55 independent measurements was ±3.5%.

Cereal Chem. 66(5):415-422

The presence of naturally occurring trypsin inhibitors in soybeans and other legume seeds has long been known (Liener and Kakade 1980). Some of these inhibitors exert toxic and antinutritional effects (Chernick et al 1948, Kakade et al 1973, Rackis et al 1985). The methods of measuring trypsin inhibitor activity (TIA) in soybean products are mainly colorimetric and based on Kunitz's (1947) original procedure in which casein was used as a substrate for trypsin. Erlanger et al (1961) introduced a synthetic substrate, benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPA), in the trypsin assay. Kakade et al (1969) recommended BAPA as a substrate for the TIA assay after evaluating casein versus BAPA. Questions concerning the reliability of the original procedure of Kakade et al (1969) led to a collaborative study organized by the American Association of Cereal Chemists and the American Oil Chemists Society (Rackis et al 1974). A modified procedure was described as a result of this collaborative study (Kakade et al 1974). Based on this modification, an AACC method was then adopted (AACC 1983).

Although reported separately, Smith et al (1980) and Hamerstrand et al (1981) modified the AACC method in a similar way by using a single inhibitor level instead of serial inhibitor levels. This modification bypasses the cumbersome data interpretation that is done by either extrapolating to zero or averaging over a range of inhibition levels. The reason for their modification is based on two observations: the patterns of enzyme activity versus inhibitor concentration are diverse, and the extrapolation method of data interpretation uses data that are not in the region in which zero-order kinetics is followed. Although another minor modification of the AACC method was also reported (Lehnhardt and Dills 1984), the above two papers established the current method for TIA assay (Rackis et al 1985). However, the current

[1]Dept. of Food Science and Human Nutrition, Michigan State University, East Lansing 48824.

© 1989 American Association of Cereal Chemists, Inc.

procedure still poses questions of accuracy and sensitivity, especially for testing samples with low TIA.

While investigating the soybean Kunitz inhibitor, Viswanatha and Liener (1954) found that a change in the order of mixing the reactants exerted a considerable influence on the extent of trypsin inhibition. In a recent publication, Liu and Markakis (1989) observed a similar reactant sequence effect while assaying the activity of two pure trypsin inhibitors—the Kunitz and Bowman-Birk (BB) inhibitors—and related this effect to limited hydrolysis of the inhibitors by the very enzyme they inhibit. In this communication, a significant modification of the current procedure is proposed that has a theoretical basis and explains the diverse inhibition patterns observed previously.

MATERIALS AND METHODS

Reagents

The assay buffer was 50 m*M* Tris buffer, pH 8.2, containing 10 m*M* $CaCl_2$. A stock trypsin solution was prepared by dissolving 10 mg of crystalline porcine trypsin (Type IX, Sigma Chemical Co., St. Louis, MO) in 50 ml of 1 m*M* HCl solution, pH about 2.5, containing 2.5 m*M* $CaCl_2$. The solution was kept at 5°C. To prepare a working trypsin solution, 2 ml of the stock solution was diluted to a total volume of 25 ml, using the above HCl solution.

A stock BAPA solution was prepared by dissolving 400 mg of BAPA (Sigma) in 10 ml of dimethyl sulfoxide. The solution was very stable even at room temperature. A working BAPA solution was prepared by diluting 0.25 ml of stock BAPA solution to a total volume of 25 ml, using the assay buffer prewarmed at 37°C. Fresh working BAPA solution was prepared for each assay.

Inhibitor Sample Preparation

The samples (soy flour, soy protein concentrate, soy isolate, cooked soybeans, raw soybeans, raw cowpeas, raw navy beans, and raw pinto beans) were ground, if necessary, and passed through a 50-mesh screen. Half a gram of sample was extracted with 50 ml of distilled water for 30 min with mechanical shaking at a speed of 200 rpm. Ten milliliters of the sample suspension was then destabilized by adding an equal volume of the assay buffer and vigorously shaking for 2–3 min before filtering through a Whatman No. 2 paper. The filtrate was then further diluted with water to the point where 1 ml gave 30–70% trypsin inhibition. This was done to keep the relative standard deviation (RSD) of TIA measured within ± 3.5%. A suitable final concentration for raw soybean samples was around 0.1 mg of dry sample per milliliter, and for heated samples, 0.5–1.5 mg/ml.

TABLE I
Procedure for Assaying Trypsin Inhibitor Activity

Mixing Sequence	Reactants	Concentration in Working Solution	Volume Needed for Assay
1st	BAPA	0.92 m*M*	2.0 ml
2nd	Sample	Causing 30–70% inhibition	1.0 ml
3rd	Enzyme	16 μg/ml	0.5 ml
4th	Acetic acid	30%	0.5 ml
Total assay volume	···	···	4.0 ml

TABLE II
Extraction of Trypsin Inhibitors from Raw and Cooked Soybeans by Different Extractants and Shaking Times[a]

Extractants	Shaking Time[b] (min) 30	60	120
Raw soybeans			
0.01*N* NaOH solution (pH 10.0)	···	···	172.2 a
0.001*N* HCl solution (pH 2.5)	162.1 ax	170.4 ay	169.4 ay
Assay buffer (pH 8.2)	169.4 bx	168.8 ax	172.6 ax
Distilled water (pH 6.5)	171.0 bx	170.0 ax	171.3 ax
Soybeans boiled 30 min			
0.01*N* NaOH solution (pH 10.0)	···	···	···
0.001*N* HCl solution (pH 2.5)	18.7 ax	19.6 axy	20.1 ay
Assay buffer (pH 8.2)	23.1 bx	23.7 bx	24.2 bx
Distilled water (pH 6.5)	24.1 bx	23.6 bx	24.3 bx

[a]Means of duplicate measurements as trypsin units inhibited per milligram of dry sample.
[b]Within a column, values followed by a and b differ significantly ($P < 0.05$); within a row, values followed by x and y differ significantly ($P < 0.05$).

Procedure

The procedure for assaying TIA is shown in Table I. The reaction was run at 37°C. Exactly 10 min after adding the trypsin solution, the reaction was stopped by injecting 0.5 ml of 30% acetic acid solution with a 1-ml syringe. The absorbance at A^s_{410} (sample reading), was a measure of the trypsin activity in the presence of the sample inhibitors. The reaction was also run in the absence of inhibitors by replacing the sample with 1 ml of water. The corresponding absorbance was symbolized as A^r_{410} (reference reading). Distilled water was used as a blank.

Calculation of TIA Values

Defining a trypsin unit as an A_{410} increase of 0.01 under the conditions of the assay, the trypsin inhibitory activity is expressed in trypsin units inhibited (TUI) per milligram of dry sample and calculated as follows:

$$\text{TUI/mg sample} = \frac{[(A^r_{410} - A^s_{410}) \times 100]/\text{ml diluted soy extract}}{(\text{mg sample/ml diluted soy extract})}$$

Alternatively, for standardization, the TIA can also be expressed in terms of international units inhibited (IUI) per gram of sample.

Statistical Analysis

The data obtained from different sample treatments were statistically analyzed using analysis of variance in a factorial design. Separation of means was conducted using the least significant difference at the 5% level of probability.

RESULTS AND DISCUSSION

Extracting the Inhibitors

Four solvents were compared for their ability to extract the greatest amount of trypsin inhibitors from both raw and cooked soybean samples and for ease of sample cleanup. 0.01*N* NaOH solution (pH about 10.0), 0.001*N* HCl solution (pH about 2.5), the assay buffer (pH 8.2), and distilled water (pH about 6.5). The ratio of dry sample to all solvents was 0.5 g/50 ml. The sample was extracted with each solvent at three times: 30, 60, and 120 min. The results are summarized in Table II and indicate that distilled water, the assay buffer, and NaOH solution are equally efficient extractants and better than the HCl solution. The NaOH extract was not destabilized by adding the assay buffer and therefore could not be filtered. The value shown in Table II for this extract was obtained by filtration after the enzyme reaction. Water is preferable to the assay buffer, because aqueous extracts are more readily destabilized by mixing with an equal volume of the assay buffer. After filtration, a clear and colorless solution is obtained, that is ready for further dilution. Since shaking for times longer than 30 min did not increase the amount of extracted inhibitors when water was the extractant, a 30-min shaking is considered adequate. In the method of Smith et al (1980), three alternatives are given: 2 min homogenization, 3 hr stirring, and overnight soaking.

Sample Cleanup Before the Reaction

In the current method, a dilute NaOH solution is used for extracting soybean samples. The extract is a rather stable suspension, and it is used as is in running the enzymatic reaction. The reaction mixture is filtered after adding acetic acid and measured photometrically. In the proposed modification, the soy sample is extracted with water, and the extract is destabilized with the assay buffer and filtered before further dilution for the

enzymic reaction. Trials were made to test whether filtering before or after the color reaction gave the same TIA values. Figure 1 shows that the two procedures produced the same inhibition value (same slope of lines connecting A_{410} to quantity of sample inhibitors). The lower color readings obtained from the samples filtered after the enzyme reaction are probably due to sorption of *p*-nitroaniline by the filter paper. Sample cleanup before the enzyme reaction not only gave the higher color reading but also made it possible to reduce the volume of the reaction mixture.

Two clarifying agents, the assay buffer (pH 8.2) and 20 m*M* acetate buffer (pH 3.5) and two filter papers (Whatman Nos. 2 and 5) were compared for the extract cleanup. The results are summarized in Table III and indicate that combining the assay buffer with No. 2 filter paper results in the highest TIA value for the soy sample.

Choosing a Proper Sample Dilution

It has been shown that when trypsin activity is plotted against levels of inhibitor, the activity deviates from linearity at high levels of inhibitor (Kakade et al 1969, Smith et al 1980, Hamerstrand et al 1981). Because of this characteristic, both the AACC and the current methods call for use of a sample dilution that results in 40–60% trypsin inhibition. By using the proposed procedure, we observed a similar curve (Fig. 1). It was further shown that the location of the curve varied with the source of enzyme and the kinds of inhibitor samples. Table IV summarizes the results, which indicate that except for the combination of porcine trypsin-BB inhibitor, the curving loci for most inhibition curves fell beyond 75% inhibition. (This feature of the trypsin inhibition curve has been attributed to a partial dissociation of the trypsin-inhibitor complex [Greene 1953].)

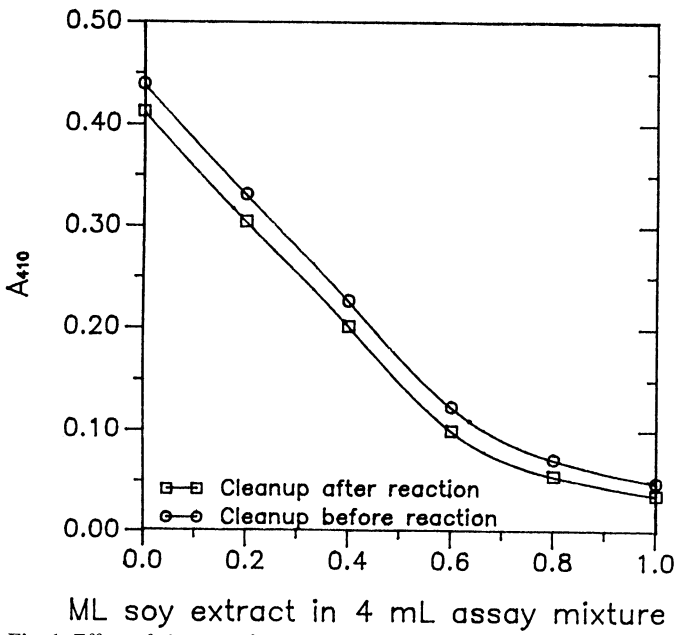

Fig. 1. Effect of cleanup of soy extracts before or after the color reaction on the trypsin inhibitor activity assay.

TABLE III
Comparison of Two Clarifiers and Two Filter Papers in Cleaning a Raw Soybean Extract for the TIA Assay (TIA as TUI/mg dry sample)[a]

Clarifiers	Filter Papers[b] No. 2	No. 5
Assay buffer (pH 8.2)	173.5 ax	167.6 ay
20 m*M* Acetate buffer (pH 3.5)	160.4 bx	152.1 by

[a]Data are means of duplicate measurements on raw Corsoy soybeans. TIA = Trypsin inhibitor activity; TUI = trypsin units inhibited.
[b]Within a column, values followed by a and b differ significantly ($P < 0.05$); within a row, values followed by x and y differ significantly ($P < 0.05$).

These curving loci differed from those reported previously. For example, when assaying raw soy extract with bovine trypsin, Kakade et al (1969) observed a curving locus at 55% of trypsin inhibition, and Hamerstrand et al (1981) reported one at 60%; in our study, the curving locus was at 75%. These differences might be due to assay system variables, such as Ca^{++} and buffer concentrations, soy sample cleanup, etc.

Theoretically, any raw sample dilution that results in less than 75% trypsin inhibition should produce the same TIA value. However, this was not observed in practice. When 55 TIA measurements were made on separate or common extracts from the same raw soybean sample using various dilutions to represent widely different levels of trypsin inhibition, the results shown in Figure 2 were obtained. TIA values corresponding to less than 30% trypsin inhibition are broadly scattered, probably because even small experimental errors are greatly enlarged when large dilution factors enter the calculations. The decline of TIA value above 75% inhibition is expected, as it is determined by the characteristic inhibition curve.

With 55 independent measurements, the relative standard deviation (RSD) was ±3.5% when the dilution was within the range of 30–70% trypsin inhibition and fell to ±3% when the dilution range was 40–60% inhibition. The convenience of sample dilution corresponding to a 30–70% trypsin inhibition outweighs the benefit of the 0.5 change in RSD; therefore, the dilution to a 30–70% inhibition is recommended.

Using Porcine Instead of Bovine Trypsin

Bovine trypsin is used for assaying trypsin inhibitors both in the AACC and the current methods, although it is unstable in alkaline solution (Buck et al 1962a). We observed that 10 min of incubation at 37°C, with pH as high as 7.5, resulted in a sharp decrease of bovine trypsin activity. Since TIA is commonly assayed at pH 8.1, which is the optimum for trypsin activity against BAPA (Erlanger et al 1961), enzyme inactivation would be expected during the assay. On the other hand, porcine trypsin, like human trypsin, is relatively stable at alkaline pH (Buck et al 1962b) and should be more suitable for assaying TIA. Moreover,

TABLE IV
Curving Loci in the Line Connecting Trypsin Activity and Inhibitor Concentration

Samples	Curving Loci as Range of % Inhibition[a] Bovine Trypsin	Porcine Trypsin
Pure Kunitz inhibitor	84–87	75–78
Pure Bowman-Birk inhibitor	84–87	64–68
Raw soybean extract	74–78	74–76
Cooked soybean extract	84–86	83–86

[a]Triplicate measurements.

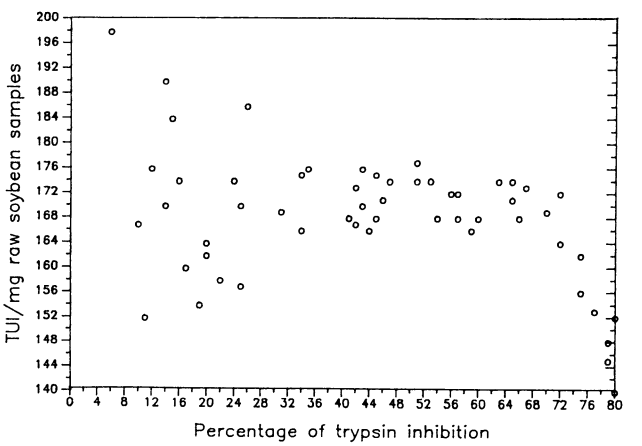

Fig. 2. Effect of degree of trypsin inhibition obtained by various dilutions of a raw soybean extract on the estimate of antitryptic activity in soybeans.

when the TIA of a soy extract was assayed with both enzymes, it was found that porcine trypsin was inhibited more than bovine trypsin (Fig. 3). In several comparative tests, the trypsin units inhibited per milliliter of soy extract tested with bovine trypsin were about two-thirds of that tested with porcine trypsin. Therefore, using porcine trypsin not only avoids autolytic enzyme inactivation during assay but also increases the sensitivity of the measurement.

Using the Enzyme-Last Test

Although the effect of sequence of mixing reactants on the extent of trypsin inhibition by soybean Kunitz inhibitor was observed long ago (Viswanatha and Liener 1954), only recently did this "reactant sequence effect" gain attention. While assaying the activity of two pure soybean trypsin inhibitors (Kunitz and BB). Liu and Markakis (1989) observed that adding trypsin last to a premix of inhibitor and substrate (the E-last test) might result in higher TIA values than adding the substrate last to a premix of the inhibitor with the enzyme (the S-last test). The difference in TIA values between the two tests depended on the premix pH and preincubation time. In this experiment, the reactant sequence effect was also observed when a raw soybean extract was assayed for TIA (Fig. 4).

In the E-last test, when the time of incubating a premix of soybean extract with BAPA or the pH of this premix was varied, the same inhibition value was obtained, indicating that the preincubation time and premix pH had no effect on the TIA assay.

In the S-last test, when the time of incubating a premix of soybean extract with either porcine or bovine trypsin was varied while the pH of the premix was fixed, the inhibition values obtained were different. The data are presented in Figure 5, in which the relative difference between the S-last and the E-last tests was expressed as

$$[(Ae - As)/Ae \times 100\%]$$

where As is the TIA obtained by the S-last test, and Ae is the TIA by the E-last test (since Ae remained constant regardless of the preincubation time, it was regarded as a reference). The results shown in Figure 5 indicate that when the premix pH was 3.5, the TIA values obtained by the S-last test were always lower than those by the E-last test, and the relative difference of the two tests was a function of preincubation time. In the first few minutes, the difference increased almost linearly with time, and after about 5 min the curve leveled off. The results also showed that the difference in TIA between the E-last and the S-last tests was greater for porcine trypsin than bovine trypsin.

Also in the S-last test, when the premix pH was varied while the time of incubating the premix was fixed at 10 min, different TIA values were obtained (Fig. 6). The results indicate that, like preincubation time, the premix pH had an effect on the TIA assay in the S-last test. As the pH increased from 2.7 to 9.0, the S-last test estimated TIA values either equal to or lower than the E-last test. There were two peaks corresponding to the largest

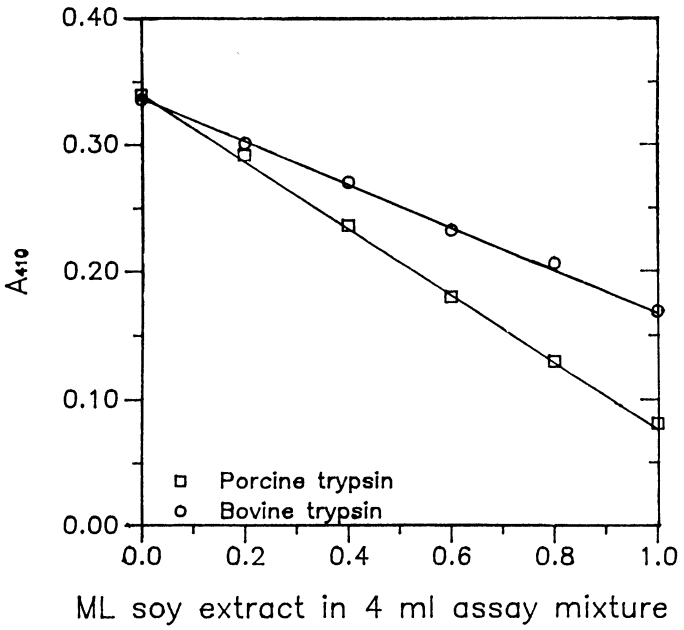

Fig. 3. Comparison of porcine and bovine trypsins for assaying trypsin inhibitor activity in soybeans.

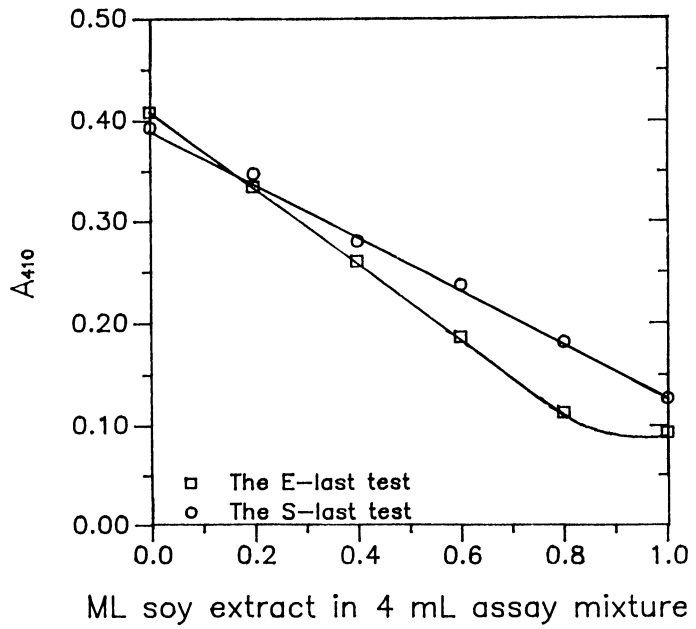

Fig. 4. Effect of the sequence of mixing the reactants on the assay of antitryptic activity in soybeans. In the S-last test, 0.5 ml of porcine trypsin solution prepared with 20 mM acetate buffer, pH 3.5, was premixed with 1.0 ml of sample solution prepared with the acetate buffer. After 3 min, 2.0 ml of BAPA solution was added and the reaction was allowed to proceed for 10 min. In the E-last test, the enzyme was added 3 min after mixing the substrate with the sample solution.

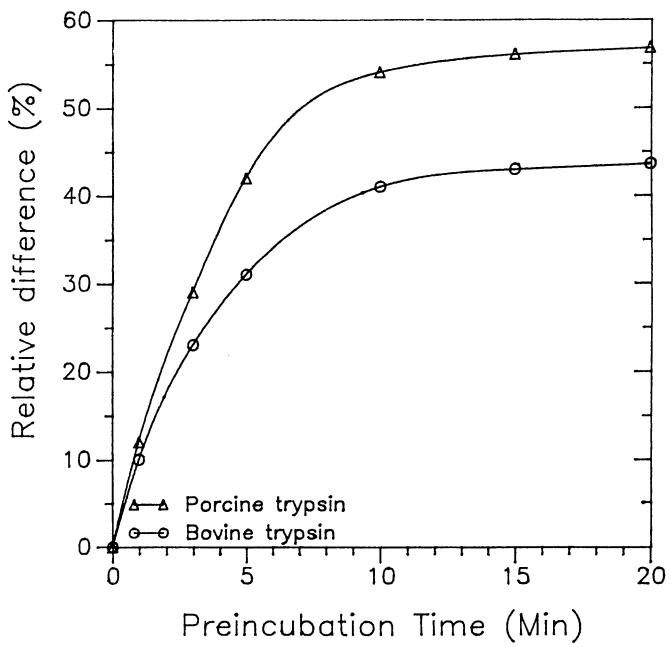

Fig. 5. Relative difference in trypsin inhibitor activity obtained by the S-last and the E-last tests as a function of the preincubation time. The relative difference is expressed as $(Ae - As)/Ae \times 100\%$, where Ae is the trypsin inhibitor activity obtained by the E-last test, and As is the trypsin inhibitor activity by the S-last test. The premix pH was kept constant at 3.5, and the preincubation time varied from 0 to 20 min.

difference between the two tests, one on the acidic side and one on the alkaline side. For the bovine trypsin, the alkaline peak was incomplete, because this enzyme is unstable above pH 7.5. Again, the difference in TIA between the E-last and S-last tests was greater for the porcine than the bovine trypsin.

The reactant sequence effect was explained in a previous paper (Liu and Markakis 1989). Under certain conditions, the lower inhibition observed in the S-last test was attributed to a limited hydrolysis of the inhibitor by the trypsin it inhibits, in accordance with the reactive site model proposed by Ozawa and Laskowski (1966). It is interesting to note that although aqueous soy extract contains both Kunitz and BB inhibitors (Liener and Kakade 1980), the pattern of the reactant sequence effect on its TIA assay was different from that of either of the two pure inhibitors. This was true particularly with bovine trypsin. For example, the changes in the relative difference between the E-last and the S-last tests as functions of preincubation time and premix pH were more pronounced for the bovine trypsin-soy extract combination (Figs. 5 and 6) than for the trypsin-Kunitz inhibitor or the trypsin-BB inhibitor combinations studied previously (Liu and Markakis 1989).

In the current method, the S-last test is used. The results obtained by this method are questionable in terms of both accuracy and resemblance to the real physiological situation (in the gut, trypsin reaches a premix of substrates and inhibitors). Since there are no preincubation time and pH effects when the E-last test is used, the proposed modification produces a uniform inhibition pattern: linear at a lower level of inhibitor and curving at a higher level. Thus the estimated values are very reproducible. In addition, when the premix pH is in the acidic or alkaline ranges, the E-last test gives higher inhibition values than the S-last test.

Reducing the Volume of the Reaction Mixture

Two different assay (reaction mixture) volumes, 4 and 8 ml, were compared for estimating the TIA of a soy extract. In the 4-ml assay, the procedure used was the same as that described under Methods. In the 8-ml assay, the same procedure was used except for doubling the volume of each reactant solution. The results obtained are shown in Figure 7. As the concentration of all reactants in the two assay systems is the same, twice the amount of soy extract present in the 8-ml assay is needed to cause the same level of trypsin inhibition as in the 4-ml assay. Thus, when TIA is expressed as trypsin units inhibited per milliliter of soy extract, the number expressing the inhibition will be twice as large using the 4 ml assay. From Figure 7, for 0.4 ml of soy extract, the value derived from the 4-ml assay is

$$(0.42 - 0.22)/0.4 \times 100 = 50 \text{ TUI/ml}$$

and for the 8 ml assay

$$(0.42 - 0.32)/0.4 \times 100 = 25 \text{ TUI/ml.}$$

Consequently, smaller quantities of trypsin inhibitors can be measured by decreasing the volume of the assay system when the concentrations of the reactants are kept unchanged.

TIA Units

Kakade et al (1969, 1974) arbitrarily defined a trypsin unit (TU) as causing an increase of 0.01 absorbance at 410 nm, and TIA was measured as trypsin units inhibited (TUI) or trypsin inhibitor units (TIU) per milligram of sample. The advantage of this expression is its independence of the purity of trypsin used in the assay. However, for comparative purposes, Kakade et al (1969) also expressed the TIA in terms of the absolute amount of pure trypsin inhibited. This was done by referring to a standard curve relating absorbance or (TU) to trypsin concentration. It was calculated that 1 μg of pure bovine trypsin has 1.9 TU. Hamerstrand et al (1981) attempted to express TIA in terms of milligrams of trypsin inhibitor per gram of sample, calculated on the assumption that 1 μg of trypsin is equivalent to 1 μg of trypsin inhibitor, whereas Smith et al (1980) stated that the expression in milligrams of trypsin inhibitor has no advantage over that in milligrams of trypsin inhibited. Since the actual molar concentration of enzyme or inhibitor is difficult to determine, and the amount of inhibitor protein does not represent its activity, in order to standardize the reporting of the inhibitor activity in the proposed method, we used the standard enzyme unit defined by the Commission on Enzymes of the International Union of Biochemistry. One international unit (IU) of enzyme is that amount that catalyzes the formation of 1 μmol of product per minute under defined conditions. As the molar absorption coefficient (a_m) of *p*-nitroaniline at 410 nm was found to be 7,760

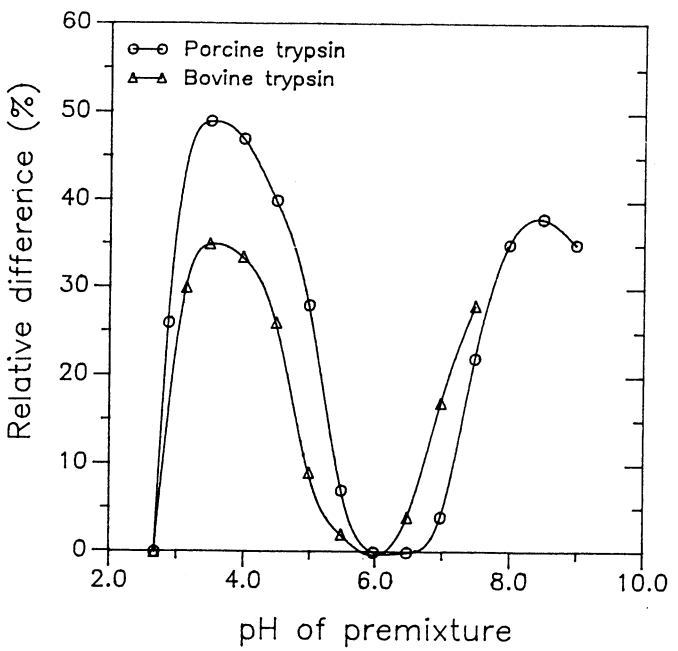

Fig. 6. Relative difference in trypsin inhibitor activity obtained by the S-last and the E-last tests as a function of the premix pH. The relative difference is expressed as $(Ae - As)/Ae \times 100\%$, where Ae is the trypsin inhibitor activity obtained by the E-last test, and As is the trypsin inhibitor activity by the S-last test. The preincubation time was kept constant at 10 min, whereas the premix pH varied from 2.7 to 9.0.

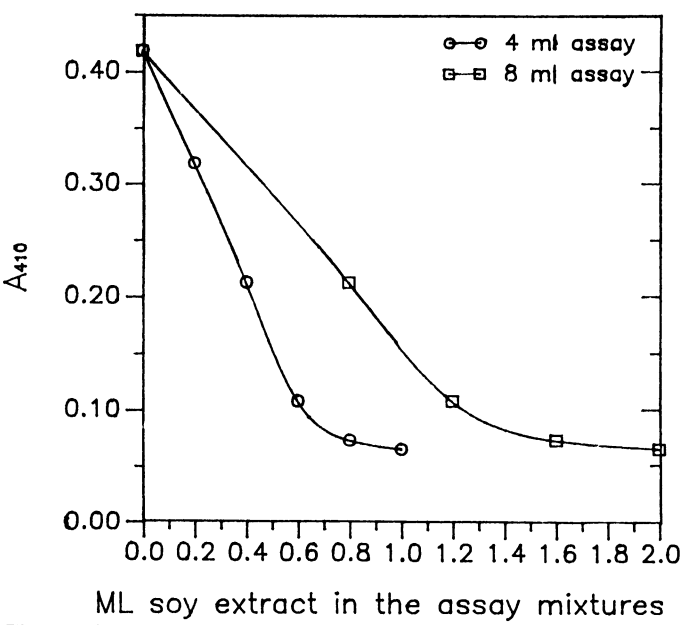

Fig. 7. Effect of the reaction mixture volume on the measurement of antitryptic activity in soybeans. In the 4-ml assay, 1 ml of sample solution was used; in the 8-ml assay, 2 ml of sample solution was used and all other reagents were doubled.

in this study, one TU is equivalent to 0.000516 IU under the assay conditions specified. We therefore express TIA in terms of both TUI (trypsin units inhibited) and IUI (international units inhibited).

Other Factors Affecting the TIA Assay

The following factors should also be considered in applying the proposed TIA assay. In general, factors affecting trypsin assay may or may not have the same effect on its inhibitor assay.

Enzyme concentration. As shown in Figure 8, the porcine trypsin employed in this test should not exceed that corresponding to $A_{410} = 0.50$ if linearity between absorbance and enzyme level is to be maintained. Within this A_{410} range, when two different amounts (6 and 8 μg) of enzyme were used to measure the TIA of the same soy extract, the parallel lines shown in Figure 9 were obtained. From these lines, the same TIA value, as TUI per milliliter of sample extract, can be derived, indicating that impurity or partial inactivation of the enzyme does not affect the assay. The independence of TIA on enzyme concentration was also addressed in the current method (Smith et al 1980, Hamerstrand et al 1981).

Reaction time. Figure 10 shows the relationship between A_{410} and reaction time. Linearity was observed for up to 12 min of reaction time, both in the absence of inhibitors (0.00 mg raw soybean /ml sample solution) and in the presence of inhibitors (0.10 and 0.15 mg raw soybean /ml). The results indicate that the rate of trypsin inhibition, expressed as TUI per milligram of dry sample per minute, was constant with the reaction time within the valid assay time range (0–12 min), whereas the TIA values, expressed as TUI per milligram of sample, increased linearly with time. For this reason, the reaction time for the TIA assay should be standardized to 10 min.

Substrate (BAPA) concentration. The apparent Michaelis constant (K_m) value for the porcine trypsin-BAPA reaction was found to be 0.96 mM at 37°C in this study. In the proposed TIA assay, the BAPA concentration would be 0.46 mM,

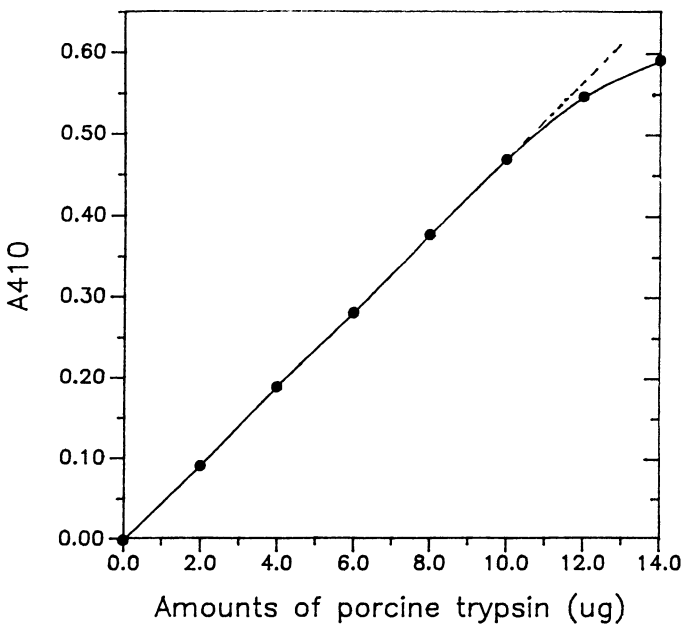

Fig. 8. Relationship between absorbance at 410 nm and amount of porcine trypsin. The reaction time was 10 min.

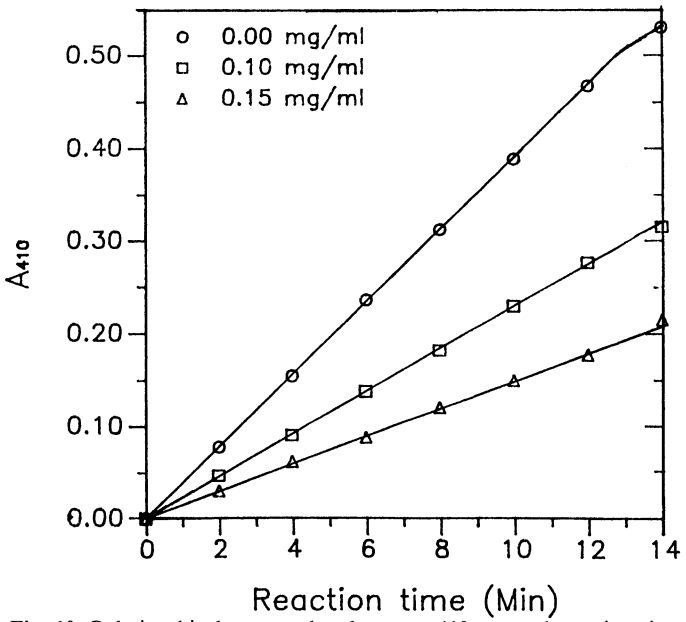

Fig. 10. Relationship between absorbance at 410 nm and reaction time in the absence or presence of inhibitors. An 8-μg sample of enzyme preparation was used in the reaction. The inhibitor samples were aqueous extracts of raw soybeans.

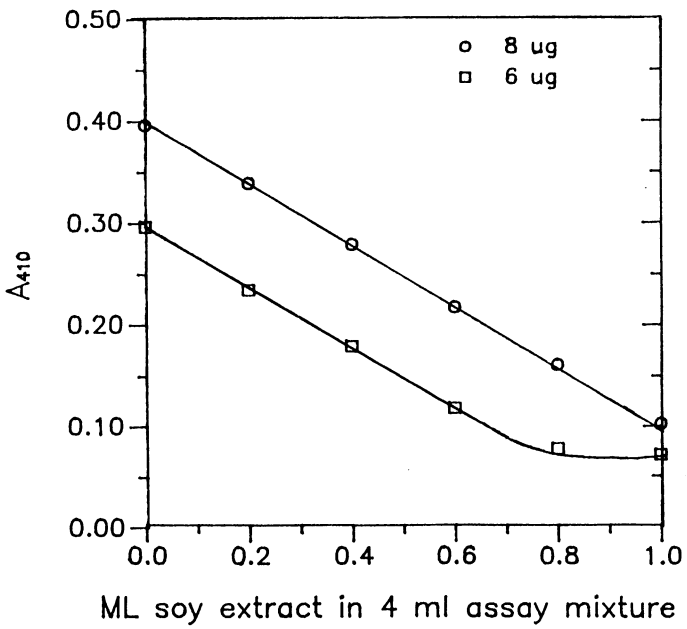

Fig. 9. Effect of porcine trypsin concentration on the assay of soybean trypsin inhibitor activity.

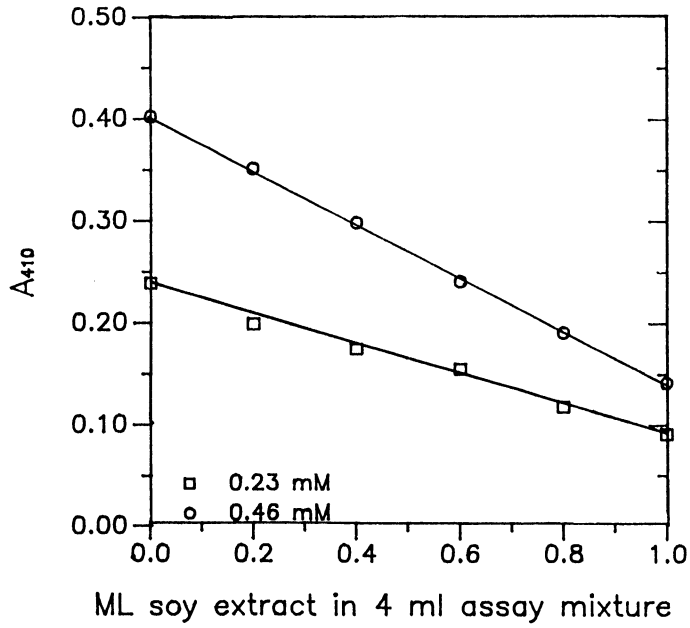

Fig. 11. Effect of substrate (BAPA) concentration on the assay of soybean trypsin inhibitor activity.

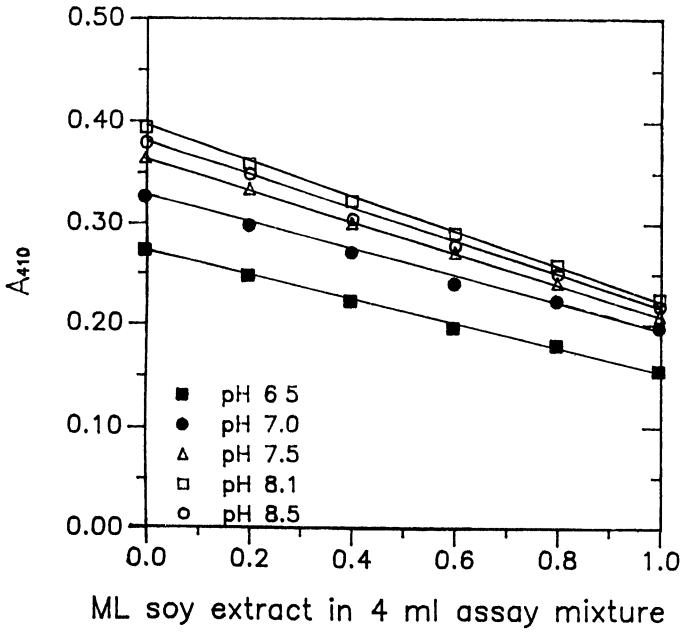

Fig. 12. Effect of assay buffer pH on the assay of soybean trypsin inhibitor activity.

TABLE V
Trypsin Inhibitor Activity in Some Commercial Soy Products and Legume Seeds Assayed by the Current and the Proposed Methods[a]

Samples	Current Method		Proposed Method	
	TUI/mg[b]	IUI/g[c]	TUI/mg[d]	IUI/g[e]
Soy protein concentrate	16.2 ± 0.8	20.9 ± 1.0	48.9 ± 1.8	25.2 ± 0.9
Soy protein isolate I	6.8 ± 0.6	8.8 ± 0.8	23.9 ± 1.1	12.3 ± 0.6
Soy protein isolate II	9.8 ± 0.6	12.6 ± 0.8	32.1 ± 0.6	16.6 ± 0.3
Cooked soybean	6.7 ± 0.4	8.6 ± 0.5	24.3 ± 1.1	12.5 ± 0.6
Raw soybean seeds	60.2 ± 1.9	77.7 ± 2.5	171.0 ± 3.4	88.2 ± 1.8
Raw cowpea seeds	8.2 ± 0.6	10.6 ± 0.8	32.3 ± 1.4	16.7 ± 0.7
Raw navy bean seeds	28.3 ± 0.9	36.1 ± 1.2	93.8 ± 0.6	48.4 ± 0.3
Raw pinto bean seeds	26.1 ± 1.2	33.5 ± 1.6	80.5 ± 2.1	41.5 ± 1.1

[a]Mean of duplicate measurements ± standard deviation.
[b]TUI = Trypsin units inhibited, where 1 TU is defined as 0.01 A_{410} under the assay conditions of the current method (pH 8.1 at 37°C with 10 ml assay volume and bovine trypsin).
[c]IUI = International units inhibited, where 1 TU is equivalent to 0.00129 IU under the assay conditions in footnote b.
[d]One TU is defined as 0.01 of A_{410} under the assay conditions of the proposed method (pH 8.1 at 37°C with 4 ml assay volume and porcine trypsin).
[e]One TU is equivalent to 0.000516 IU under the assay conditions in footnote d.

corresponding to about one half of the Michaelis constant. As the BAPA concentration affects the trypsin assay, so does it affect the TIA assay. Figure 11 shows that for two different BAPA concentrations, 0.23 and 0.46 m*M*, the lines connecting A_{410} and amounts of inhibitors are not parallel, a fact that emphasizes the significance of standardizing the BAPA concentration in the TIA measurement. In addition, since BAPA decomposed slowly with time, causing variation of the TIA value, it is recommended that a fresh working BAPA solution be used (Lehnhardt and Dills 1984).

Calcium ion concentration. Ca^{++} is known to stabilize trypsin (Buck et al 1962a). We observed that when Ca^{++} was added at two concentrations to the assay buffer, 5 and 10 m*M*, the TIA values were not significantly influenced, but its presence at the 5 m*M* level is recommended for protection of the enzyme from inactivation. Lehnhardt and Dills (1984) observed that the presence of Ca^{++} reduced not only autolytic trypsin inactivation but also the effect of phytate on the TIA assay.

pH of the assay buffer. In this study, the optimum pH for hydrolysis of BAPA by porcine trypsin was found to be 8.1, which resembled that by bovine trypsin (Erlanger et al 1961). In order to determine the optimum buffer pH for the TIA assay, the following five pH levels were tried: 8.5, 8.1, 7.5, 7.0, and 6.5. The results are summarized in Figure 12, and indicate that the A_{410} versus inhibitor quantity lines were not exactly parallel and the one corresponding to pH 8.1 led to the greatest TIA value (highest slope of the line).

Application of the Proposed Method to Some Soy Products and Legume Seeds

The TIA in some commercial soy products and legume seeds was measured according to both the current and the proposed procedures. The results are presented in Table V. Comparison of the two methods indicates that the proposed procedure estimates 1) much higher values when the TIA is expressed as TUI per milligram of sample, and 2) higher values when the TIA is expressed as IUI per gram of sample. The proposed method also reduces the relative standard deviation of the estimates. In summary, the proposed modification for measuring TIA in soybean products has a theoretical basis (Liu and Markakis 1989) and a practical significance. It can eventually be used for measuring TIA in many other proteinaceous food products.

ACKNOWLEDGMENTS

This is Michigan Agricultural Experiment Station Journal Article 12,730. The authors appreciate the partial support of this work by The Coca-Cola Company.

LITERATURE CITED

AMERICAN ASSOCIATION Of CEREAL CHEMISTS. 1983. Approved Methods of the AACC. Method 71-10, approved November 1973. The Association: St. Paul, MN.

BUCK, F. F., VITHAYATHIL, A. J., BIER, M., and NORD. F. F 1962a. On the mechanism of enzyme action LXXIII. Studies on trypsins from beef sheep and pig pancreas. Arch. Biochem. Biophys. 97:417-424.

BUCK, F. F., BIER, M., and NORD, F. F. 1962b. Some properties of human trypsin. Arch. Biochem. Biophys. 98:528.

CHERNICK, S. S., LEPKOVSKY, S. S., and CHAIKOFF. I L. 1948. A dietary factor regulating the enzyme content of the pancreas: Changes induced in size and proteolytic activity of the chick pancreas by the ingestion of raw soybean meal. Am. J. Physiol. 155:33.

ERLANGER, B. F., KOKAWSKY, N., and COHEN, W. 1961. The preparation and properties of two new chromogenic substances of trypsin. Arch. Biochem. Biophys. 95:271-278.

GREENE, N. M. 1953. Competition among trypsin inhibitors J. Biol Chem. 205:535.551.

HAMERSTRAND, G. E., BLACK, L. T., and GLOVER, J. D. 1981. Trypsin inhibitors in soy products: Modification of the standard analysis procedure. Cereal Chem. 58:42-45.

KAKADE, M. I., SIMONS, N., and LIENER, I. E. 1969. An evaluation of natural vs. synthetic substances for measuring the antitryptic activity of soybean samples. Cereal Chem. 46:518-526.

KAKADE, M. L., HOFFA, D. E., and LIENER, I. E. 1973. Contribution of trypsin inhibitors to the deleterious effect of unheated soybean fed to rats. J. Nutr. 103:1772.

KAKADE, M. L., RACKIS, J. J., McGHEE. J. E., and PUSKI. G. 1974. Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. Cereal Chem. 51:376-382.

KUNITZ, M. 1947. crystalline soybean trypsin inhibitor II: General properties J. Gen. Physiol. 30:291-310.

LEHNHARDT, W. L., and DILLS, H. G. 1984. Analysis of trypsin inhibitors in soy products: Evaluation of methodology and improvements. (Abstr.) J. Am. Oil Chem. Soc. 61:691.

LIENER, I. E., and KAKADE, M. L. 1980. Protease inhibitors. In: Toxic Constituents of Plant Foodstuffs, 2nd ed. I. E. Liener, ed. Academic Press: New York.

LIU, K., and MARKAKIS, P. 1989. Trypsin inhibition assay as related to limited hydrolysis of inhibitors. Anal. Biochem. 178:159-165.

OZAWA, K., and LASKOWSKI, M., JR. 1966. Reactive site of trypsin inhibitor. J. Biol. Chem. 241:3955.

RACKIS, J. J., GUMBMANN, M. R., and LIENER, I. E. 1985. The USDA trypsin inhibitor study. I. Background, objectives, and procedural details. Qual. Plant Foods Hum. Nutr. 35:213-242.

RACKIS, J. J., McGHEE, J. E., LIENER, I. E., KAKADE, M. L.,

and PUSKI, G. 1974. Problems encountered in measuring trypsin inhibitor activity of soy flour: Reports of a collaborative analysis. Cereal Sci. Today. 19:513-516.
SMITH, C., MEGEN, W. V., TWAALFHOVEN, L., and HITCHCOCK, C. 1980. The determination of trypsin inhibitor levels in foodstuffs. J. Sci. Food Agric. 31:341-350.
VISWANATHA, T., and LIENER, I. E. 1954. Inhibition of trypsin: I. Sequence of mixing the reactants. J. Biol. Chem. 210:97-108.

[Received August 25, 1988. Revision received March 12, 1989. Accepted May 8, 1989.]