Trypsin Inhibitors in Soy Products: Modification of the Standard Analytical Procedure

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ABSTRACT

The standard AACC method for determining trypsin inhibitor in soy products, based on the tryptic hydrolysis of a synthetic substrate, benzoyl-DL-arginine-*p*-nitroanalide hydrochloride, produced data in the nonlinear portion of the absorbance-sample size curve (above 60% inhibition of trypsin). These values, when converted to trypsin inhibitor units per ml and extrapolated to zero concentration, tended to give erroneously high values for trypsin inhibitor content. To obviate these problems, the trypsin inhibitor was determined from a single dilution of a sample extract that inhibited at least 40 but no more than 60% of the trypsin. The inhibitor content was calculated from the differential absorbance readings and reported in "pure" or absolute units as milligrams of trypsin inhibitor per gram of sample. Values obtained by the modified procedure, although approximately 20% lower than those obtained by the standard method, were considered a more accurate representation of the trypsin inhibitor actually contained in the sample. In addition, based on 18 replicate sets of data on the same soy sample, the modified procedure was more reproducible, giving a standard deviation of ± 2.4 units vs ± 4.2 units for the standard method.

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The standard AACC method for determining the trypsin inhibitor (TI) content of soy products (AACC 71-10) is based on use of a synthetic substrate, benzoyl-DL-arginine-*p*-nitroanalide hydrochloride (BAPA). This method evolved, primarily, from the work of Kakade et al (1969, 1974), who evaluated and compared the synthetic substrate, first introduced by Erlanger et al (1961), with a natural substrate, casein, which previously had been the generally accepted standard method (Kunitz 1947). Kakade concluded that the synthetic substrate was the more convenient and reliable method of assaying TI content of soy products, provided the competitive nature of the inhibition was taken into consideration. This was accomplished by extrapolating the TI activity, expressed in arbitrary trypsin inhibitor units (TIU) per milliliter of extract, to zero concentration of the inhibitor. Although this method of sampling and technique of data interpretation were shown to have good reproducibility among collaborators using identical samples (Kakade et al 1974), concern that the extrapolation procedure could lead to erroneous values for trypsin inhibition content of soy products has been widespread[2] (Egbert et al 1975). Laboratory observations and a review of the literature show that the standard extrapolation method of data interpretation uses data that are not in the region in which zero order kinetics are followed. Accordingly, this report presents a detailed analysis of the method currently used for data collection and interpretation and gives data to support the procedural changes being recommended.

[1] Mention of firm names or trade products does not imply endorsement or recommendation by the USDA over other firms or similar products not mentioned.
[2] Private communications.

MATERIALS AND METHODS

Tris-Buffer

Tris(hydroxymethyl) aminomethane (1.21 g) and 0.59 g of $CaCl_2 \cdot 2H_2O$ were dissolved in 180 ml of distilled water. The pH was adjusted to 8.2 with 1 *N* HCl (10–15 drops) and made up to 200 ml with distilled water. This solution, prewarmed to 37° C for BAPA formulation, was stable up to 8 hr.

BAPA

BAPA (0.080 g) was dissolved in 2 ml of dimethyl sulfoxide and diluted to 200 ml with tris buffer. The solution was stable up to 4 hr.

Trypsin Solution

Trypsin (0.0040 g) was weighed into a 200-ml volumetric flask and diluted to 200 ml with 0.001 *N* HCl. Although the solution may be stored for as long as a month at 5–10° C, a fresh solution was made with each run.

Sample Extract

A soy sample (1.00 g) was extracted with 50 ml of 0.01 *N* NaOH (the pH adjusted, when required, to 8.4–10.0) for 3 hr. Stirring sufficient to keep the sample in suspension was maintained. This suspension was then diluted so that 2 ml of the sample extract inhibited 40–60% of the trypsin used as a standard in the analysis. The appropriate dilutions were determined from either a preknowledge of the heat treatment of the sample or from a urease analysis, which is a reflection of the heat treatment of the soy product. If the value obtained did not fall within the specified range of inhibition, the analysis was repeated with the correct dilution.

Procedure

To each of four test tubes, 2-ml aliquots of the diluted sample extract were added with a wide-tip pipette. A fifth tube was prepared for the trypsin standard by adding 2 ml of distilled water.

To three of the four tubes containing the sample extract and the tube containing distilled water, 2 ml of the trypsin solution was added, and the tubes were placed in a constant temperature bath (37° C) for 10 min. Five milliliters of BAPA solution (prewarmed to 37° C) was rapidly blown into each tube. The contents were stirred immediately on a vortex mixer, and the tubes were replaced in the constant temperature bath. The reaction was terminated *exactly* 10 min later by blowing in 1 ml of 30% acetic acid with immediate mixing with a vortex mixer. A sample blank (the fourth tube containing sample extract) was prepared by the same procedure except that the trypsin solution was added *after* the reaction was terminated by the addition of acetic acid.

The absorbance of each solution was determined at 410 nm against the sample blank. Values obtained from each of the three sample extracts were subtracted from the trypsin standard. These values were averaged, and the trypsin inhibitor content was determined from the following relationship:

$$\text{TI, mg/g of sample} = \frac{\text{differential absorbance}}{0.019 \times 1{,}000} \times \text{dilution factor} \quad (1)$$

RESULTS AND DISCUSSION

Typical absorbance data obtained by the standard method, AACC 71-10, is shown in Fig. 1 (curve AB). Rather than using only the four specified aliquots of sample extract (0.6, 1.0, 1.4, and 1.8 ml), aliquots were taken at 0.1-ml increments up to a maximum diluted sample extract size of 2.0 ml. One gram of soy was diluted so that 1 ml of sample contained 600 μg (dilution factor, 1,667).

The plot of sample size vs the differential absorbance (standard–sample) at 410 nm yields a linear relationship up to approximately 60% inhibition of the trypsin present. Beyond this point (at approximately 0.72 ml of sample extract in this particular set of data), the TI activity deviates from linearity, attributable to the partial dissociation of the TI complex (Green 1953).

To obtain an expression of TI activity, the differential absorbance readings are first converted to TIU; one such unit is defined as that giving an increase of 0.01 in absorbance. These values are then divided by aliquot size to yield TIU per milliliter for each sample extract used in the analysis, and the data are extrapolated (line CD) to zero concentration (point C). The TI activity is expressed in TIU per gram of sample and is obtained from the following relationship:

$$\text{TIU/g} = \frac{\text{Y intercept (TIU/ml)} \times \text{dilution factor}}{\text{sample weight, g}}$$

If only the TIU per milliliter data points specified in the standard method are considered in Fig. 1, the extrapolation procedure would appear to be a logical and appropriate method for determining TI activity from absorbance data. However, several problems are encountered when one attempts to use the extrapolation procedure. Depending on how much of the trypsin is inhibited by each aliquot of the diluted sample extracts used in the analysis, wide variation can be found in the slope of the extrapolated line (if generation of such a line is even possible). In the example shown in Fig. 1, the sample extract was diluted so that the 0.6-ml aliquot would inhibit approximately 50% of the trypsin present. Thus, the remaining sample aliquots (1.0, 1.4, and 1.8 ml) produce differential absorbance readings that are in the nonlinear or upper portion of the absorbance "curve." When these values are converted to TIU per milliliter, data points are obtained that are more amenable to the extrapolation procedure. If the sample extract is diluted so that a lower range of inhibition of trypsin is obtained (TIU per milliliter data points shifted toward the y axis), the data generated probably cannot be effectively interpreted by the extrapolation procedure. Below approximately 50% inhibition, TIU per milliliter data points are widely scattered, which seriously diminishes the validity of the extrapolation procedure. Two factors contribute to the scattering of data. First, obtaining a uniform and representative sampling is difficult when small sample aliquots are used; secondly, any experimental error in absorbance values is increasingly magnified as the sample size is diminished. Thus, with a 0.1-ml aliquot, a change in absorbance of 0.010 units will result in a change of 10 TIU/ml, whereas with a 1 ml aliquot, a change of 0.010 in absorbance units will change TIU per milliliter by only one unit.

Although the sample uniformity and the disproportionate effect that the small sample sizes have on TIU per milliliter make difficult the achievement of any degree of reproducibility when using the standard method, they were not the primary consideration in our decision to use another method of data analysis. The data show that the relationship between sample size and differential absorbance is linear up to approximately 60% inhibition of the trypsin present in the standard solution. This is also supported by others[3] (Kakade et al 1969, 1974). If this portion of the absorbance curve is linear, then the TIU per milliliter, when plotted against sample size, should have 0 slope (line EF) at levels of inhibition below 60%. If the values

[3]Private communications.

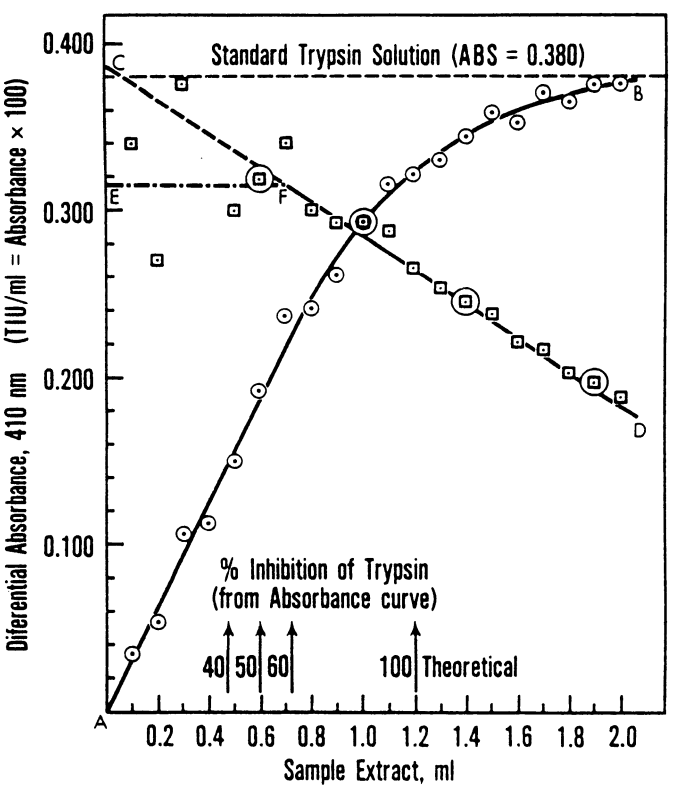

Fig. 1. Typical absorbance data obtained from raw soy samples with benzoyl-DL-arginine-*p*-nitroanalide hydrochloride as substrate. O = Differential absorbance values (standard–sample), ⊡ = trypsin inhibitor units (TIU) per milliliter calculated from absorbance values, ⊡ = sample sizes specified in AACC method 71:10 to be used for extrapolation to zero concentration. **AB,** differential absorbance "curve"; **CD,** extrapolation of TIU/ml to zero concentration (**C**); **EF,** theoretical 0-slope line of sample size vs TIU per milliliter.

are taken from the nonlinear portion of the "curve," as illustrated, the TIU per milliliter line (CD) will have a negative slope and could give erroneously high TI values.

The data presented in Fig. 2 illustrate both the disproportionate effect that the smaller sample sizes have on TIU per milliliter and the linear relationship in the absorbance "curve" (AB). These data were obtained by analyzing triplicate samples taken at 0.1-ml increments from 0.2 to 1.0 ml. The average as well as the high and low values in each set were plotted against absorbance. Each of these values was then converted to TIU per milliliter and plotted against sample size. Although, for the reasons cited, reliable data is difficult to obtain at the lower levels of inhibition, the data points for TIU per milliliter produce an excellent fit for the theorethical 0-slope line (DE), which intersects the y axis at 29.5 TIU/ml. Linear regression analysis of the data points from 0.2 through 0.8 ml produces a line nearly identical with the theoretical line. It has a slope of −0.18 and intersects the y axis at 29.5 TIU/ml.

Procedural Modifications

In view of the difficulty we had in obtaining reproducible data with the extrapolation procedure and because of questions raised by others in the field, we used a method of interpretation based directly on the differential absorbance readings obtained within a specified range of inhibition. Because 1 μg of pure trypsin has been shown to have an activity equivalent to 0.019 absorbance units (Kakade et al 1969), the amount of TI present in a sample can be determined from the relationship:

$$\text{TI, mg/g of sample} = \frac{A_{std} - A_{sam}}{0.019 \times \text{sample wt., g}} \times \frac{\text{dilution factor}}{1{,}000 \times \text{sample size, ml}} \quad (3)$$

If a l-g (db) soy sample is used, as is routine, then the equation can be simplified to:

$$\text{TI, mg/g of sample} = \frac{A_{std} - A_{sam}}{19} \times \text{dilution factor} \quad (4)$$

Because the TI activity is calculated in terms of "pure" trypsin, the purity of the trypsin enzyme used in these analyses is not critical in respect to TI calculations. The only restriction is that the standard trypsin solution (containing no soy) should not produce an absorbance at 410 nm that exceeds 0.450 absorbance. Beyond approximately 0.450 absorbance, the relationship between trypsin and absorbance deviates from Beers Law.

Implementation of the modified method requires only minimal changes in the standard AACC method. Instead of the four dilutions specified, only one sample dilution is used, one that will yield a level of trypsin inhibition in the 40–60% range. The lower level of 40% has been arbitrarily selected simply to minimize the influence that small changes in absorbance obtained at the lower levels of inhibition will have on the final TI value. Although only a single dilution is required, replication of the analysis improves both accuracy and reproducibility. If no preknowledge exists as to the approximate amount of TI contained in the sample, an estimate of the TI activity can be obtained from a simple urease analysis (AACC method 22-90). The destruction of urease approximately parallels destruction of TI in cooked soy samples. The amount of TI in the sample, expressed as milligram of TI per gram of sample is determined from equation 4.

Comparison of Methods

The absorbance and TIU per milliliter data presented in Fig. 1 has been replotted in Fig. 3 to illustrate the difference in the two methods of data interpretation. When the specified differential

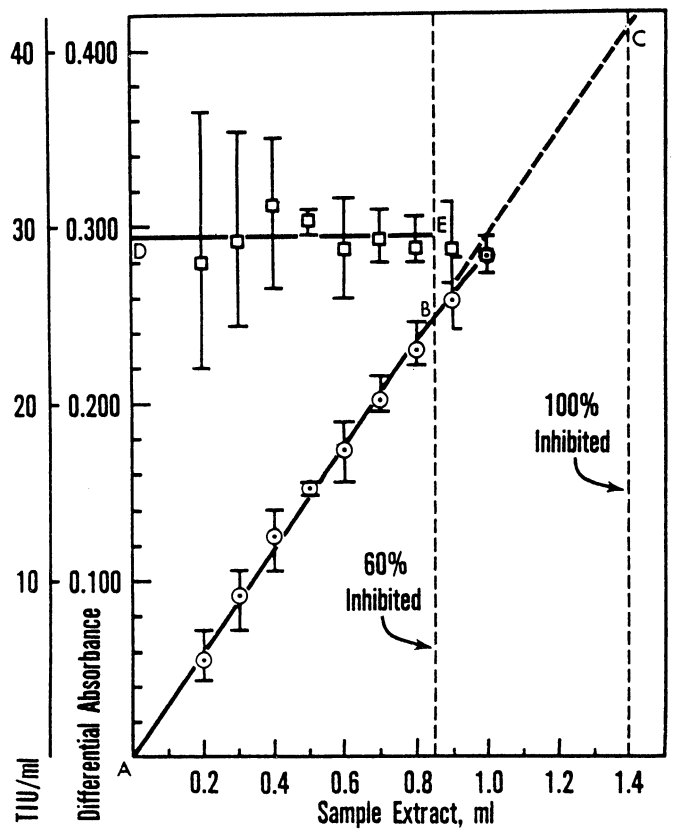

Fig. 2. Disproportionate effect that small sample sizes have in conversion of absorbance data to trypsin inhibitor units (TIU) per milliliter. ⊙ = Differential absorbance values (high, low, and average), ⊡ = TIU per milliliter calculated from absorbance values (high, low, and average). **AB,** differential absorbance "curve"; **BC,** extension of linear portion of absorbance curve to 100% inhibition (**C**); **DE,** theoretical 0-slope line of sample size vs TIU per milliliter.

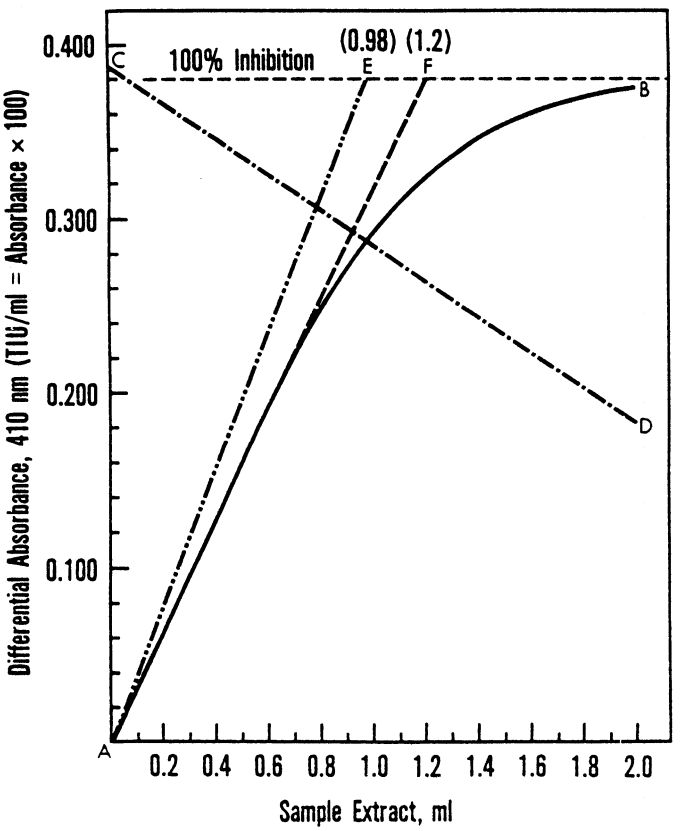

Fig. 3. Quantity of soy extract required to inhibit equivalent amounts of trypsin as determined from two methods of data interpretation, AACC method 71-10 (point E) and modified procedure (point F). **AB,** differential absorbance "curve"; **AE,** line derived from extrapolated tripsin inhibitor units (TIU) data; **AF,** extension of linear portion of absorbance "curve"; **CD,** extrapolation of TIU per milliliter to zero concentration (**C**).

absorbance data are converted to TIU per milliliter and extrapolated to zero concentration (line CD), a TI activity of 38.9 TIU/ml (point C) or 34.1 mg of TI per gram of sample is obtained. By the modified procedure, the amount of TI can be obtained directly from any point on the linear portion, or projection of the linear portion, of the absorbance curve. Based on a soy concentration of 600 μg per ml (dilution factor, 1,667), a value of 27.8 mg of TI per gram of sample is obtained from equation 3. This value is 6.2 mg lower than that of the extrapolation procedure. Depending on which procedure is used, the theoretical relationship between TI contained in the sample and absorbance is represented by either line AE (derived from the extrapolated TIU per milliliter data) or line AF, which is merely an extension of the linear portion of the absorbance curve. In this particular set of data, the standard trypsin solution produces an absorbance of 0.380, which, by definition, is also the value that will be obtained when 100% of the trypsin present is inhibited by the soy sample. Because 1 ml of the diluted soy extract yields a value of 38.9 TU by extrapolation (0 concentration), then 100% inhibition will, theoretically, be obtained at 0.98 ml of diluted extract (point E). With the single point determination, the amount of TI theoretically required to inhibit all the trypsin is valued with 1.2 ml of the diluted extract (point F).

Before deciding on the procedural changes in TI analysis, we obtained 18 replicate sets of data on a uniform soy sample. A control sample had been routinely included whenever the TI analysis was performed. These data have been interpreted by both methods. By extrapolation, the average amount of TI contained in the soy sample was calculated to be 34.0 mg of TI per gram of sample with a standard deviation of ± 4.2. With the standard extrapolation method, even though a good fit was observed in all sets of data between the TIU per milliliter data points and a line extrapolated to zero concentration, the reproducibility was not as good as that obtained from the single level determination. Using only the absorbance data from the one aliquot of diluted sample extract in each set that inhibited at least 40 but no more than 60% of the trypsin, the average amount of TI contained in the sample was calculated to be 28.2 mg of TI per gram of sample with a standard deviation of ± 2.4.

CONCLUSIONS

In theory, the basic BAPA method for determination of the TI activity in soy products was not appreciably altered. Modifications were concerned primarily with the method used for interpretation of experimental data. Problems inherent in the specified extrapolation procedure of data intrepretation can be minimized by use of a single-level TI analysis in which the TI activity falls within specified limits (40–60%). In a study of 18 replicate sets of data, the single level determination was not only more reproducible but also produced values for TI activity that are considered to be more accurate. In both routine and specially controlled analysis, the TI activity as determined by the standard method was approximately 20% higher than that obtained by the modified procedure.

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