Determination of Trypsin Inhibitor Activity of Soy Products: A Collaborative Analysis of an Improved Procedure[1]

M. L. KAKADE[2], J. J. RACKIS[3], J. E. McGHEE[3], and G. PUSKI[4]

ABSTRACT

A more accurate and reproducible procedure is described for measurement of trypsin inhibitor activity of soybean products than the method developed by Kakade in 1969 for measuring antitryptic activity in raw soybeans. Because the modified procedure is particularly suitable in determining trypsin inhibitor activity of heat-processed samples, it is recommended for use in evaluating the heat destruction of trypsin inhibitors in soybean samples.

Rackis et al. (1) previously reported that trypsin inhibitor values of raw and toasted soy flours, examined by a group of collaborators, varied widely when the original procedure of Kakade et al. (2) was followed. Statistical analyses showed that the relative standard deviation between collaborators was ±48%. This collaborative study has been continued to see if the problem could be corrected by a judicious choice of experimental conditions. Here we describe a modified procedure that emerged from these experiments; modifications include extracting soybean samples at pH 8.4 to 10.0 rather than at pH 7.6 and using an uncentrifuged extract instead of a centrifuged one. Some other minor modifications were also incorporated in the improved procedure to make it more accurate and reproducible.

MATERIALS AND METHODS

Materials

Tris-buffer (0.05M, pH 8.2) containing 0.02M $CaCl_2$: 6.05 g. *tris* (hydroxymethylamino methane) (Nutritional Biochemicals, Cleveland, Ohio) and 2.94 g. $CaCl_2:2H_2O$ dissolved in 900 ml. of water. The pH was adjusted to 8.2, and the volume brought to 1 liter with water.

Substrate Solution

Forty milligrams of benzoyl-DL-arginine-*p*-nitroanilide (BAPA) hydrochloride (Nutritional Biochemical Corp.) was dissolved in 1 ml. of dimethyl sulfoxide and diluted to 100 ml. with *tris*-buffer prewarmed to 37°C. The BAPA solution was prepared daily and kept at 37°C. while in use.

Trypsin Solution

In 200 ml. 0.001M HCl was dissolved 4 mg. of accurately weighed trypsin (2X crystallized, salt-free, Worthington Biochemicals Corp., Freehold, N.J.). This solution can be stored in the refrigerator for 2 to 3 weeks without appreciable loss in activity.

---

[1]Joint American Association of Cereal Chemists/American Oil Chemists' Society Committee on Soybean Trypsin Inhibitor Analysis.

[2]Land O'Lakes, Inc., Minneapolis, Minn. 55440.

[3]Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill. 61604. Mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

[4]Central Soya Co., Inc., Chicago, Ill. 60639.

Soybean Samples

Both raw and toasted, dehulled, defatted soy flours were evaluated for their trypsin-inhibitor activity. To prepare toasted soy flour, raw flakes were treated with live steam in a preheated autoclave for 30 min. at 100°C. Other soy products came from commercial sources. All samples were ground to pass through a 100-mesh screen.

Preparation of Soy Samples for Assay

One gram of finely ground sample (100 mesh) was extracted with 50 ml. of 0.01N NaOH. With raw soy flour an extraction time was 1 hr., whereas a longer time was needed to extract the maximum amount of trypsin inhibitor activity from heat-treated samples (see **Results and Discussion** for details). The pH of the suspension was usually 9.5 to 9.8 (if pH is below 8.4, extraction should be repeated with a stronger NaOH solution so that pH of the suspension is within 8.4 to 10.0). This suspension should be diluted to the point where 1 ml. produces trypsin inhibition of 40 to 60%. This limitation is needed to reduce the relative standard deviation (1).

Procedure

Portions (0, 0.6, 1.0, 1.4, and 1.8 ml.) of soybean suspension were pipetted into duplicate sets of test tubes and adjusted to 2.0 ml. with water. After 2 ml. of trypsin solution was added to each test tube, the tubes were placed in a water bath at 37°C. To each tube, 5 ml. of BAPA solution previously warmed to 37°C. was added; exactly 10 min. later the reaction was terminated by adding 1 ml. of 30% acetic acid. After thorough mixing, the contents of each tube was filtered (Whatman No. 3) and the absorbance of the filtrate was measured at 410 nm. against a reagent blank. The reagent blank was prepared by adding 1 ml. of 30% acetic acid to a test tube containing trypsin and water (2 ml. each) before the 5 ml. of BAPA solution was added. Because the soybean samples showed no appreciable absorption at 410 nm., no sample blank had to be prepared. If desired, a sample blank may be prepared by adding 5 ml. of BAPA solution to 2 ml. of the sample extract, incubating the mixture at 37°C. for 10 min., and then adding 1 ml. of acetic acid followed by the addition of 2 ml. of trypsin. The blank readings for any intermediate concentration of the sample can be calculated by simple mathematical calculation (e.g., if the sample blank reading, when read against reagent blank, was 0.1 absorbance, then the sample blank reading of 1 ml. of extract would be 0.05 absorbance and so on).

Expression of Activity

One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance units at 410 nm. per 10 ml. of the reaction mixture under the conditions used herein. Trypsin inhibitor activity is expressed in terms of trypsin units inhibited (TIU).

RESULTS AND DISCUSSION

Effect of pH and Centrifugation

As shown in Table I, much higher trypsin inhibitor values were obtained with moderately heated and with toasted soy flours when uncentrifuged extracts of

TABLE I. EFFECT OF pH AND CENTRIFUGATION ON TRYPSIN INHIBITOR ACTIVITY OF DEFATTED SOY FLOURS[a]

	pH of Extract					
	1.8		7.6		8.4	
Soy Flour[b]	Centrifuged	Uncentrifuged	Centrifuged	Uncentrifuged	Centrifuged	Uncentrifuged
Unheated	72.8	73.5	72.5	73.1	72.5	73.1
Moderately heated[c]	14.3	18.0	16.3	18.0	18.8	19.5
Toasted	1.9	2.8	2.8	3.6	4.9	5.8

[a] According to the procedure of Kakade et al. (2) activity expressed as trypsin units inhibited (TIU) per mg.sample.

[b] Samples supplied by J. J. Rackis, Northern Regional Research Laboratory.

[c] Heated at 100° C. for 9 min.

TABLE II. SUMMARY OF COLLABORATIVE TESTS OF SOYBEAN TRYPSIN INHIBITOR ACTIVITY (ANALYSIS PERFORMED INDEPENDENTLY)

		TIU mg. Soy Flour[a]	
Collaborator	Extract	Raw, extrapolated value	Toasted, average value
1	A	87.6	6.3
	B	89.1	6.3
2	A	96.7	6.9
	B	90.7	6.7
3	A	96.0	4.5
	B	94.8	4.3

[a] Amsoy soybeans, 1970 crop, preparation 1. Extraction time, 1 hr.

samples extracted at pH 8.4 were analyzed. No significant differences were observed in trypsin inhibitor activity of unheated samples due to variation in pH of extraction and to use of centrifuged and uncentrifuged extracts. Evidently heat treatment induces some denaturation changes in trypsin inhibitors whereby the amino acid residues at reactive sites become inaccessible for the formation of the trypsin-trypsin inhibitor complex (3). Apparently the modifications introduced in our improved procedure measure the inhibitory activity of insoluble trypsin inhibitors and expose their amino acid residues for interaction with trypsin.

In 1972, Wang et al. (4) reported similar observations; they found trypsin inhibitor activity increased in centrifuged extracts of boiled, fermented soybeans compared to similar extracts from boiled, unfermented soybeans. They therefore suggested that soybean trypsin inhibitors exist in bound form which can be released by *Rhizopus oligosporus* fermentation. However, it may not be necessary to postulate the existence of bound trypsin inhibitors in soybeans. It may be that proteases of *R. oligosporus* solubilized the heat-denatured trypsin inhibitors and expose the amino acid residues to inhibition by interaction with trypsin.

Trypsin Inhibitor Activity Analysis

Two series of analyses by the modified procedure were made. In the first series, the assay was carried out independently and each collaborator prepared his own extract and reagents. In the second series (on-the-spot assay), the collaborators assayed the same extracts using the same stock solutions of trypsin and BAPA. In both series, the collaborators were required to dilute the extracts until 1-ml. aliquots inhibited trypsin about 50%. Dilution factors used in the first series varied between 60 to 66.6 for raw soy samples and 60 to 66.6 for toasted samples.

The independent collaborative tests are summarized in Table II. The trypsin inhibitor values are in excellent agreement although a somewhat greater variation resulted in toasted soy flour. The relative standard deviation between collaborator was ±5%.

TABLE III. SUMMARY OF COLLABORATIVE TESTING OF TRYPSIN INHIBITOR ANALYSIS (ON-THE-SPOT ASSAY PERFORMED AT THE NORTHERN REGIONAL RESEARCH LABORATORY)

		TIU mg. Soy Flour[a]	
Collaborator	Extract	Raw, extrapolated value	Toasted, average value
1	A	109.4	8.3
	B	107.8	7.6
2	A	101.6	8.5
	B	101.6	8.7
3	A	96.9	10.8[b]
	B	109.4	10.9[b]
4	A	110.9[b]	10.1[b]
	B	106.3[b]	10.2[b]

[a] Amsoy soybeans, 1970 crop, preparation 2.

[b] Extraction time, 5 hr.; otherwise 1 hr. extraction.

Graphic extrapolation to zero inhibitor level was used to obtain trypsin inhibitor activity (2) values for raw soy flour. With toasted soy flour extracts, the plot of TIU per ml. extract as a function of level of inhibitor did not give a negative linear correlation. Consequently trypsin inhibitor activity values for toasted soy flour were established by averaging the TIU per ml. for each level of inhibitor solution taken for analysis. The averaging technique was used to calculate TIU per mg. in subsequent assays of toasted soy flour and other heat-treated samples. Results of on-the-spot assay are given in Table III. Once again the agreement between collaborators was excellent.

During these tests, much higher trypsin inhibitor activity was observed with 5-hr. extracts of toasted soy flour than with those extracted for 1 hr. The effect of time of extraction was, therefore, investigated further. As shown in Table III, there were no significant differences in trypsin inhibitor activity of raw soy flour extracted for 1 or 5 hr. However, inhibitor activity of toasted samples was 27% higher for the 5-hr. extract as compared to the 1-hr. extract. On the basis of these results, another experiment was performed to examine the effect of time of extraction on the release of trypsin inhibitor activity from toasted soy flour. The TIU per mg. of toasted samples extracted for 1, 3, 5, and 20 hr. was 5.4, 7.9,8.0, and 6.7, respectively. Therefore, extraction of the toasted sample for about 3 hr. is necessary to gain maximum values.

Trypsin Inhibitor Activity of Commercial Products

Trypsin inhibitor values for commercial soy products are summarized in Table IV. For comparative purposes, values obtained by the original procedure (2) are also given. Except for sample 4, all show two- to sevenfold increases in TIU per mg. when assayed by the modified procedure as compared to values by the original one.

Statistical Analyses

The logarithm of the TIU per mg. was the measurement analyzed. Variations associated with soy treatment combinations, collaborators, and extraction procedures were examined. Precision estimates were determined for collaborators analyzing common extracts and separate extracts. Data were also analyzed from assay lines of milliliters of soy extract vs. TIU per ml. used in estimating the intercept.

Table V shows the pooled standard deviations for deviations about the assay lines, for experiments involving common abstracts, and for those involving separate extracts. The respective relative standard deviations are 5, 2, and 5% of the observed value.

In the final analysis, it may be argued that since BAPA is not a natural substrate, the TIU thus obtained may not represent "true" values. However, it was shown in a previous publication (2) that the changes in TIU obtained with protein substrate (casein) parallel changes observed with BAPA as substrate. This consideration, plus the simplicity of the procedure itself, has prompted us to adopt the BAPA method for the determination of trypsin inhibitor activity of soybean samples.

CONCLUSIONS

On the basis of previous results obtained (1) and those described here, the inherent problems associated with the determination of trypsin inhibitor activity in

TABLE IV. COMPARISON OF TRYPSIN INHIBITOR ACTIVITY OF COMMERCIAL SOY PRODUCTS USING ORIGINAL AND MODIFIED PROCEDURES[a]

Sample	Original Procedure[b]	Modified Procedure
Toasted soy flour	5.3	9.7
Soy protein concentrate	2.4	12.0
Soy protein isolate	4.8	8.5
Soy protein isolate	18.8	19.8
Specially processed soy flour for calf feeding[c]	0.9	3.8
Specially processed soy flour for calf feeding[d]	0.7	4.4

[a]Extraction time, 1 hr.
[b]Kakade et al. (2).
[c]Nutramine®, Cargill, Minneapolis, Minn.
[d]Soyassim®, Societe' Industrielle des Oleagineaux, France.

TABLE V. VARIATION ESTIMATES OF TRYPSIN INHIBITOR ASSAY STUDIES

Source of Variation	Rel. Std. Dev. %
Deviation about assay lines[a]	5
Collaborators with common extract[b]	2
Collaborators with separate extracts[c]	5

[a]Error involved in converting the change in trypsin units to TIU per ml. from the milliliter of soy used for assay, such as 0.6, 1.0, 1.4, and 1.8 ml. soy. The relative standard deviation for the standard trypsin curve is less than 5%.
[b]On-the-spot assay at Northern Regional Research Laboratory.
[c]Extracts and assays made in laboratories of individual collaborator.

soy products can be overcome by a judicious choice of experimental conditions. The greatest problems are associated with analysis of heat-treated soy flours and other processed soy protein products (Table IV) that contain appreciable amounts of denatured protein constituents.

To obtain accurate and reproducible trypsin inhibitor values, the four major conditions that must be observed include: a) extract soy samples at pH 8.4 to 10.0; b) use uncentrifuged extracts; c) dilute extracts taken for analysis so that 1 ml. inhibits trypsin in the range of about 40 to 60%; and d) extract for at least 1 hr. depending upon the conditions under which the soy product was processed. With raw soy products, a 1-hr. extraction with 0.01N sodium hydroxide is sufficient. However, to reach maximum trypsin inhibitor values for toasted soy flour, an extraction of about 3 hr. is required.

As shown in Table IV, commercially available soy protein products also exhibit much greater trypsin inhibitor activity when assayed by the modified procedure.

However, these assays were made before the effect of time of extraction on the release of trypsin inhibitor activity was observed; an extraction time of 1 hr. was used. Additional analyses are required to establish the optimum extraction time needed to gain maximum trypsin inhibitor values for these products.

Acknowledgments

Statistical analyses were performed by W. F. Kwolek, Biometrician, North Central Region, Agricultural Research Service, U.S. Department of Agriculture.

Literature Cited

1. RACKIS, J. J., McGHEE, J. E., LIENER, I. E., KAKADE, M. L., and PUSKI, G. Problems encountered in measuring trypsin inhibitor activity of soy flour. Report of a collaborative analysis. Cereal Sci. Today (in press).
2. KAKADE, M. L., SIMONS, N., and LIENER, I. E. An evaluation of natural versus synthetic substrates for measuring the antitryptic activity of soybean samples. Cereal Chem. 46: 518 (1969).
3. LIENER, I. E., and KAKADE, M. L. Protease inhibitors. In: Toxic constituents of plant foodstuffs, ed. by I. E. Liener, p. 7 (1969).
4. WANG, M. L., VESPA, J. B., and HESSELTINE, C. W. Release of bound trypsin inhibitors in soybeans by *Rhizopus oligosporus.* J. Nutr. 102; 1495 (1972).

[Received July 11, 1973. Accepted September 5, 1973]