Measuring Trypsin Inhibitor in Soy Meal: Suggested Improvements in the Standard Method

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ABSTRACT

The parameters involved in the Trypsin Inhibitor Assay (AACC method 71-10) were examined. The peak absorbance of the difference spectrum is at 385 nm rather than 410 nm as presently used; absorbance is 40% greater at the lower wavelength. The formation of product during the incubation of *N*-benzoyl-DL-arginine *p*-nitroanilide was not linear with time. The effect this has on the numbers derived from the analysis was explored, and it is shown that the inhibitor concentration in a soy extract is underestimated by about 5%. A better method of treating the data derived from the assay is given, namely making a direct linear fit to the absorbance found in the presence of varying amounts of inhibitor. Michaelis-Menten parameters for the reaction of porcine trypsin with substrate at 25°C, pH 8.2, 10 m*M* $CaCl_2$ are determined. For the reaction with the L-isomer (substrate) $K_M = 2.20$ m*M* and $k_{cat} = 8.92$ sec^{-1}. The D-isomer is a competitive inhibitor with $K_i = 2.33$ m*M*. Methods for expressing the amount of trypsin used per assay tube in SI units by performing a titration assay on stock trypsin and by a rate assay using *N*-benzoyl-L-arginine ethyl ester are presented. Certain modifications in the present standard method of trypsin inhibitor assay are suggested that allow determination of inhibitor concentration in soy samples in SI units (moles per gram) rather than the present arbitrary TI units.

Measuring trypsin inhibitor, a major antinutritional factor in soy meal, is of importance to soy processors who are concerned with providing a high-quality product for animal feed. At this time the standard method uses *N*-benzoyl-DL-arginine *p*-nitroanilide as a chromogenic substrate for trypsin, and the ability of aliquots of soy meal extract to inhibit the activity of trypsin towards this substrate is utilized to estimate the amount of trypsin inhibitor in a soy meal sample (AACC 1983; Kakade et al 1969, 1974). The amount of *p*-nitroaniline formed during a 10-min incubation is measured spectrophotometrically, and the absorbance values in the presence and absence of soy extract are used in calculations that give a number for trypsin inhibitor units (TIU) per gram of original soy sample.

The calculations used to derive TIU from absorbance measurements have been questioned (Hamerstrand et al 1981). They are somewhat convoluted, requiring more numerical manipulation than seems necessary. Furthermore, each assay requires several trial dilutions, attempting to arrive at a dilution such that the standard aliquot of 2 ml will give 40–60% inhibition of trypsin. Finally, the definition of one TIU is arbitrary, being the amount of inhibitor that produces a decrease of 0.01 in absorbance.

In this paper I will discuss some of the theoretical and actual shortcomings in the present standard assay method, show how the experimental data can be treated in a theoretically sound manner, and indicate how to calculate the concentration of trypsin inhibitor in SI units, i.e., moles per gram of sample.

MATERIALS AND METHODS

Materials

N-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPA), *N*-benzoyl-L-arginine *p*-nitroanilide hydrochloride (B-L-APA), *N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester hydrochloride (Z-Lys-pNP), *N*-benzoyl-L-arginine ethyl ester hydrocholoride (BAEE), dimethylsulfoxide (DMSO), glacial acetic acid (HOAc), and crystallized porcine trypsin were all obtained from Sigma Chemical Co. Citric acid, tris-(hydroxymethyl)-aminomethane (Tris), 1*N* HCl, 1*N* NaOH, and $CaCl_2$ were purchased from Fisher Scientific. Enzyme-active defatted soy flour was obtained from ADM Company.

Buffers and stock solutions. A stock solution of 1*M* Tris-HCl, 0.2*M* $CaCl_2$ buffer contained 12.1 g of Tris, 2.9 g of $CaCl_2 \cdot 2H_2O$, and 37 ml of 1*N* HCl in 100 ml total volume. A 20-fold dilution of this stock had a pH of 8.2, 0.05*M* Tris-HCl, 0.01*M* Ca^{++}, and was used for all the enzyme assays to be described. For the titration assay of trypsin, the buffer was 0.05*M* citric acid, adjusted to pH 3.0 with 1*N* NaOH. BAPA stock solution was 40.0 mg dissolved in 1.00 ml of DMSO, 92 m*M* BAPA. For B-L-APA, the stock solution was 20.0 mg in 1.00 ml of DMSO, 46 m*M*. The stock solution of trypsin titration substrate Z-Lys-pNP was 60 m*M*, 26.3 mg in 1.00 ml of DMSO. BAEE stock solution was 150 m*M*, 51.4 mg in 1.00 ml of DMSO.

Trypsin stock solution. The stock solution for the titration assay was 50 mg trypsin per milliliter in 0.001*N* HCl or in 0.05*M* citrate buffer pH 3.0. Dilutions for measuring absorbance at 280 nm and for enzyme assays were made in 0.001*N* HCl.

Soy extract. To 0.2 g enzyme-active defatted soy flour was added 9.9 ml of water and 0.1 ml 1*N* NaOH. After 3 hr with intermittent stirring, 0.1 ml of the suspension was added to 9.9 ml of pH 8.2 buffer and filtered through a 0.45-μm filter.

Enzyme Initial Rate Measurements

Rate measurements were made in a total volume of 3 ml at 25°C in a double-beam Spectronic 2000 spectrophotometer. For each run, 8–10 absorbances were recorded. Because the rate of product formation slows down during the run due to substrate depletion, a program written in QuickBasic for a personal computer was used to obtain the initial rate. After entering the data pairs, a quadratic least squares fit was made of Abs as a function of t, and Abs at zero time was found. Each Abs was corrected for this blank value, the data pairs (Abs_{corr}/t, t) were calculated, and a linear least squares fit was made to find the extrapolated value of Abs_{corr}/t at time zero (Stauffer 1989). These initial rates, in terms of absorbance units per minute, were later converted to rates in millimoles per liter per minute by dividing by the appropriate ϵ for the substrate in question.

Standard Trypsin Inhibitor Assay

The assay was run according to the standard method, with two slight exceptions. The diluted soy extract was filtered before the runs were made, thus obviating the need for filtering each sample after stopping the reaction with 30% aqueous HOAc (Liu and Markakis 1989b). The volumes used were half that specified in the standard method. The blank tube (HOAc added before enzyme) was placed in the reference beam of the spectrophotometer and all other readings were made relative to it.

Computer Simulation of Enzyme Reaction Progress

The formation of product during the course of the enzyme-catalyzed reaction can be readily modeled with a program for the personal computer that carries out a numerical integration of the Michaelis-Menten equation: $\Delta[P]/\Delta t = V[S]/(K + [S])$.

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Input from the keyboard consists of K, V, initial $[S]$, the size of Δt, how frequently to print out the current value of $[P]$, and how long to run the program. Typical inputs for a run in this work might have $V = 0.07$, $K = 2.44$, $[S] = 0.257$, $\Delta t = 0.01$, print out every 100 cycles and run until $t = 10$. The calculations are: $\Delta[P] = \Delta t V[S]/(K + [S])$; $[P] = [P] + \Delta[P]$; $[S] = [S] - \Delta[P]$; $t = t + \Delta t$; and the cycle is repeated.

RESULTS AND DISCUSSION

The Reaction of Trypsin with BAPA

Wavelength of measurement. In their original publication regarding the use of BAPA as a chromogenic substrate for trypsin, Erlanger et al (1961) recommended measuring the absorbance of *p*-nitroaniline at 410 nm and gave a value for $\epsilon = 8.8$ mM^{-1} cm^{-1}. The product *p*-nitroaniline has an absorbance peak at 380 nm ($\epsilon = 13.5$ mM^{-1} cm^{-1}), but the substrate anilide also absorbs modestly at this wavelength, hence the authors recommended reading at a wavelength at which BAPA is transparent. In re-examining the product of complete hydrolysis of BAPA by trypsin, I found that the peak of the difference spectrum (the reference cuvette contained 0.20 mM BAPA; the sample beam contained 0.10 mM *N*-benzoyl-D-arginine *p*-nitroanilide, 0.10 mM *p*-nitroaniline, and 0.10 mM *N*-benzoyl-L-arginine) occurs at 385 nm with a value of $\epsilon = 11.1$ mM^{-1} cm^{-1} (Fig. 1). In comparing absorbances of several samples during the course of this work, I found that A_{385} is 40% higher than A_{410}. This point is not insignificant. A spectrophotometer error of 1 nm at 385 nm introduces a relative error of less than 0.1% (0.001/1.114), whereas a similar instrumental error at 410 nm gives a relative error of more than 3.0% (0.025/0.788). Reading absorbance of product from hydrolysis of BAPA at 385 nm increases sensitivity by 40% and markedly decreases spectrophotometer error in the method.

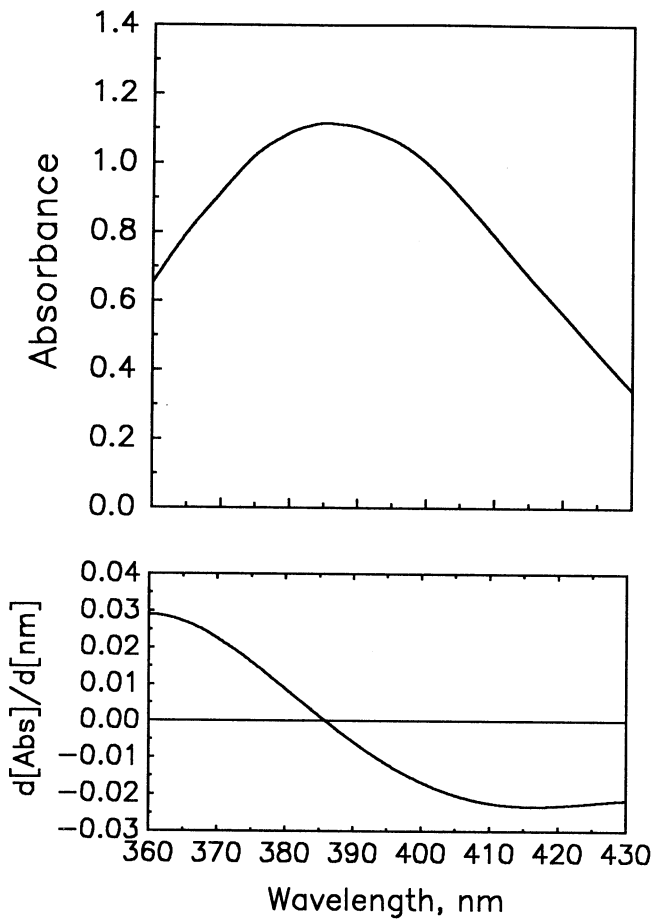

Fig. 1. Difference absorption spectrum of BAPA and enzyme-hydrolyzed product. Reference cell contained 0.2 mM *N*-benzoyl-DL-arginine *p*-nitroanilide. Sample cell contained 0.1 mM each *N*-benzoyl-D-arginine *p*-nitroanilide, *N*-benzoyl-L-arginine, and *p*-nitroaniline (0.2 mM BAPA reacted with trypsin until hydrolysis was complete). Both solutions were in pH 8.2 buffer, 0.05M Tris-HCl, 0.01M $CaCl_2$.

Michaelis-Menten parameters. The D-isomer of BAPA is a competitive inhibitor of trypsin (Erlanger et al 1961). In considering the kinetics of the reaction, it is important to correct for this factor, but the correction requires a knowledge of K_i for the D-isomer as well as the true K_M for the L-isomer substrate. To determine these two values as well as to obtain a value for k_{cat} for the reaction, I measured the initial rate of hydrolysis at 25°C in 0.05M Tris buffer, pH 8.2, 0.01M in Ca^{++} at several concentrations of BAPA and also of B-L-APA and used the HYPER computer program (Cleland 1970, 1979; Stauffer 1989) to estimate V_{app} (maximum rate) and K_{app} (substrate concentration at half that rate) for each substrate (Fig. 2).

In considering the significance of the equation parameters, it is important to remember that in the measurements with BAPA, as substrate concentration increases so does inhibitor concentration (the reaction with B-L-APA does not have this complication). Since the inhibition by D-isomer is competitive, and in this case $[I] = [S_0]$ in all runs, we have:

$$v = \frac{V_{max}[S_0]}{K_M(1 + [I]/K_i) + [S_0]} = \frac{V_{max}[S_0]}{K_M + K_M[S_0]/K_i + [S_0]} \quad (1)$$

$$v = \frac{V_{max}[S_0]}{K_M + [S_0](1 + K_M/K_i)} = \frac{V_{app}[S_0]}{K_{app} + [S_0]} \quad (2)$$

where $V_{app} = V_{max}/(1 + K_M/K_i)$ and $K_{app} = K_M/(1 + K_M/K_i)$.

Treating the data in this fashion, it is not necessary to use the isolated D-isomer of BAPA as an inhibitor to find K_i as was done by Erlanger et al (1961).

For the experiment with B-L-APA $V_{max} = 73.3$ μM min^{-1} (standard error [SE] 8.7) and $K_M = 2.20$ mM (SE 0.39). From the run with BAPA, $V_{app} = 37.1$ μM min^{-1} (SE 2.5) and $K_{app} = 1.15$ mM (SE 0.15). From the ratios

$$V_{max}/V_{app} = 1 + K_M/K_i = 1.976;\ K_i = 2.41$$

and

$$K_M/K_{app} = 1 + K_M/K_i = 1.913;\ K_i = 2.25$$

I found a value for K_i of 2.33 mM at 25°C, pH 8.2. In the standard method assay $[I] = [S_0] = 0.257$ mM. K_{app} in this assay equals $K_M(1 + 0.257/2.33) = 2.44$ mM, calculated at 25°C. Grant and Hermon-Taylor (1979) found 2.65 mM for K_{app} using BAPA.

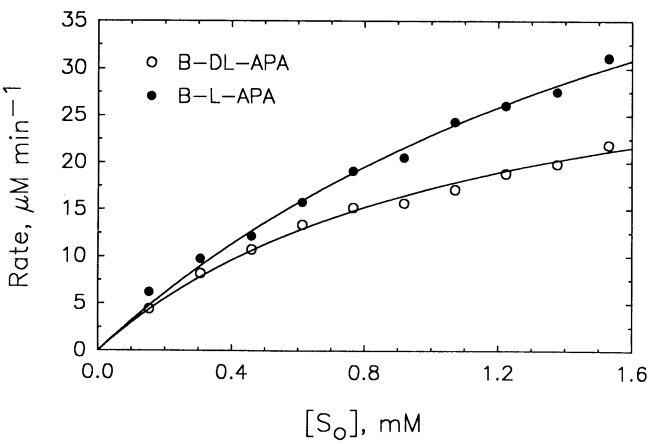

Fig. 2. Initial rates of hydrolysis of *N*-benzoyl-DL-arginine *p*-nitroanilide (B-DL-APA) and *N*-benzoyl-L-arginine *p*-nitroanilide (B-L-APA) by 0.21 μM porcine trypsin at 25°C, 0.05M Tris-HCl buffer pH 8.2, 10 mM in $CaCl_2$.

Erlanger et al (1961) reported K_M = 0.94 mM on B-L-APA and K_i = 0.80 mM for the D-isomer, both results at 15°C. Using an activation energy of 8,800 cal (Harmon and Niemann 1949) for the binding constants, these values at 25°C are 1.55 and 1.32 mM, respectively.

In these runs $[E_0] = 0.137\ \mu M$, so from $V_{max} = k_{cat}[E_0]$ I calculate k_{cat} = 535 min^{-1}, or 8.92 sec^{-1}. Grant and Hermon-Taylor (1979) reported k_{cat} of 2.72 sec^{-1} for the DL racemate, which multiplied by the factor 1.976 equals 5.37 sec^{-1}. Erlanger et al (1961) found a value of 0.611 at 15°C. Using 11,200 cal for the activation energy E_a (Schwert and Eisenberg 1949), we can calculate this value to be 2.45 sec^{-1}; the authors assumed the trypsin they were using was 100% active, but a more likely estimate of 50% activity would make their value for $k_{cat} \approx 4.9\ sec^{-1}$.

TABLE I
Formation of Product from BAPA as a Function of Time of Reaction

Time (min)	A_{385}	Product Concentration (μM)	
		Experimental	Calculated
1	0.043	3.8	3.8
2	0.082	7.4	7.5
3	0.124	11.2	11.2
4	0.163	14.7	14.8
5	0.205	18.5	18.4
6	0.243	21.9	21.9
7	0.282	25.4	25.4
8	0.320	28.8	28.8
9	0.358	32.3	32.2
10	0.395	35.6	35.6

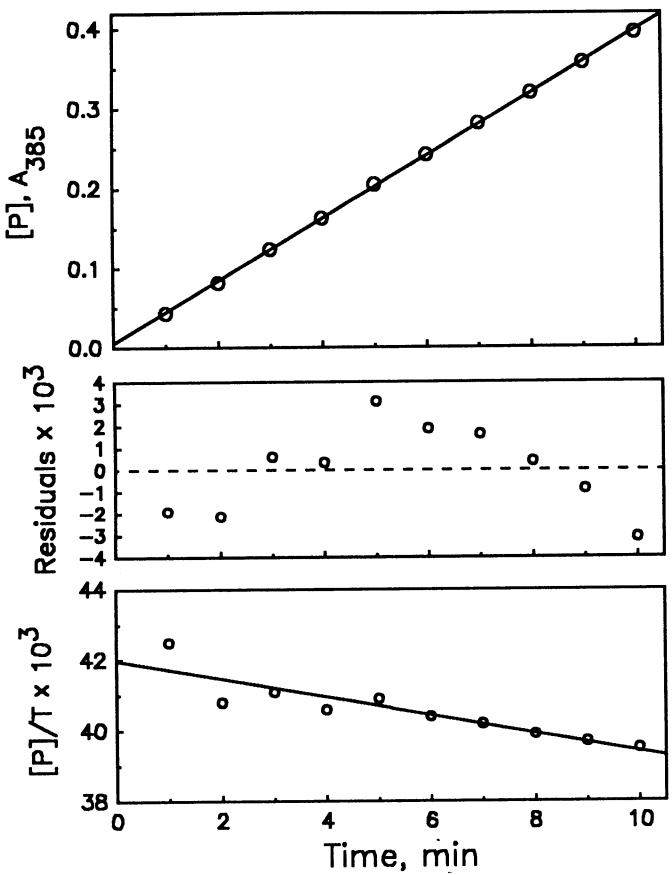

Fig. 3. Analysis of the time course of hydrolysis by trypsin of BAPA at pH 8.2, 25°C showing gradual slowing of the reaction due to substrate depletion. $[S_0]$ = 0.257 mM, $[E]$ = 0.077 μM. The upper frame shows the experimental data with the straight line fitted by linear least squares. The middle frame plots the residuals of each point from the LLS line. The lower frame is a plot of $[P]/t$ (i.e., rate to that time) versus time, showing the decrease in apparent rate.

For the standard assay at 37°C, using the values of E_a of 11.2 kcal for rate and 8.8 kcal for binding, the results from the present work are estimated to be k_{cat} = 1,110 min^{-1}, K_M = 3.83 mM, K_i = 4.06 mM, and K_{app} = 4.07 mM ($[I]$ = 0.257 mM).

Nonlinearity of the enzyme reaction. Because $[S_0]$ is below K_{app}, one would expect the instantaneous rate to decrease during the reaction due to substrate depletion. In fact this is what happens. To 2.95 ml of Tris buffer at 25°C was added 33.3 μl of a stock enzyme ($[E] = 0.244\ \mu M$), and the reaction was started by adding 16.7 μl of BAPA stock in DMSO. The A_{385} was recorded at 1 min intervals for 10 min (Table I). The absorbance values are plotted in the top frame of Figure 3. The line represents the linear least squares fit, and appears to depict a high degree of linearity. Plotting the residuals from the fit (Fig. 3, middle frame) shows a definite "hump,", characteristic of a set of data that is better fit by a quadratic regression. This is made even more apparent when $[P]/t$ is plotted versus time (Fig. 3, bottom frame). If one just measures A_{385} at the end of the reaction, then the "rate" would appear to be 0.0395 absorbance units (AU) per minute, while the actual initial velocity of the reaction is 0.042 AU/min. The overall "rate" is only 94% of the initial rate. In Table I A_{385} is converted to $[P]$ in micromoles per liter, and a numerical integration was done to see if the decrease in $[P]/t$ is strictly due to substrate depletion. Using K = 2.44, $[S_0]$ = 0.257, and V = 0.0402, the values of $[P]$ listed in the table were calculated. The agreement is excellent; no cause (e.g., product inhibition or enzyme denaturation) other than substrate depletion need be invoked. While the point may seem minor, in fact it has implications for the nature of the plot of rate versus amount of trypsin inhibitor in the standard method assay.

Three similar runs were also made incorporating amounts of soy extract that gave approximately 25, 50, and 75% inhibition. In all cases the progress curves could be fit exactly in the same way as the uninhibited curve, changing only V to correspond to the degree of inhibition. The presence of inhibitor does not affect the nature of the decrease in rate due to substrate depletion.

Analysis of Data from High-Affinity Inhibitor Assays

Theory. The mathematical relationships applying to enzyme reactions in the presence of a high-affinity inhibitor of the enzyme have been developed and published (Goldstein 1944, Bieth 1974,

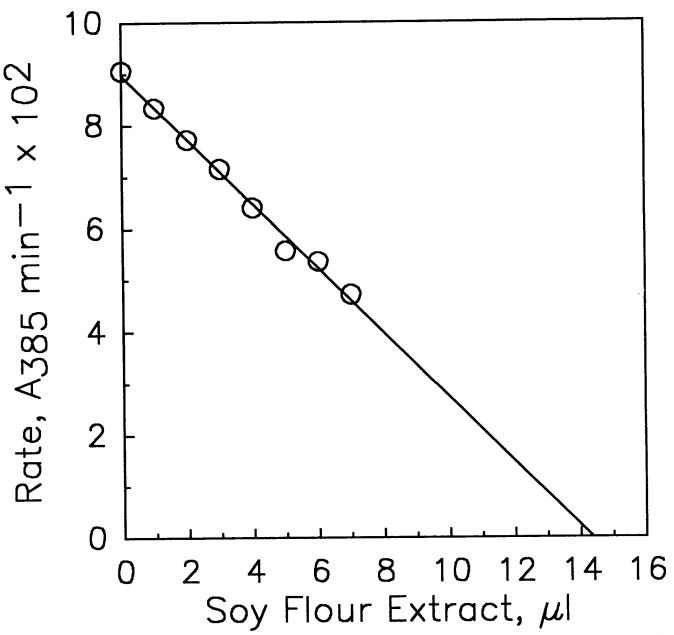

Fig. 4. Inhibition by an extract of soy flour of the initial rate of hydrolysis of BAPA by trypsin at pH 8.2, 0.01M $CaCl_2$, 25°C. Total reaction volume was 3 ml, $[E] = 0.165\ \mu M$, $[S_0] = 0.257$ mM. The initial rate was determined by extrapolating $[P]/t$ versus t to zero time to correct for the decrease in rate due to substrate depletion. The straight line was fitted using linear least squares statistics.

Stauffer 1989). The analysis of data consisting of rates measured in the presence of various amounts of soy extract, as we are considering here, is straightforward. No arbitrary assignments need be made in defining units of trypsin inhibitor activity.

The key point in the analysis is that the total concentration of inhibitor ($[I_t]$) is roughly equal to total concentration of enzyme ($[E_t]$) so that the formation of enzyme-inhibitor complex (EI) significantly depletes the inhibitor concentration. Denoting the concentrations of free inhibitor and free enzyme by $[I]$ and $[E]$ respectively, we have:

$$K_i = [E][I]/[EI] = ([E_t] - [EI])([I_t] - [EI])/[EI] \quad (3)$$

$$= [E_t][I_t]/[EI] - [E_t] - [I_t] + [EI] \quad (4)$$

The measured rates are proportional to the amount of free enzyme in the assay tube, and we can take $v_0 = [E_t]$, $v_i = [E]$, and $v_0 - v_i = [EI]$. Substituting these into equation 4 gives:

$$K_i = [I_t]v_0/(v_0 - v_i) - [I_t] - v_0 + (v_0 - v_i) \quad (5)$$

The terms containing $[I_t]$ are gathered, and the last two terms are collected and multiplied by $[E_t]/v_0$ (equals 1) to give:

$$K_i = [I_t][v_i/(v_0 - v_i)] - [E_t]v_i/v_0 \quad (6)$$

Rearranging this gives:

$$v_i = v_0 - [I_t]v_i/(K_i + [E_t]v_i/v_0) \quad (7)$$

$[E_t]$ is usually around $1 \times 10^{-7} M$, and K_i for soy trypsin inhibitors is of the order of $1\text{–}5 \times 10^{-10} M$ (Liener and Kakade 1980) so $[E_t]v_i/v_0 >> K_i$, and we arrive at the final equation:

$$v_i = v_0 - [I_t]v_0/[E_t] \quad (8)$$

A plot of v_i versus $[I_t]$ is a straight line with y-intercept v_0, a slope of $-v_0/[E_t]$, and an x-axis intercept of $[E_t] = [I_t]$. The units for $[I]$ do not affect this plot. If, for example, they were milliliters of (diluted) soy extract, and the amount of enzyme present in each assay tube was expressed in arbitrary assay units, e.g. "BAEE units," then $[I_t]$ would be reported in "BAEE units per milliliter." As will be discussed below, it is just as easy to use absolute terms, picomoles for trypsin and microliters for the amount of undiluted soy extract, calculating the value of $[I_t]$ in the extract in micromolar units. This should improve the reproducibility of the assay between laboratories, in addition to bringing the method in line with accepted SI designation.

Two points should be raised about gathering the data for making this plot. The first relates to choosing the levels of inhibition to use. My experience is that with soy flour extract the plot is linear up to at least 50% inhibition. Hamerstrand et al (1981) reported similar data as did Liu and Markakis (1989b). On the other hand, with other inhibitor sources the plot may become nonlinear at somewhat lower degrees of inhibition (Richardson 1981). Collaborative work to modify the present method would be needed to give better guidance for selecting the points to make the linear least squares (LLS) fit.

The second point is more fundamental; the data are used directly for the LLS fit as was done, for example, by Smith et al (1980). There is no reason to plot the difference $v_0 - v_i$ versus amount of inhibitor as the standard method prescribes. Presumably the experimental error of the inhibitor-free determination is no less than that of each of the assays containing inhibitor. Taking the difference just doubles the error in each point, which is then used to find the concentration $[I]$ (Hamerstrand et al 1981). Rather, the control determination is treated merely as one of the set of data pairs; it should have no more weight in determining the best straight line than any other point. (This point is exemplified in the high-enzyme run shown below.) Also, it should be noted that the x-axis intercept is not measured graphically. Rather, from the parameters of the LLS straight line $y = A(0) + A(1) \times x$, the x-intercept is calculated, xint $= -A(0)/A(1)$.

Initial rate (v_0) measurements. As is apparent in the theoretical development presented above, the rates are considered to be initial velocities, i.e., $d[P]/dt$ when substrate concentration is 0.257 mM. It is quite possible to use methods that assay trypsin inhibitor based on initial rates. Figure 4 shows the results of one such experiment. After placing 0–0.7 ml of soy flour extract (diluted 1:100 in buffer) and Tris buffer to a total of 2.93 ml in the cuvette, 0.05 ml of stock trypsin solution followed by 16.7 μl of stock BAPA in DMSO was added to start the reaction, and the initial rate was determined as described under Methods. The stock trypsin concentration was 9.9 μM, so each reaction cuvette contained 495 pmol of enzyme. From the LLS fit to the data, the x-axis intercept was 14.4 μl, so the absolute concentration of trypsin inhibitor in the original soy extract was $[I_t] = 495/14.4 = 34.4$ μM.

Comparison of v_0 with 10-min assays. How does using the 10-min incubation assay compare with the more theoretically correct initial rate assay? This is discussed on a straight theoretical basis, and also using experimental data. The theoretical comparison is shown in Figure 5. Using 2.44 mM for K and 0.257 mM for $[S_0]$, I found empirically that a value of 0.07 min^{-1} for V would result in conversion of 23% of the substrate to product in 10 min; in the standard method about 19% conversion occurs in the uninhibited assay. I chose an x-axis intercept of 14 μl, so each μl of inhibitor decreases V by 0.005, as indicated by the dotted line in Figure 5. Then I used the numerical integration program to find out how much product was formed in 10 min at each value of V from 0.07 min^{-1} down to 0.04 min^{-1}. These are the circled points in Figure 5. The LLS fit to the calculated points gave an x-axis intercept of 15.07 μl, some 7.6% higher than the theoretical value. The points describe a very slight downward bend (apparent from the residuals of the LLS fit), and if the calculations were extended to lower values of V the value of P would of course become 0 at 14 μl.

The same effect is seen in the data shown in Figure 6 and presented in Table II. The assays were run by the standard method, modified slightly as noted above. For run 1 (squares in Fig. 6) I used somewhat more enzyme than I should have, obtaining 40% conversion of substrate to product in the uninhibited tube. The slope of the fitted line is −0.0438, or 4.38 TIU/μl using

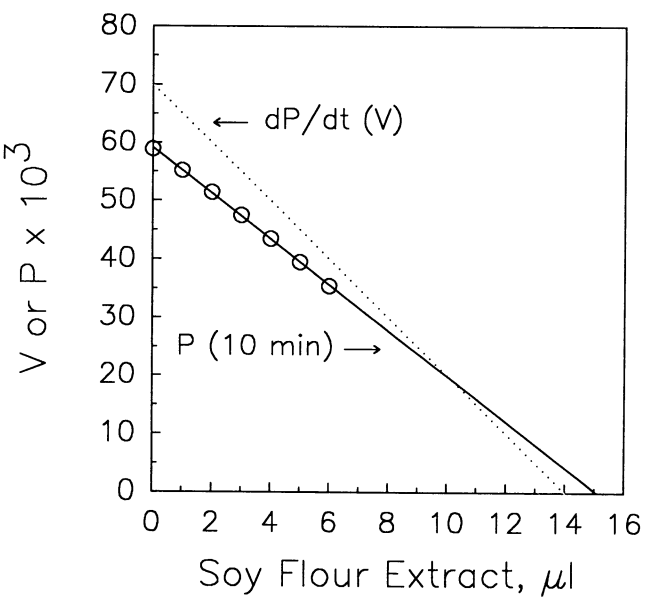

Fig. 5. Theoretical calculations of effect of soy trypsin inhibitor on hydrolysis of BAPA by trypsin, using initial rate (dP/dt) or amount of product formed during 10 min of reaction (P) as the measure of activity. The values of P (circles) were obtained by computer integration of the Michaelis-Menten equation, using initial parameters $K_{app} = 2.44$ mM, $[S_0] = 0.257$ mM, and $V = 0.070$, 0.065, 0.060, etc. min^{-1} to correspond to various degrees of inhibition.

the definition according to the standard method. For run 2 (circles in Fig. 6) I adjusted the amount of enzyme to correspond to that which is recommended by Kakade et al (1974) and had 27% conversion of substrate in 10 min. The slope of the line is −0.0462, 4.62 TIU/μl, 5.5% higher than with the higher level of enzyme. The x-axis intercepts are 23.5 and 15.2 μl, respectively, which, together with the respective amounts of enzyme per assay tube, yields inhibitor concentrations of 26.2 and 26.3 μM. The agreement is much better than that based on line slopes.

From each A_{385} datum it is possible to calculate the initial rate using the integrated rate (Henri) equation:

$$V = \left[[P] + K_{app}\ln\left([S_0]/\{[S_0] - [P]\}\right)\right]/t \qquad (9)$$

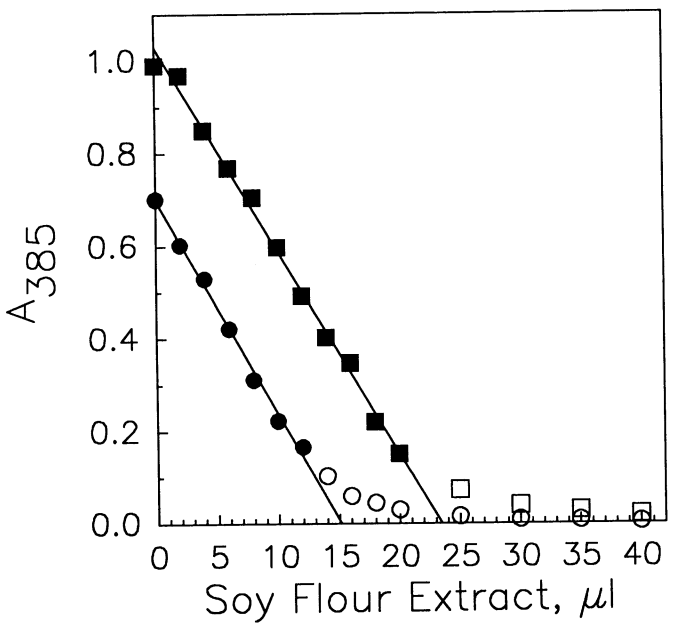

Fig. 6. Standard method assay of trypsin inhibitor activity of a soy flour extract. Volumes of reactants were half that specified in the standard method. Tubes represented by squares each contained 615 pmol of trypsin, while tubes represented by circles each contained 400 pmol of enzyme. Aliquots of soy flour extract plus buffer plus BAPA were attemperated at 37°C, and 0.1 ml of stock trypsin solution was added at zero time. After 10 min incubation, 30% HOAc was added to stop the reaction. Dilutions of soy flour extract were used in setting up the experiments, but the abscissa is given in terms of microliters of initial extract present per assy tube. The straight lines were fitted to the solid data points in each run, using linear least squares statistics.

TABLE II
Standard Method Assay of Soy Trypsin Inhibitor at Two Levels of Added Trypsin

Soy Extract (μl)	High Level of Trypsin		Low Level of Trypsin	
	A_{385}	Calculated v_0	A_{385}	Calculated v_0
0	0.991	1.204	0.702	0.800
2	0.969	1.171	0.604	0.674
4	0.850	1.000	0.530	0.583
6	0.769	0.889	0.422	0.455
8	0.705	0.804	0.312	0.329
10	0.599	0.668	0.223	0.232
12	0.493	0.538	0.167	0.172
14	0.403	0.433	...	...
16	0.348	0.370	...	...
18	0.221	0.229	...	...
20	0.152	0.156	...	...
A_0	1.029	1.227	0.700	0.787
A_1	−0.0438	−0.0549	−0.0462	−0.0540
x, μl	23.5	22.3	15.2	14.6
E_0, pmol	615	615	400	400
[I], μM	26.2	27.6	26.3	27.4

and the Michaelis-Menten equation:

$$v_0 = V[S_0]/(K_{app} + [S_0]) \qquad (10)$$

The initial parameters are $K_{app} = 4.07$ mM (calculated for 37°C), $[S_0] = 0.257$ mM, $t = 10$ min, and $\epsilon = 11.1$ mM^{-1} cm^{-1}. The input is A_{385}, which, divided by ϵ, converts $[P]$ to millimolar units; V is calculated from equation 9; v_0 is calculated from equation 10, and this is multiplied by ϵ to give v_0 in terms of ΔA_{385} min^{-1}. The calculated values are listed in Table II. The parameters for the LLS fits, the x-axis intercepts, and the calculated $[I]$ are also listed. As in Figure 5 note that the x-axis intercepts for the standard method points are higher than the x-axis intercepts for the initial rate data, by 5.1 and 4.1% for the two runs. This means that the calculated inhibitor concentrations are lower in the standard method by the same percentage.

Suggestions for Improving the Trypsin Inhibitor Assay

Determining absolute trypsin concentration. In order to calculate trypsin inhibitor concentration in SI units, the trypsin concentration must also be known in such units. This means that the primary standard assay must be a well-characterized titration assay that gives a result in units such as micromoles per liter. The one usually used for trypsin employs Z-Lys-pNP as the titrating substrate (Bender et al 1965, 1966) in citrate buffer, pH 3. The method is straightforward and can be done with any spectrophotometer that records absorbance at 340 nm. With 1 mM Z-Lys-pNP, the "burst" amounts to 91% of the total amount of active trypsin present (Bender et al 1966), so this correction must be applied.

The crystallized porcine trypsin that I used assayed as being 87% active trypsin by this method, based upon a molecular weight of 23,560 (Sober 1970, p. C-10) and a factor of 1.50 for the absorbance at 280 nm of a 1 mg/ml trypsin solution in a 1-cm path length cuvette (Sober 1970, p. C-91). Actually, for the purpose of assaying trypsin inhibitor, the percentage activity is not needed; only the molar concentration of active enzyme in the stock solution is important. The stock trypsin solution is about 50 mg/ml in 0.001N HCl. Bender et al (1965) recommend making the stock in the pH 3 citrate buffer. I found that such stocks gave an abnormally large "burst," with the calculated "purity" of the trypsin being around 140%. This curious behavior is, as far as I know, unexplained. In 0.001N HCl the enzyme is fairly stable at 5°C, losing less than 10% activity per week and millimolar HCl is the preferred solvent.

During routine work, trypsin concentration can be determined by running an initial rate assay in the normal way. An excellent substrate for this purpose is 0.5 mM BAEE, where one follows the increase in absorbance at 253 nm. The K_M at pH 8.2 is 0.02 mM (Bender et al 1966) or 0.017 mM (Grant and Hermon-Taylor 1979), so the $[S_0] \approx 25 \times K_M$, which means substrate depletion is not a serious factor during the run. At 253 nm $\epsilon = 1.15$ mM^{-1} cm^{-1} (Schwert and Takenaka 1955), so total hydrolysis of substrate would produce an increase of 0.57 in A_{253}. If the change is limited to 0.2 absorbance units, the rate is linear with time as judged by the residuals to a LLS fit. From a large number of runs made at 25°C, pH 8.2, 0.05M Tris-HCL, 0.01M Ca^{++}, I determined k_{cat} as 24.3 ± 1.2 sec^{-1} (1,400 ± 70 min^{-1}). Grant and Hermon-Taylor (1979) report a value of 30.5 sec^{-1} at pH 8.4, 0.01M $CaCl_2$. Calcium ion is a known activator of trypsin, and in its absence Bender et al (1966) found k_{cat} to be 11.5 sec^{-1}. At $[S_0]$ of 0.5 mM $v_0 = 0.96\ k_{cat}[E]$. Including the value of ϵ, one can calculate that $[E]$, $\mu M = 0.644 \times \Delta A_{253}$ min^{-1}.

A more convenient but less desirable substrate from the theoretical standpoint is the substrate solution for the standard method assay, BAPA at $[S_0] = 0.257$. In this case, the rate is not linear with time, and the data are treated as described under Methods to find the initial rate ΔA_{385} min^{-1}. The substrate is 16.7 μl of BAPA stock in DMSO or 1.67 ml of BAPA in buffer as per the standard method plus additional pH 8.2 buffer to a total volume of 2.9 ml. The reaction is initiated by adding 0.1 ml of trypsin. From several runs at 25°C I found that $v_0 = 0.0946$

$k_{cat}[E] = 49.1 \pm 1.0$ min$^{-1}[E]$. Using $\Delta\epsilon = 11.1$ mM^{-1} cm^{-1}, this gives $[E]$, $\mu M = 1.835 \times \Delta A_{385}$ min^{-1}. One can also estimate the enzyme concentration in a standard assay tube from the absorbance of the control. For example, in the two runs shown in Figure 6, in the high level $[E] = 0.137$ μM (615 pmol/4.5 ml) and $A_{385} = 1.029$, whereas in the lower level $[E] = 0.089$ (400 pmol/4.5 ml) and $A_{385} = 0.700$. The correspondence is not exact, but this is a good internal check to make sure that a decimal point hasn't been slipped or an improper dilution made during routine operation.

Summarizing, the titration with Z-Lys-pNP is the primary standard for determining active enzyme concentration. The assay with BAEE is an excellent secondary standard, and the initial rate assay using BAPA is a useful day-to-day standard for monitoring the stability of concentrated stock solution stored in the cold. It is definitely not the case that "the actual molar concentration of enzyme or inhibitor is difficult to determine" (Liu and Markakis 1989b).

Filtration of diluted soy extract. Kakade et al (1974) reported that filtering the soy extract before running the assay resulted in decreased inhibitor concentration. Liu and Markakis (1989b) investigated the extraction of inhibitor and found that 30 min extraction using water or buffer was as effective as the 3 hr extraction using 0.01N NaOH specified in the standard method, and that filtering the extract before beginning the assay did not change the concentration of inhibitor. I made a few preliminary trials of this sort, and my observations are in agreement with their report.

Relationship of TIU to absolute inhibitor concentration. As indicated above (Fig. 6 and Table II), when we know the molar amount of active trypsin used in the assay tube, it is a simple matter to calculate the molar concentration of trypsin inhibitor in the soy flour extract. To calculate the ratio between the trypsin inhibitor unit as defined in the present standard method and the molar concentration of inhibitor in extract, I will refer to Table II as an illustration. One trypsin inhibitor unit equals an amount that will increase by 0.01 the quantity $A_{410,o}-A_{410,i}$. If the soy extract contains 1 TIU per microliter, the slope of the line fitted directly to the data is -0.01, or -0.014 if the absorbance measurement is at 385 nm. The soy flour extract in Table II contained 3.2 TIU/μl, taking the average of the slopes of the two assays that used the standard 10-min incubation. By dividing the picomoles of trypsin per assay tube by the x-intercept, the molar concentration of trypsin inhibitor was 26.24×10^{-12} mol/μl. Thus, at pH 8.2, 0.01M $CaCl_2$, 37°C, 1 TIU $= 8.2 \times 10^{-12}$ mol of trypsin inhibitor.

Three points need to be made about this relationship. First, as long as the molar stoichiometry of the trypsin-inhibitor reaction is 1:1, the actual molecular weight of the inhibitor is irrelevant. One mole of inhibitor reacts with one mole of enzyme whether the molecular weight is 21 kDa (Kunitz inhibitor) or 8 kDa (Bowman-Birk inhibitor). Second, since the incubation assay underestimates inhibitor concentration by about 5%, 1 TIU actually equals 8.6×10^{-12} mol of inhibitor. Third, the definition of TIU is for assay at 37° C and is strongly temperature dependent, whereas the determination of absolute inhibitor concentration from the x-intercept of the LLS fit is independent of temperature. A change in the temperature of the assay will affect the rate of enzyme reaction proportionately the same in all the assay tubes, so the y-intercept and the slope will both change in the same direction but the x-intercept will remain the same, as will the estimation of the molar concentration of inhibitor in the extract being assayed.

Methodology changes recommended. An old, sound adage is "If it ain't broke, don't fix it!" While the present standard method for trypsin inhibitor assay is not "broke," it is apparent that many researchers feel that it could at least use some preventive maintenance, as discussed by Liu and Markakis (1989b). The following points should be taken into account by any collaborative group that may be involved in such an examination of the method with a view to updating it.

1) Make the absorbance readings at 385 nm. While the sensitivity is increased by 40%, this is not the main reason for this recommendation. The overriding consideration ought to be for reproducibility between laboratories as affected by spectrophotometric error. Liu and Markakis (1989b) found a value of ϵ_{410} for *p*-nitroanilide of 7.76 mM^{-1} cm^{-1}. Erlanger et al (1961) reported 8.8 for this value, and in this work I found a value of 7.93. If we assume that the spectrophotometer used by Liu and Markakis was accurately set at 410 nm, then my instrument setting was in truth 409.4 nm, and the one used by Erlanger et al was set at 405.5 nm. Discrepancies of this size are not unrealistic in day-to-day laboratory operation. Put another way, an instrumental error of 5 nm will produce an error of 15% in calculating TIU by the present method. A similar 5 nm error in the 385 nm region will produce a 0.5% error in TIU.

2) Standardize the stock trypsin solution in absolute molarity concentration units. This is not difficult to do. A particular laboratory should initially run a titration assay using Z-Lys-pNP and then make initial rate assays using BAEE to establish their own factor for converting BAEE "units" to trypsin concentration. (I do not recommend using any other reported conversion factor without at least checking it out.) Once that is done, the establishment of trypsin concentration would be a simple routine asay, performed at the start of each day's work.

3) Measure the amount of *p*-nitroanilide formed during a 10-min incubation with trypsin in the presence of several aliquots of soy extract, chosen to give 0–50% inhibition. Fit a straight line to these data using linear least squares methods, calculate the intercept on the x-axis, and use this value plus the known number of moles of trypsin per assay tube to calculate the concentration of trypsin inhibitor in the original soy extract in micromolar units. This procedure has two advantages over the present standard assay, one philosophical and one practical. The first advantage is that the measurements are in SI units, free of arbitrary definitions, and comparable among various approaches to measuring trypsin inhibitor. The practical advantage is that the measurement becomes independent of temperature, thus removing one more source of experimental error between laboratories.

4) Add the enzyme last to initiate the 10-min incubation period as proposed by Liu and Markakis (1989b). Viswantha and Liener (1954) and Liu and Markakis (1989a,b) state that this produces a larger slope to the plot of rate versus amount of soy extract compared with the present method of adding substrate last, while Birk (1967) claims this is not so. Leytus et al (1984) analyzed the kinetics that govern the interaction of enzyme-substrate-inhibitor during the early stages of the reaction, and from that analysis adding the enzyme last should produce less rather than more apparent inhibition. I am presently investigating the transient kinetics of this system and have confirmed their predictions using BAEE but find that with BAPA the pre-steady state deviations are negligible (*unpublished*). In any event, my reason for this recommendation is practical, not theoretical. Trypsin in 0.001N HCl inactivates more rapidly at 37°C than at 5°C. By maintaining the stock trypsin solution at the lower temperture during the day's work and initiating the assay by adding a small aliquot (say, 0.1 ml) to the attemperated solution of inhibitor plus substrate, the amount of enzyme inactivation following the standardization runs at the beginning of the day would be negligible. This minimizes one more source of experimental error.

5) The 10-min incubation assay is much more convenient than initial rate assays when a large number of samples is to be run, so I recommend continuing to use it. For practical purposes, e.g., comparing the effectiveness of various treatments in decreasing trypsin inhibitor in soy meals or for production quality control, this is perfectly adequate. A determination of the exact amount by which this procedure underestimates the true amount of inhibitor present could be the subject of a collaborative study, and the revised official method could include a note that states the extent of underestimation, so that researchers who wish to compare measurements by different methods of molar amounts of trypsin inhibitor could correct the results from the incubation assay by this amount.

LITERATURE CITED

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

BENDER, M. L., KILLHEFFER, J.V., JR., and ROESKE, R. W. 1965. The determination of the normality of a trypsin solution by a specific titration. Biochem. Biophys. Res. Commun. 19:161.

BENDER, M. L., BEGUE-CANTON, M. L., BLAKELY, R. L., BUBACHER, L. J., FEDER, J., GUNTER, C. R., KEZDY, F. J., KILLHEFFER, J. V., MARSHALL, T. H., MILLER, C. G., ROESKE, R. W., and STOOPS, J. K. 1966. The determination of the concentration of hydrolytic enzyme solutions, α-chymotrypsin, trypsin, papain, elastase, subtilisin and acetylcholinesterase. J. Am. Chem. Soc. 88:5890.

BIETH, J. 1974. Some kinetic consequences of the tight binding of protein-proteinase inhibitors to the proteolytic enzymes and their application to the determination of dissociation constants. Pages 463-469 in: Proteinase Inhibitors. Proc. Int. Res. Conf., 2nd (Bayer Symp. V) H. Fritz, H. Tschesche, L. J. Greene, and E. Truscheit, eds. Springer-Verlag: New York.

BIRK, Y. 1967. Chemistry and nutritional significance of proteinase inhibitors from plant sources. Ann. N. Y. Acad. Sci. 146:388.

CLELAND, W. W. 1970. Steady state kinetics. Pages 1-65 in: The Enzymes, 3rd ed. P. D. Boyer, ed. Academic Press: New York.

CLELAND, W. W. 1979. Statistical analysis of enzyme kinetic data. Methods Enzymol. 63:103.

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

GOLDSTEIN, A. 1944. Mechanism of enzyme-inhibitor-substrate reactions. Cholinesterase-eserine-acetylcholine system. J. Gen. Physiol. 27:529.

GRANT, D. A. W., and HERMON-TAYLOR, J. 1979. Hydrolysis of artificial substrates by enterokinase and trypsin and the development of a sensitive specific assay for enterokinase in serum. Biochim. Biophys. Acta 567:207.

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

HARMON, K. M., and NIEMANN, C. 1949. The hydrolysis of *N*-benzoyl-L-argininamide by crystalline trypsin. J. Biol. Chem. 178:743.

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

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.

LEYTUS, S. P., TOLEDO, D. L., and MANGEL, W. F. 1984. Theory and experimental method for determining individual kinetic constants of fast-acting, irreversible proteinase inhibitors. Biochim. Biophys. Acta 788:74.

LIENER, I. E., and KAKADE, M. L. 1980. Protease inhibitors. Pages 7-71 in: Toxic Constituents of Plant Foodstuffs. I. E. Liener, ed. Academic Press: New York.

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

LIU, K., and MARKAKIS, P. 1989b. An improved colorimetric method for determining antitryptic activity in soybean products. Cereal Chem. 66:415.

RICHARDSON, M. 1981. Protein inhibitors of enzymes. Food Chem. 6:235.

SCHWERT, G. W., and EISENBERG, M. A. 1949. The kinetics of the amidase and esterase activities of trypsin. J. Biol. Chem. 179:665.

SCHWERT, G. W., and TAKENAKA, Y. 1955. A spectrophotometric determination of trypsin and chymotrypsin. Biochim. Biophys. Acta 16:570.

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.

SOBER, J. A. 1970. Handbook of Biochemistry, 2nd ed. CRC Press: Cleveland, OH.

STAUFFER, C. E. 1989. Enzyme Assays for Food Scientists. Van Nostrand Reinhold: New York.

VISWANATHA, T., and LIENER, I. 1954. Inhibition of trypsin: I. Sequence of mixing the reactants. J. Biol. Chem. 210:97.

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