

An Evaluation of Natural vs. Synthetic Substrates for Measuring the Antitryptic Activity of Soybean Samples¹

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ABSTRACT

The casein digestion method of Kunitz for the measurement of trypsin inhibitor activity of soybean extracts has been modified to obtain more accurate and reproducible results. The modification involved the use of 2% casein (instead of 1%) and the mathematical transformation of absorbance readings (A) at 280 m μ to A^{3/2}. The use of the synthetic substrate, benzoyl-DL-arginine-p-nitroanilide (BAPA), proved to be a convenient and reliable method of assaying trypsin inhibitor activity provided one takes into account the competitive nature of the inhibition. The latter effect could be largely compensated for by extrapolating the trypsin inhibitor activity, expressed as trypsin units inhibited per ml. extract, to zero concentration of the inhibitor. Although a series of soybean samples could be ranked in the same relative order of inhibitor activity by using either casein or BAPA, the quantity of trypsin inhibited was consistently higher when BAPA was employed as the substrate.

Casein has been widely used as a substrate for measuring the trypsin inhibitor activity of natural trypsin inhibitors such as those which occur in soybeans and other legumes. The most common method employing casein is the one originally described by Kunitz (1) which involves the spectrophotometric determination of the breakdown products produced by a given concentration of trypsin in the presence and absence of the inhibitor. However, as pointed out by several investigators (2,3) and according to our own experience, the fact that the rate of hydrolysis of casein by trypsin does not follow zero order kinetics under the conditions defined by Kunitz (1) detracts from the reliability of this method and poses problems of reproducibility.

On the other hand, the tryptic hydrolysis of a synthetic substrate such as benzoyl-DL-arginine-p-nitroanilide (BAPA), first introduced by Erlanger et al. (4), does follow a zero order reaction, and one obtains, within broad limits, a linear relationship between the quantity of p-nitroaniline released and the concentration

¹This study was supported by Agricultural Research Service, U.S. Dept. of Agriculture, Grant No. 12-14-100-9180 (71), administered by the Northern Utilization Research and Development Division, Peoria, Illinois.

of the active enzyme. Although BAPA has been used for assaying the antitryptic activity of soybean fractions (5), a critical evaluation of this technique in our laboratory has indicated that some caution must be exercised in the interpretation of the results obtained by this method, particularly if meaningful comparisons are to be made from one soybean sample to another.

The purpose of the present communication is to describe in detail certain modifications of the methods employing either casein or BAPA which were found to be necessary for obtaining reliable and reproducible data on the trypsin inhibitor activity of soybean samples.

EXPERIMENTAL

Trypsin Inhibitor Activity Using Casein Substrate

Materials. Phosphate buffer (0.1M, pH 7.6): 23.3 g. $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 1.8 g. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ were dissolved in 900 ml. water. The pH was adjusted to 7.6 and the final volume brought to 1 liter.

Casein solution (1 or 2%): 1 or 2 g. of casein (Hammersten quality, Nutritional Biochemicals Corp., Cleveland, Ohio) was suspended in 80 ml. of the phosphate buffer and completely dissolved by heating on a steam bath for 15 min. The solution was cooled, made to 100 ml. with buffer, and stored in the refrigerator when not in use.

Stock trypsin solution: 4 to 5 mg. of accurately weighed² trypsin (2X crystallized, salt-free, Worthington Biochemical Corp., Freehold, N.J.) was dissolved in 100 ml. 0.001M HCl. This solution can be stored in the refrigerator for 2 to 3 weeks without appreciable loss in activity.

Preparation of Soybean Samples. Samples of mature soybeans were ground in a Wiley mill to pass through a 100-mesh screen and extracted with 10 vol. of petroleum ether (b.p. 60° to 70°C.) at room temperature. One gram of the meal was suspended in 19 ml. water, and the pH of the suspension adjusted to 7.6. After mechanical shaking for 1 hr., the suspension was centrifuged, and 1 ml. of the supernatant was diluted to 50 ml. with phosphate buffer.³ The protein content⁴ of the diluted extract was determined by the method of Lowry et al (6).

Procedure. Trypsin standard curve: 0.2 to 1.0 ml. of the stock trypsin solution was pipetted into a triplicate set of test tubes (one set for each level of trypsin) and the final volume of each tube adjusted to 2 ml. with the phosphate buffer. The tubes were set in a water bath at 37°C. To one of the triplicate tubes was added 6 ml. 5% (w./v.) trichloroacetic acid; this tube serves as a blank (see below); 2 ml. of the casein solution, previously brought to 37°C., was added to each tube. The tubes were allowed to remain at 37°C. for exactly 20 min., at which time the reaction was stopped by adding 6 ml. of 5% trichloroacetic acid to the experimental tubes. After standing for 1 hr. at room temperature, the suspension was filtered, and the absorbance of the filtrate was measured at 280 μ against the blank.

²Alternatively, the concentration of trypsin (mg./ml.) may be determined by multiplying the absorbance of the trypsin solution at 280 μ by the factor 0.65 (Worthington Enzyme Manual, 1967, p. 4-67).

³When assaying samples of processed soybean preparations in which the trypsin inhibitor activity may be partially destroyed, the original extract may be used directly or should be diluted to the point where 1 ml. produces an inhibition of at least 80%.

⁴The amount of protein extracted by this procedure will depend on the physical properties of the soybean product being examined. In view of the uncertainty that may attend the extractability of the protein, it may at times be advisable to express the trypsin inhibitor activity on the basis of the protein content of the extract (see, for example, Table IV).

Trypsin inhibitor activity: 0.2- to 1.0-ml. aliquots of the soybean extract were pipetted into a triplicate set of test tubes (one set for each level of extract), and the volume brought to 1.0 ml. with the phosphate buffer; 1 ml. of the stock trypsin solution was added to each tube, and the tubes were placed in the water bath at 37°C. The remainder of the procedure was the same as that described in the preceding paragraph.

Expression of Activity. One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance units at 280 $m\mu$ in 20 min. per 10 ml. of the reaction mixture under the conditions set forth herein. Trypsin inhibitor activity is defined as the number of trypsin units inhibited (TUI).

Trypsin Inhibitor Activity Using BAPA Substrate

Materials. Tris buffer (0.05M, pH 8.2) containing 0.02M CaCl_2 : 6.05 g. tris-(hydroxymethyl) aminoethane (Sigma 7-9, Sigma Chemical Co., St. Louis, Mo.) and 2.94 g. $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ were dissolved in 900 ml. water. The pH was adjusted to 8.2, and the volume brought to 1 liter with water.

BAPA solution: 30 mg. BAPA·HCl (Nutritional Biochemicals Corp.) was dissolved in 1 ml. dimethylsulfoxide and diluted to 100 ml. with the tris buffer prewarmed to 37°C. Care should be taken to dissolve all of the BAPA in dimethylsulfoxide since traces of undissolved crystals may cause precipitation to occur upon standing (4). The BAPA solution was freshly prepared daily and kept at 37°C. while in use.

Trypsin solution: See casein method.

Preparation of Soybean Sample. Same as described under casein method except that water was used to make the 1:50 dilution of the original soybean extract.⁵

Procedure. Trypsin standard curve: 0.2 to 1.0 ml. of the stock trypsin solution was pipetted into a triplicate set of test tubes, and the volume made up to 2 ml. with water. The test tubes were placed in a water bath at 37°C.; 1 ml. of 30% acetic acid was added to one of the triplicate tubes to serve as a blank. To each tube was then added 7 ml. of BAPA solution, previously warmed to 37°C., and, exactly 10 min. later, the reaction was terminated by adding 1 ml. of 30% acetic acid to each of the experimental tubes. After thorough mixing the absorbance of each solution was measured at 410 $m\mu$ against the appropriate blank.

Trypsin inhibitor activity: 0.2 to 1.0 ml. of the soybean extract was pipetted into a triplicate set of test tubes and the final volume adjusted to 1.0 ml. with water; 1 ml. of the stock trypsin solution was added to each of the tubes, which were then assayed as described in the previous paragraph.

Expression of Activity. One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance units at 410 $m\mu$ per 10 ml. of the reaction mixture under the conditions defined herein. Trypsin inhibitor activity is defined as the number of trypsin units inhibited (TUI).

RESULTS AND DISCUSSION

The standard trypsin curve obtained by using the 1% casein solution (final concentration of casein in the digestion mixture, 0.5%) recommended by Kunitz

⁵See footnote 3. Protein may precipitate upon the addition of 30% acetic acid (see "procedure" below) when dilutions of less than 1150 are employed. In this case the solutions should be filtered prior to reading the absorbance.

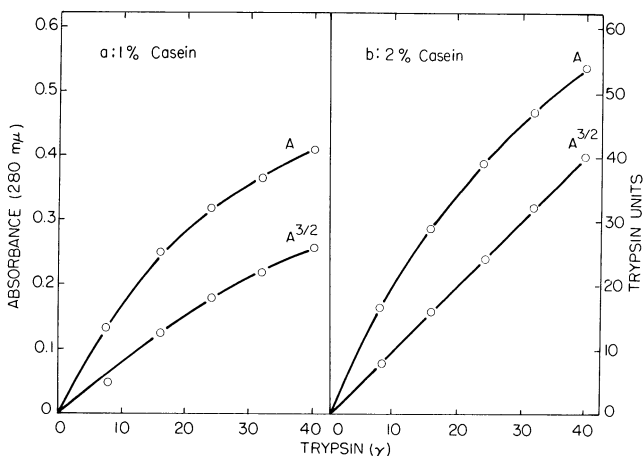


Fig. 1. Standard trypsin curves. (a). 1% casein as substrate; (b). 2% casein as substrate. Curves A were obtained by plotting absorbance readings (A) directly against trypsin concentration. Curves $A^{3/2}$ were obtained by mathematical transformation of absorbance readings (7).

(1) is shown in Fig. 1a. In an attempt to convert this curvilinear response to a linear one, the absorbance readings (A) were transformed to $A^{3/2}$ as originally proposed by Miller and Johnson (7). However, it will be noted from the upper curve of Fig. 1a that this deviation from linearity still persisted at higher levels of enzyme concentration. Since this effect has been attributed to limited substrate concentration (3,7), the concentration of the casein was increased to 2% (final concentration in digestion mixture, 1%) with the results shown in Fig. 1b. Under these conditions a linear relationship could now be obtained between $A^{3/2}$ and the range of enzyme concentrations employed in this study. Such a curve now permits one to obtain consistent values for the specific activity of the trypsin preparation compared with the original Kunitz procedure which gives decreasing values for specific activity with increasing concentrations of trypsin (see Table I). Thus, with the Kunitz procedure a fivefold increase in enzyme concentration caused almost a 50% decrease in the calculated specific activity of trypsin, whereas the specific activity values obtained by the modified procedure remained fairly constant over the same range of enzyme concentrations.

TABLE I. THE SPECIFIC ACTIVITY OF TRYPSIN WITH CASEIN AS THE SUBSTRATE

Level of Trypsin γ	Specific Activity ^a	
	Kunitz Procedure ^b	Present Modification ^c
8	1.65	0.98
16	1.57	0.96
24	1.31	1.01
32	1.14	0.99
40	1.03	1.00

^aTrypsin units (as defined in the text) per gamma of trypsin.

^bSubstrate was 1% casein. Specific activities were calculated from data presented in Fig. 1a (curve A).

^cSubstrate was 2% casein. Specific activities were calculated from data presented in Fig. 1b (curve $A^{3/2}$).

TABLE II. DETERMINATION OF THE TRYPSIN INHIBITOR ACTIVITY OF A SOYBEAN EXTRACT WITH CASEIN AS SUBSTRATE

Level of Soybean Extract	Absorbance ^a (A)	3/2 A	TU ^b	TUI ^c	TUI/ml.
ml.					
0	0.542	0.399	40.0
0.2	0.479	0.331	33.1	6.9	34.5
0.4	0.394	0.247	24.7	15.3	38.2
0.6	0.279	0.148	14.8	25.2	42.0
0.8	0.179	0.076	7.6	32.4	40.5
1.0	0.099	0.031	3.1	36.9	36.9
Average					38.4

^aAverage of duplicate determinations.

^bTrypsin units as defined in the text for casein method.

^cTrypsin units inhibited.

The linear relationship between the rate of hydrolysis of BAPA and trypsin concentration reported by Erlanger et al. (4) has been confirmed in the present study (Fig. 2). Because of this linear relationship, the specific activity of trypsin may be calculated at any level of trypsin, at least within the limits in which this linearity has been established experimentally.

The data and calculations pertaining to the measurement of the trypsin inhibitor activity of a sample of raw soybeans using either casein (modified procedure) or BAPA as trypsin substrate are shown in Tables II and III, respectively. It is evident that, in the case of casein, the values for trypsin inhibitor activity, expressed as TUI per ml. of extract, are quite consistent and independent of the level of inhibitor extract tested. On the other hand, with BAPA as the substrate, the trypsin inhibitor activity becomes progressively less as the level of inhibitor extract was increased. The reason for the difference in the response of inhibitor activity to the level of inhibitor being assayed by these two methods becomes more apparent if one plots percent inhibition as a function of level of soybean extract as is done in Fig. 3. It is evident that, with casein as the substrate, trypsin inhibitor activity decreases in direct proportion to the level of inhibitor up to the point of about 80% inhibition. With BAPA as the substrate, deviation from linearity commences at a lower level of inhibition, namely, about 55%. Hence, if casein is to be used as the substrate, trypsin inhibitor activity, when expressed on a per ml. basis, should remain fairly

TABLE III. DETERMINATION OF THE TRYPSIN INHIBITOR ACTIVITY OF A SOYBEAN EXTRACT WITH "BAPA" AS SUBSTRATE

Level of Soybean Extract	Absorbance ^a	TU ^b	TUI ^c	TUI/ml. extract
ml.				
0	0.480	48.0		
0.2	0.384	38.4	9.6	48.0
0.4	0.283	28.3	19.7	49.2
0.6	0.197	19.7	28.3	47.2
0.8	0.129	12.9	35.1	43.9
1.0	0.078	7.8	40.2	40.2
Extrapolated value ^d				54.0

^aAverage of duplicate determinations.

^bTrypsin units as defined in the text for BAPA method.

^cTrypsin units inhibited.

^dSee Fig. 5 and explanation in the text.

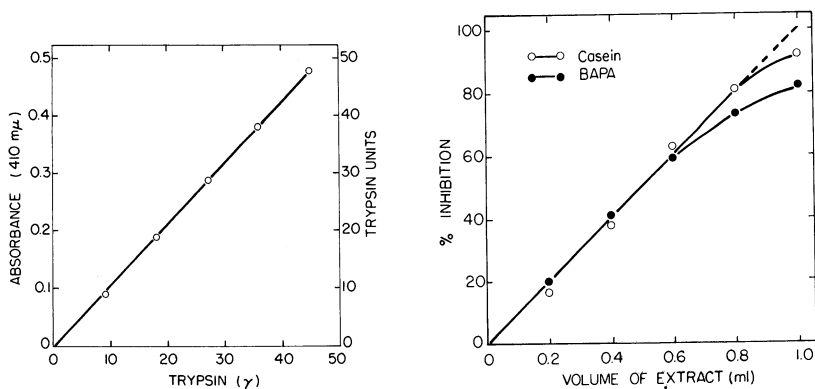


Fig. 2 (left). Standard trypsin curve using BAPA as substrate.

Fig. 3 (right). Inhibition of trypsin activity as a function of the level of crude soybean extract using casein (o --- o) or BAPA (● --- ●) as substrate. Dashed lines are extrapolated from linear portion of each curve.

constant up to a level of about 80% inhibition, whereas with BAPA the trypsin inhibitor activity, similarly expressed, becomes progressively less once inhibition has exceeded a value of about 55%.

It was of interest to compare the inhibitor activity of a crystalline preparation of the Kunitz soybean trypsin inhibitor (Worthington) with casein and BAPA as trypsin substrates. As shown in Fig. 4, unlike the crude extract, the curves relating percent inhibition to level of inhibitor on both substrates were identical.

The fact that trypsin inhibitor activity deviates from linearity at high levels of inhibitor concentration has been attributed to the partial dissociation of the trypsin-inhibitor complex (3,8). It would appear from the curves shown in Fig. 3 for the crude extract that the magnitude of this dissociation is greater in the case of BAPA than with casein. The reason for this apparent difference in dissociation is not clear since the dissociation constant (K_i) of the enzyme-inhibitor complex should be independent of the nature of the substrate (8), as was in fact

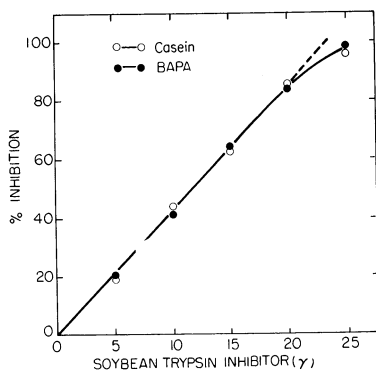


Fig. 4. Inhibition of trypsin activity as a function of the level of the Kunitz soybean trypsin inhibitor, using casein (o --- o) or BAPA (● --- ●) as substrate. Dashed line is extrapolated from linear portion of the curve.

demonstrated with the pure inhibitor in Fig. 4. The explanation for the anomalous effect of substrate on the apparent K_i of the crude extract probably lies in the fact that the crude extract contains several inhibitors (9), each of which may possess a characteristic K_i value. For example, the K_i of the Kunitz inhibitor is 2×10^{-10} M (8) whereas the K_i for the AA inhibitor of Birk has been reported to be 5.6×10^{-8} M (10). The affinity of trypsin for the two inhibitors is much greater than its affinity for natural substrates such as casein [$K_m = 10^{-2}$ M (8)] so that the inhibition is essentially noncompetitive (11). On the other hand, synthetic substrates have a much greater affinity for trypsin [$K_m = 10^{-5}$ M in the case of benzoyl-L-arginine ethyl ester (12)] than does the protein substrate, so that the former may now compete with the inhibitor for any free enzyme at a given instant. Thus as the K_m value more nearly approaches the K_i of the AA inhibitor one might expect to observe a competitive type of inhibition with this inhibitor which might not necessarily be apparent with the Kunitz inhibitor. (Frattali (13) has reported recently that trypsin combines with the AA inhibitor in a nonstoichiometric fashion, indicative of competitive inhibition, with p-toluenesulfonyl-L-arginine methyl ester as a substrate. When assayed under identical conditions, the Kunitz inhibitor combined stoichiometrically with trypsin, giving a curve similar to the one shown in Fig. 4 herein.) It is evident, therefore, that the presence of inhibitors other than the Kunitz inhibitor in the crude extract could clearly influence the type of kinetic picture one would observe with such a complex system.

When the trypsin inhibitor activity (TUI/ml. extract) determined by the BAPA method was plotted as a function of the level of inhibitor solution, a negative linear correlation was obtained as shown in Fig. 5. By extrapolating such a curve to zero level of inhibitor solution, one should obtain a value which more nearly approaches the true inhibitory activity of the soybean extract.

Trypsin inhibitor measurements were made on extracts from several different varieties of soybeans both by the modified casein procedure and by the BAPA method. In the latter instance, graphic extrapolation to zero inhibitor level was employed to obtain trypsin inhibitor activity. Although expression of trypsin

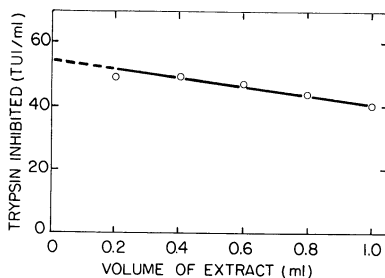


Fig. 5. Trypsin inhibitor activity (TUI/ml. extract), determined by the BAPA method, in relation to level of crude soybean extract. Curve was constructed from data recorded in Table III. Extrapolated curve, as represented by broken line, intercepts 0 ml. at 54 TUI/ml.

TABLE IV. COMPARISON OF TRYPSIN INHIBITOR ACTIVITY^a OF SOYBEAN SAMPLES USING CASEIN AND BAPA METHODS

Soybean Sample ^b	Protein Extracted ^c %	Casein Method		BAPA Method	
		Series A ^d	Series B ^e	Series A ^d	Series B ^e
PI 189,898	92	46.6	49.3	65.8	60.8
Minsoy	88	51.4	52.6	67.3	65.0
PI 153,251	90	56.8	57.2	72.6	70.7
PI 189,876	92	57.5	60.3	75.1	72.4
PI 153,240	95	59.7	62.8	75.9	77.3
PI 189,900	92	63.6	63.6	77.3	79.0
PI 238,924	87	65.1	72.5	83.6	89.7
PI 257,428	95	73.4	75.8	96.4	93.8
PI 257,438	93	78.2 ^f	82.6	98.9 ^f	107.5
Kunitz inhibitor		0.89 ^f	...	1.02 ^f	...

^aExpressed as gamma trypsin inhibited per milligrams protein in crude soybean extract.

^bNumbers refer to strain designation of soybean samples grown in 1964 and provided by Jean Lambert of the Department of Agronomy and Plant Genetics.

^cBased on N content of soluble extract prepared as described in text and N content of whole soybean sample.

^dAverage of trypsin inhibitor activity determined at five different levels of crude extract.

^eAverage of trypsin inhibitor activity determined at three different levels of crude extract.

^fMilligrams trypsin inhibited per milligram inhibitor; value shown is the average of the activity obtained with five different levels of the inhibitor.

inhibitor activity in terms of trypsin units inhibited has the advantage that the activity is independent of the purity of the trypsin, it may be desirable for comparative purposes to express the inhibitory activity in terms of the absolute amount of pure trypsin inhibited. This can be done by referring to a standard curve relating absorbance (or trypsin units) to trypsin concentration (Fig. 1b, curve A^{3/2}, and Fig. 2). Unfortunately most commercial preparations of crystalline trypsin are far from pure (14), so that it becomes necessary to establish the purity of the trypsin being used. In this study the purity of the enzyme was determined by active site titration using p-nitrophenyl-N-benzyloxycarbonyl-L-lysinate (15) and was found to be 56% (16). On this basis, 1 γ of "pure" trypsin was calculated to have an activity of 1.79 TU by the casein method and 1.90 TU by the BAPA method. The trypsin inhibitor activities of nine samples of soybeans, expressed as the γ of "pure" trypsin inhibited per mg. protein in the crude extract, are recorded in Table IV. The inhibitor activity of the purified Kunitz inhibitor is included for comparison.

Table IV shows that the several varieties of soybeans which were tested can be arranged in the same order of increased amount of trypsin inhibitor activity irrespective of which method of assay was employed. It is important to note, however, that the amount of trypsin inhibited with BAPA as the substrate was consistently higher (about 25 to 30%) than the value obtained with casein as the substrate. This difference was also noted to a lesser extent with the purified Kunitz inhibitor. Although 1.02 mg. of trypsin was inhibited by 1 mg. of the inhibitor when assayed on BAPA, only 0.89 mg. was inhibited when casein was employed as the substrate⁶. In their review of trypsin inhibitors, Laskowski and Laskowski (11) point out that other investigators have likewise observed that the inhibitor activity

⁶Assuming molecular weights of 23,800 (ref. 16) and 21,700 (ref. 10) for trypsin and the Kunitz inhibition respectively, 1 mg. of the latter should theoretically inhibit 1.10 mg. of trypsin.

of the soybean trypsin inhibitor was always higher on synthetic substrates than on natural protein substrates. In a more recent review, Fritz et al. (17) note that the proteinase inhibitors from mammalian tissues exhibit a similar phenomenon which they are inclined to attribute to an interference with the formation of the enzyme inhibitor complex as a result of the steric hindrance caused by the larger molecules of natural substrates. If this effect is also true for the soybean inhibitors, then the BAPA method should provide a more accurate means of assessing the true inhibitor content of soybeans. This consideration, plus the simplicity of the procedure itself, has prompted us to adopt the BAPA method for the routine determination of the trypsin inhibitor activity of soybean samples.

Acknowledgment

The authors wish to thank Dr. Jean Lambert of the Department of Agronomy and Plant Genetics for providing the samples of soybeans used in this study.

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[Received November 4, 1968. Accepted March 12, 1969]