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ASSAYS FOR TRYPSIN AND CHYMOTRYPSIN INHIBITION BY PROTEIN INHIBITORS

by

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ABSTRACT

ASSAYS FOR TRYPSIN AND CHYMOTRYPSIN INHIBITION BY PROTEIN INHIBITORS

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For determining the antitryptic activity of soybean products, the current colorimetric method has been modified as follows: a) water rather than dilute alkali is used for extracting the inhibitors, b) the aqueous extract is destabilized with Tris buffer and filtered before, rather than after, the reaction, c) porcine rather than bovine trypsin is used, d) the enzyme, not the substrate, is added last to the reaction mixture, and e) the assay volume is reduced from 10 to 4 ml. The proposed modification is more sensitive and reliable than the current method. The relative standard deviation (RSD) was ± 3.5% (n=55).

For assaying chymotrypsin inhibitor activity, a colorimetric method is developed, using benzoyl-L-tyrosine-p-nitroanilide as a substrate. Since the inhibition curve (enzyme activity, A_{385}, vs. concentration of inhibitor, [I]) fits the reverse ratio function, $y = 1/(a + bx)$, linearity of $1/A_{385}$ (1/y) vs. [I] (x) is obtained. Accordingly, one chymotrypsin inhibitor unit (CIU) is defined as a 0.01 increase of $(A^{o}_{385}/A_{385} - 1)$, where A^{o}_{385} is the enzyme activity when [I]=0. The method, although involves mathematical data conversion, is relatively simple and reliable. The RSD was ± 4.8% (n=22).

In the assays of trypsin and chymotrypsin inhibition by soybean protease

inhibitors, two procedures were used, the common procedure in which substrate is added to mixture of inhibitor and enzyme, and the new procedure in which enzyme is added to mixture of inhibitor and substrate. The inhibition value of the common procedure was either equal to or lower than that of the new procedure, depending on the premix pH and preincubation time, while the value of the new procedure were constant regardless of the premix pH and the preincubation time. When the premix pH was jumped from the acidic or alkaline ranges to near neutral, the sequence effect was abolished completely for trypsin inhibition and partially for chymotrypsin inhibition. These observations are in accordance with the reactive site model proposed by Ozawa and Laskowski, Jr. (1966, J. Biol. Chem. 241, 3955) and suggest an instantaneous binding between inhibitors and enzymes, which may become a complement to the standard mechanism. For assaying protein inhibitors of proteases, the new procedure is preferable to the common procedure.

DEDICATION

To my parents

&

Grandparents

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LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

A_{385}	Absorbance at 385 nm.
A_{410}	Absorbance at 410 nm.
BB	Bowman-Birk (soybean inhibitor).
BAPA	Benzoyl-DL-arginine-p-nitroanilide.
BTpNA	Benzoyl-L-tyrosine-p-nitroanilide.
CIA	Chymotrypsin inhibitory activity
CIU	Chymotrypsin inhibitor units as is defined under Materials & Methods.
E	Enzyme.
I	Virgin inhibitor with a reactive site peptide bond intact.
I^*	Modified inhibitor with a reactive site peptide bond hydrolyzed.
IU	Internatioal units. One IU of enzyme is that amount which catalyzes the formation of 1 umole of product per min under defined conditions.
IUI	Internatioal units inhibited.
K_{cat}	Hydrolysis rate constant for a peptide bond.
K_{hyd}	Equilibrium constant of peptide hydrolysis.
TI	Trypsin inhibitor.
TIA	Trypsin inhibitory activity.
TU	Trypsin units as defined under the Materials and Methods.
TUI	Trypsin units inhibited.

INTRODUCTION

Substances which are capable of inhibiting the proteolytic activity of certain enzymes are ubiquitous (1,2). Some of them are proteins in nature. Protease inhibitors have gained the attention of scientists in many disciplines: (a) nutritionists, because of the possible toxic and antinutritional effects of these inhibitors to humans and animals (2-4), (b) enzymologists, because inhibitors can be used as a natural tool to probe the active center of enzymes (5-7), and (c) protein chemists, because the reaction of these inhibitors with enzymes provides a model system for studying protein-protein interactions (8-10). However, the greatest amount of research has been directed to soybean protease inhibitors because of their possible influence on the nutritive value of soybean protein, one of the the most important sources of vegetable proteins. (2-4,11,12).

Most protease inhibitors, such as the soybean Kunitz and Bowman-Birk (BB) inhibitors, are trypsin and/or chymotrypsin inhibitors. To study these inhibitors, we often perform inhibition assays. This research is comprised of two parts. The first part deals with modifications of the currently used method for measuring trypsin inhibitor activity (TIA) in soybean products (13,14). The second part deals with a proposed method for assaying chymotrypsin inhibitor activity (CIA) using a synthetic substrate (15). This thesis challenges the traditional view that preincubation of inhibitor with enzyme is necessary for obtaining equilibrium data in an inhibition assay (1,13,14,16-20) by demonstrating an effect of the reactant mixing sequence on the inhibition assay. This reactant sequence effect is attributed to limited hydrolysis of inhibitors by the very enzyme they inhibit, in accordance with a standard mechanism

proposed by Ozawa and Laskowski, Jr. for protein inhibitors of serine proteases (21).

LITERATURE REVIEW

I. Proteases

Proteases are relatively small proteins (25-35 KD), which cleave other proteins at peptide bonds. All proteases utilize a general acid-base type of cleavage mechanism, but the side chains that act as the acid and base differ. Based on these differences, proteases are grouped into four classes: metallo proteases, carboxyl or acid proteases, thio proteases and serine proteases (22).

II. Serine Proteases

Serine proteases comprise a large group of proteases which use the hydroxyl group of a serine residue as a Lewis acid during cleavage action towards proteins. A common test for these enzymes is the inhibition of hydrolase activity by the reaction of the serine residue with diisopropyl-phosphofluoridate (DFP) (22).

The catalytic mechanism

The active site of a typical serine proteinase is made up of two regions: (1) the catalytic site, and (2) the substrate binding site(s). The catalytic site is composed of residues Ser^{195}, His^{57} and Asp^{102} (chymotrypsin numbering). These three residues form a hydrogen binding system often referred to as the catalytic triad or the charge relay system. The catalytic triad and the catalytic mechanism is depicted in Fig. 1. The substrate (ester or amide) carbonyl carbon undergoes a nucleophilic attack by the hydroxyl group of Ser^{195},

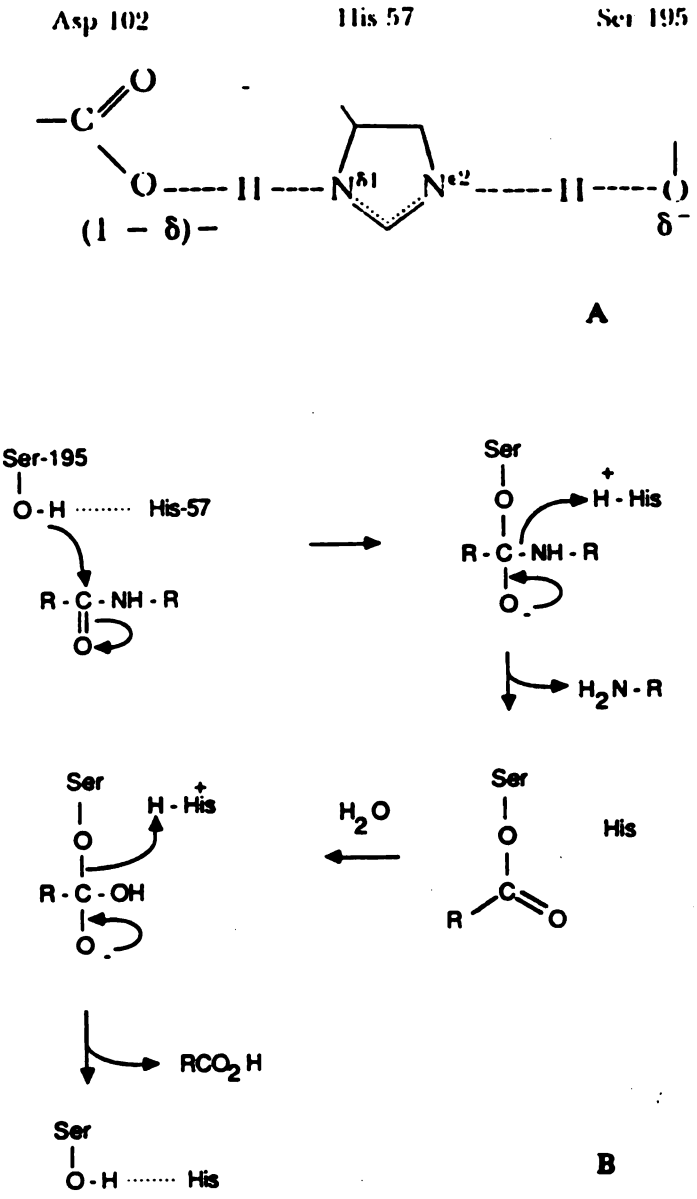

Fig. 1 The catalytic triad (A) and the catalytic mechanism (B) of a serine protease (22). In B, the catalytic residue Asp^{102} is not shown.

which leads to the formation of an acyl enzyme intermediate. His^{57} functions as a catalytic base by assisting in the transfer of a proton from the serine hydroxyl to the substrate leaving group. Asp^{102} is believed to stabilize the His^{57} conformation or His^{57} tautomer (23).

The substrate binding site, which is made up of the primary structure and of the overall three dimentional structure of the enzyme, determines the substrate specificity of a serine protease.

Trypsin and chymotrypsin

Trypsin and chymotrypsin, the two major proteolytic enzymes of the pancreas, belong to serine proteinase group. They are believed to be similar in terms of catalytic mechanism. The central difference between the two lies in their specificity. Trypsin cleaves a peptide bond most efficiently on the carboxyl side of positively charged amino acids (Lys and Arg). Chymotrypsin favors the peptide bonds on the carboxyl side of large amino acids (Trp, Phe, Tyr and Leu) (24).

The mechanism of inhibition

Thompson (25) initially proposed that the inhibition of porcine pancreatic elastase by some tight-binding peptide aldehydes was a result of the formation of a hemiacetal linkage between the aldehyde carbonyl of the inhibitor and the active site serine of the enzyme. This tetrahedral adduct was presumed to be similar to the tetraaldehydral intermediate formed during peptide or ester bond hydrolysis (Fig. 2). This inhibition mechanism of serine protease was supported by many studies with other serine proteases and their inhibitors (6,7,26).

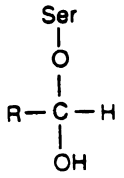

Fig. 2. Structure of the hemiacetal adduct formed between a peptide aldehyde (R-CHO) and the active site serine of a serine protease.

Trypsin from different biological sources

Trypsin from various biological sources have been found to differ in certain aspects (27,28). Human, bovine, ovine and porcine trypsins are quite similar in pH (8.0-8.2) and temperature (45-50 C) optima, Michaelis-Menten constants and kinetics of esterolytic activity. However, porcine trypsin is found to have a lower isoelectric point and higher resistance to bases than ovine and bovine trypsins. Human trypsin resembles porcine trypsin more than the other two. Another important variation among trypsins is in the extent of inhibition by some trypsin inhibitors. For example, unlike bovine trypsin, human trypsin is not inhibited by chicken ovomucoid, bovine and porcine pancreatic inhibitors, or the soybean Kunitz inhibitor (29).

III. Protein Inhibitors of Proteases

Protease inhibitors are substances which, when added to a mixture of protease and substrate, bind to the enzyme and produce a decrease in the rate of substrate cleavage. Protein inhibitors of proteases are ubiquitous. All but one

of these inhibitors act on serine proteases. The sole exception is the complex between the potato carboxypeptidase inhibitor and carboxypeptidase A, which is a zinc metalloprotease (30).

The major common element in the structures of these inhibitors is the primary contact regions or the reactive site loop. The formation of the loop is highly complementary to the surface of the enzymes and resembles that of an oligopeptide substrate. When bound to the serine proteinase active site, these reactive site loops project out from the inhibitor, so that they are accessible to the active site of proteolytic enzymes (30).

IV. Protease Inhibitors from Soybeans

The protein inhibitors that have been isolated from soybeans fall into two main categories, the Kunitz soybean inhibitor and the Bowman-Birk (BB) inhibitor.

Kunitz inhibitor

The Kunitz soybean inhibitor was first isolated and crystallized by Kunitz (31,32). The isolation involves extracting soybeans with water and precipitating the inhibitor with alcohol. It has a MW between 20-25 KD, with a specificity directed primarily toward trypsin. The inhibitor was shown to combine with trypsin in a stoichiometric fashion, i.e., 1 mole of inhibitor inactivates 1 mole of enzyme. The complete amino acid sequence of the inhibitor was established by Koide et al. (33). It consists of 181 amino acid residues and two disulfide bonds, with a reactive site at residues Arg^{63} and Ile^{64}.

Bowman-Birk inhibitor

The soybean BB inhibitor was first described by Bowman (34) as an acctone-insoluble factor in contrast to the alcohol-insoluble factor, which was later recognized as the Kunitz inhibitor. Isolation of the BB inhibitor involves extracting beans with 60% alcohol and precipitating the inhibitor with acetone. Later, Birk (35) and Birk et al. (36) resumed investigation of the acetone-insoluble factor and succeeded in purifying and characterizing the inhibitor. Cumbersome descriptive terms have then been used to refer to this protein: acetone-insoluble factor, purified AA inhibitor and trypsin and a-chymotrypsin inhibitor.

The complete amino acid sequence of the BB inhibitor was determined by Odani and Ikenaka (37). It is a single polypeptide chain of 71 amino acids including seven disulfide bonds. Its MW is about 8 KD. It is capable of inhibiting both trypsin and chymotrypsin at independent reactive sites; the trypsin reactivc site being located at residues Lys^{16} and Ser^{17}, and the chymotrypsin reactive site being located at residues Leu^{44} and Ser^{45}. The BB inhibitor is generally considered more heat stable than the Kunitz inhibitor (35). However, a recent study showed that this is true only for purified forms. *In situ*, the BB inhibitor appeared to be more heat labile than the Kunitz inhibitor (38).

V. A Standard Mechanism of Inhibition

Most protein inhibitors of serine proteases appear to interact with the enzyme they inhibit according to a standard mechanism of Laskowski, Jr. (39). They bind to the enzymes as if they were good substrates, but very tightly, and are cleaved very slowly at a peptide bond referred to as the reactive site (21). The model has stemmed from the observations of many workers, especially Michael

Laskowski, Jr., its chief proponent. The detailed characteristics of the mechanism are described as follows (1,10).

1. Incubation of the inhibitor with catalytic amounts of enzyme leads to specific hydrolysis of one peptide bond, the reactive site peptide bond of the inhibitor (40). Thus, the reaction between inhibitor and trypsin is better represented by the scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} E + I^* \qquad [1]$$

where E is a serine protease, I is a virgin inhibitor whose reactive site peptide bond is intact, and I^* is a modified inhibitor whose reactive site peptide bond has been cleaved.

2. The newly formed COOH terminal in the modified inhibitor was shown to be arginine and the newly formed NH_2-terminal, isoleucine. The two peptide chains of the modified inhibitor are strongly held together by one or more disulfide bridges. The equilibrium constant for virgin to modified inhibitor conversion is close to unity at neutral pH (41-43).

3. Reduction and carboxymethylation of the modified inhibitor produced two fragments. The smaller fragment is composed of 64 amino acids, has the original NH_2-terminal of aspartic acid of the virgin inhibitor and the newly formed COOH-terminal of arginine. The larger fragment, is composed of 134 amino acids, has a newly formed NH_2-terminal belonging to isoleucine and the original COOH-terminal of leucine (Fig. 3).

4. Both virgin and modified inhibitors are active but the modified inhibitor reacts with the enzyme much more slowly than the virgin inhibitor. The stable enzyme-inhibitor complex is the same chemical substance whether formed from virgin or from modified inhibitor. Removal of either the newly formed COOH

terminal amino acid residue or the newly formed NH_2 terminal amino acid residue form modified inhibitor causes loss of activity (40).

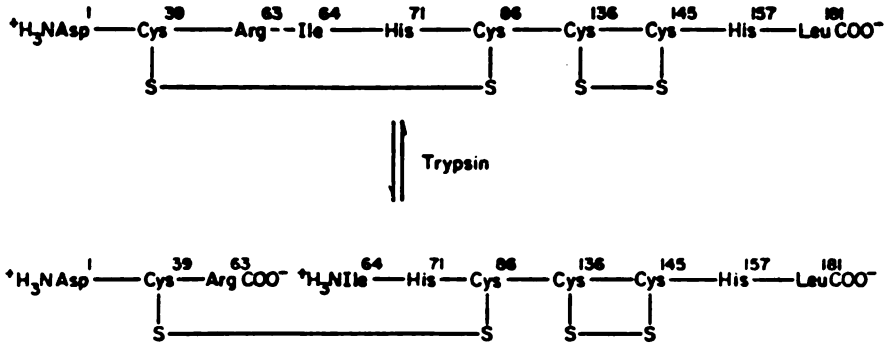

Fig. 3 Schematic diagram of the tryptic conversion of virgin into modified soybean trypsin inhibitor according to Ozawa and Laskowski, Jr. (21). The complete amino acid sequence of the inhibitor was determined by Koide et al. (44).

5. The complex involves extremely close fit between the reactive site of the inhibitor and the active site of the enzyme. The conformation of the residue in the inhibitor interacting with the enzyme is closely similar in various inhibitors even through the inhibitors themselves are not conformationally similar. Examples of these inhibitors include the soybean BB inhibitor (45,46), chicken ovomucoid (21), the bovine pancreatic secretory inhibitor (47,48) and the lima bean trypsin inhibitor (18).

Of special interest are those protease inhibitors which have the unique capacity to inhibit both trypsin and chymotrypsin at independent, non-overlapping binding sites. They have been termed "double-headed" (49). Turkey ovomucoid (50), lima bean trypsin inhibitor (51), and BB soybean inhibitor (35)

have been cited as examples of double-headed inhibitors. Studies with soybean BB inhibitor have shown that partial proteolysis with trypsin followed by carboxypeptidase B hydrolysis resulted in loss of trypsin inhibitory activity (TIA) without affecting chymotrypsin inhibitory activity (CIA), while partial proteolysis with chymotrypsin resulted in loss of CIA without any effect on the TIA (45,46). This is true also with the lima bean inhibitor (18).

VI. Nutritional Implications of Protease Inhibitors

Osborne and Mendel (52) made the first significant observation that soybeans had to be heated in order to support the growth of rats. An assumption is that trypsin inhibitor (TI) is responsible for growth depression by reducing the digestibility of proteins. Later on, another observation was reported by Desikachar and De (53), that soybean diets containing predigested protein or free amino acids still retard the growth of rats. This was later confirmed by Liener (4). This observation indicated that inhibition of proteolysis by TI was not the sole factor responsible for growth depression. At the same time, the third significant finding was made by Chernick et al. (3). They found that raw soybeans as well as TI itself could cause hypertrophy of the pancreas of chicks. Nesheim et al (54) made a similar observation with rats.

Since the pancreas is responsible for the production of most enzymes required for the digestion of food, any dietary components which affect pancreatic function may markedly influence the availability of nutrients from the diet. Experiments with rats have demonstrated that pancreatic enzyme secretion is controlled by a negative feedback mechanism (Fig. 4). The amount of pancreatic secretion is determined by the level of free trypsin and/or chymotrypsin present in the intestine. As the level of trypsin goes below a

threshold level, the pancreas is induced to produce more enzymes. The TI evokes increased pancreatic enzyme secretion by forming inactive trypsin-TI complex. This results in endogenous loss of essential amino acids being secreted by a hyperactive pancreas. The loss of methionine and cysteine in this way would be particularly acute since soybean protein is deficient in these amino acids. On the other hand, intact soybean proteins were found to account for about 60% of the growth inhibitory and pancreatic hypertrophic effects due to their resistance to enzymatic attack unless denatured by heat (56). Therefore, it would appear that the TI and the refractory nature of the soybean protein act through a common mechanism described in Fig. 4 to inhibit the growth of rats.

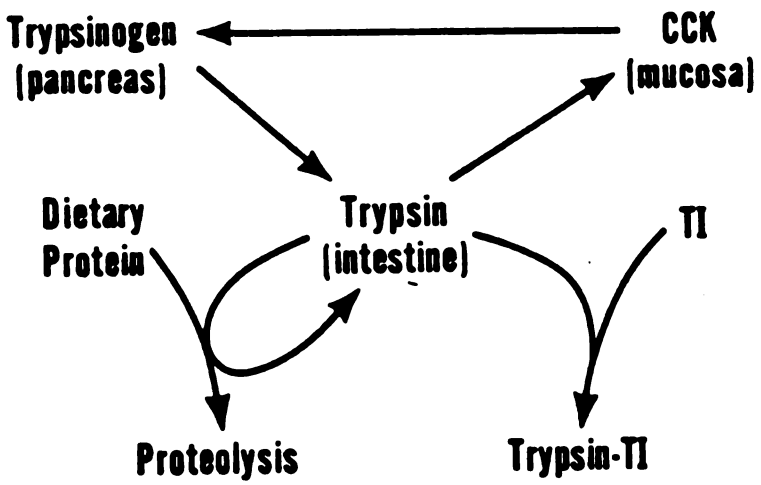

Fig. 4 Regulation of the secretion of trypsin by the pancreas.
CCK, cholecystokinin; TI, trypsin inhibitor (55).

More recent studies showed that short-term feeding of raw soy flour and purified TI also caused pancreatic hypertrophy and hyperplasia in certain monogastric animals while prolonged exposure to high levels of TI in raw soy ultimately led to pancreatic nodular hyperplasia and acinar cell adenoma in rats

(12). This confirmed the adverse nutritional effect of TI in food.

VII. Current Methods for Trypsin Inhibition Assay

Although various methods of column chromatography, affinity chromatography and electrophoresis have proved valuable for isolation and characterization of diverse TI (57,58), these methods are not suitable for quantification. At present, methods for TI assay are mainly colorimetric although a fluorometric assay (59), immunoelectrophoresis assay (58) and enzyme linked immunoassay (60) have been introduced.

The original colorimetric method employing casein, a natural substrate, was first described by Kunitz (32). It involves the spectrophotometric determination of hydrolysis of casein by a given concentration of trypsin in the presence and absence of the inhibitor. However, the rate of hydrolysis was later reported not to follow zero order kinetics under the condition defined by Kunitz (61). Erlanger et al (62) introduced a synthetic substrate, benzoyl-DL-arginine-p-nitroanilide HCl (BAPA), for assaying trypsin activity. They found that the hydrolysis rate of BAPA by trypsin not only followed zero order kinetics but also could be followed colorimetrically since the p-nitroaniline released is chromagenic.

In 1969, Kakade et al. (16) made an evaluation of natural vs. synthetic substrate for measuring TIA in soybean samples and concluded that the use of the synthetic substrate, BAPA, proved to be a convenient and reliable method provided the competitive nature of the inhibition was taken into consideration, that is, trypsin inhibitor activity deviates from linearity at high levels of inhibitor concentration. This was accomplished mainly by introducing an extrapolation procedure for data interpretation, in which TIA was expressed in

arbitrary trypsin units inhibited (TUI) per ml of the extract at zero concentration of the inhibitor (Fig. 5). Questions concerning both the accuracy and reliability of this method led to a collaborative study organized by the American Association of Cereal Chemists and the American Oil Chemists' Society (63). A modified procedure was reported as a result of this collaborative study (20). Based on the modified procedure the standard AACC method for determining the TIA of soybean products was adopted (64).

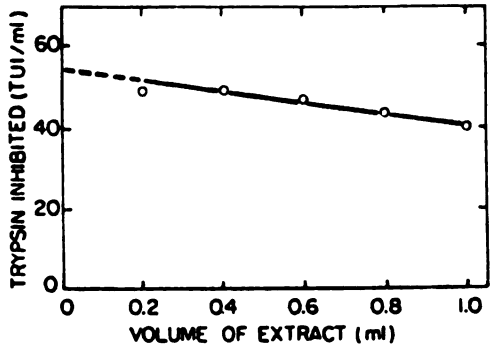

Fig. 5 Trypsin inhibitor activity (TUI/ml extract) in relation to level of crude soybean extract. Extrapolated curve, as represented by the broken line, intercepts y-axis (0.0 ml) at 54 TUI/ml extract (16).

Although reported separately, Smith et al. (13) and Hamerstrand et al. (14) modified the AACC method in a similar way by using a single inhibitor level instead of serial inhibitor levels. This modification bypasses the cumbersome data manipulation which is done by either extrapolating to zero or averaging over a range of inhibition levels. The reason for their modification is based on two observations: a) the patterns of the relationship between enzyme activity vs. inhibitor concentration are diverse, and b) the extrapolation method of data

interpretation uses data that are not in the region in which zero order kinetics is followed. Although another minor modification of the AACC method was also reported (65), the above two papers established the current method for TIA assay (6). However, since the modifications are limited only to data interpretation and the reasons behind the diverse patterns of inhibition curves observed remain unknown, the current method still poses questions of accuracy and sensitivity, especially for testing samples with low TIA.

VIII. Current Methods for Chymotrypsin Inhibition Assay

For measuring chymotrypsin inhibitor activity (CIA), a linear relationship between enzyme activity and enzyme concentration is an important prerequisite for obtaining reliable and reproducible measurements. Use of casein or denatured hemoglobin for measuring chymotrypsin activity was originally proposed by Kunitz (32). It was soon found to give a curvilinear response between enzyme activity and enzyme concentration (66). Several modifications of the method were proposed, including a mathematical transformation (67) and Ca^{++} incorporation (68). However, Kakade et al. (17) pointed out that these modifications likewise failed to produce a linear relationship and therefore, they modified the casein method by judicious choice of experimental conditions. The method not only involves cumbersome procedures, but also poses a question of reliability.

Several simple synthetic substrates have been proposed for assaying chymotrypsin, including acetyl-L-tyrosine ethyl ester (ATEE) (69), N-carbobenzyl-L-tyrosine-p-nitrophenyl ester (CTNE) (70), and N-acetyl-L-tyrosine-p-nitroanilide (ATpNA) and N-benzoyl-L-tyrosine-p-nitroanilide (BTpNA) (15).

Among the methods using a synthetic substrate, the method of Martin et al.(70) is most sensitive. As little as 5 mμg (5×10^{-9} g) enzyme can be

detected. However, lack of specificity (trypsin, thrombine, plasmin and papain are also very active against CTNE) and a need for the spontaneous substrate hydrolysis correction restrains it from gaining popularity. The method of Schuert and Takenaka (69) has the same sensitivity to that of Bundy (15) (1.5-15 ug enzyme can be tested), but the latter is simpler since p-nitroaniline released is a chromogenic substance and can be readily followed at 385 nm with spectrophotometers not having an ultraviolet attachment.

When used for the chymotrypsin assay, these synthetic substrates have advantages over natural substrates because of simplicity and easy achievement of a linear response, although they are less water-soluble and require the presence of an organic solvent in the reaction mixture. BTpNA is frequently used for CIA assay (38,71), however, so far no detailed report has been given regarding procedure and conditions of the assay, inhibitor titration curve and factors affecting the assay.

MATERIALS AND METHODS

Reagents

Crystalline porcine and bovine trypsins, crystalline bovine a-chymotrypsin, soybean Kunitz and BB inhibitors, benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA) and benzoyl-L-tyrosine-p-nitroanilide (BTpNA) were purchased from Sigma Co. (St. Louis, MO).

Part 1. TRYPSIN INHIBITION ASSAY

I Methodology

i. Buffer and solutions

The assay buffer was 50 mM Tris buffer containing 10 mM $CaCl_2$, pH 8.2.

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

A stock trypsin solution was prepared by dissolving 10 mg of crystalline porcine trypsin in 50 mL of 1 mM HCl solution, pH about 2.5, containing 2.5 mM $CaCl_2$. The solution was kept at 5 C. For preparing a working trypsin solution, 2 mL of the stock solution was diluted to a total volume of 25 mL, using the above HCl solution.

Stock inhibitor solutions were prepared by dissolving 5 mg soybean BB or Kunitz inhibitor in 50 mL water. Working inhibitor solutions were made by diluting 2 mL of the stock solutions to a total volume of 25 mL, using water.

ii. Inhibitor sample preparation

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

iii. Procedure

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

Table 1. Procedure for assaying TIA in legume products

Sequence of Mixing	Reactants	Concentration in working solution	Volume needed for assay
1st	BAPA	0.92 mM	2.0 mL
2nd	Sample	Causing 30-70% inhibition	1.0 mL
3rd	Enzyme	16 ug/mL	0.5 mL
4th	Acetic acid	30%	0.5 mL
Total assay volume			4.0 mL

iv. Calculating trypsin inhibition

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

$$\text{TUI/mg sample} = \frac{[(A^{O}_{410} - A^{S}_{410}) \times 100]/\text{mL diluted soy extract}}{(\text{mg sample/mL diluted soy extract})}$$

Alternatively, for standardization, the TIA can also be expressed in terms of International Units Inhibited (IUI) per g sample. One IU of enzyme is the amount that catalyzes the formation of 1 μ mole of product per min under difined conditions. One TU is equal to 0.000516 IU on the basis that the molar

absorption coefficient (a_m) at 410 nm is 7760.

II. Procedures for Studying the Effect of the Reactant Mixing Sequence on the TIA assay

i. Buffers and solutions

Three preincubation buffers were used: 20 mM acetate buffer, 20 mM Tris buffer and mixture of the two to reach pH values from 2.7 to 9.0.

Other buffer and solutions are referred to Methodology section (p. 17).

ii. Procedures

All preincubations of the inhibitors (0-2 μg) with enzymes (8 μg) in the S-last test, or of the inhibitors with BAPA (0.8 mg) in the E-last test, were carried out in one of the three preincubation buffers to reach pH values from 2.7 to 9.0, with total volume of 1.5 mL. After a specified time period of incubation, 2 mL of the assay buffer was added to the premix. This brought the pH of the assay system to 8.1±0.2. Immediately following this step, 20 μL of the BABA solution in the S-last test or 20 μL of the enzyme solution in the E-last test, were added to start the enzymic reaction. The reactions were allowed to proceeded for 10 min and stopped by injecting 0.5 mL of 30% acetic acid solution.

iii. Calculating trypsin inhibition

Since different doses of the inhibitor were used to measure the activity of the inhibitor by each test, a titration curve (A_{410} vs. dose of inhibitor) could be plotted. Linearity was generally obtained over lower dose ranges. The slope of the straight line was taken as the inhibition value.

Part 2. CHYMOTRYPSIN INHIBITION ASSAY

I. Methodology

i. Buffer and solutions

Tris buffer, 50 mM,pH 8.2, containing 10 mM $CaCl_2$ was used as an assay buffer.

A stock chymotrypsin solution was prepared by dissolving 20 mg of crystalline chymotrypsin in 50 mL of 1 mM HCl solution, pH about 2.5, containing 2.5 mM $CaCl_2$. The solution was kept at 5 C. To prepare a working enzyme solution, 2 mL of the stock solution was diluted to a total volume of 25 mL, using the above HCl solution.

A stock BTpNA solution was prepared by dissolving 15 mg BTpNA in 25 mL acetone. The solution was stored at 5 C. A working BTpNA solution was freshly prepared by diluting 5 ml of the stock solution to a total volume of 25 mL, using the assay buffer prewarmed at 37 C and kept at that temperature.

A stock inhibitor solution was prepared by dissolving 5 mg soybean BB inhibitor in 50 mL water. A working inhibitor solution (4 µg/mL) was made by diluting 2 mL of the stock solution to a total volume of 25 mL using water.

ii. Inhibitor sample preparation

Refer to the preparation procedure for trypsin inhibition assay (p. 18).

iii. Assay procedure

One mL of the inhibitor solution, which results in 35-65% chymotrypsin inhibition, was pipetted to a test tube and addition of 2 mL BTpNA solution

followed. The enzymic reaction was started by adding 0.5 mL of the enzyme solution and allowed to proceed for 10 min at 37 C. The reaction was stopped by injecting 0.5 mL of 30% acetic acid solution with an 1 mL syringe. The absorbance at 385 nm, A_{385}, was a measure of the chymotrypsin activity. The reaction was also run in the absence of inhibitors, by replacing the inhibitor solution with 1.0 mL water. The corresponding absorbance was symbolized as A^{o}_{385}. Water was used as a blank for all color measurements.

iv. Chymotrypsin inhibitor unit (CIU)

Under the assay conditions specified in this study (10 min, 4 mL reaction mixture, pH=8.1 at 37 C, with BTpNA as a substrate), one chymotrypsin inhibitor unit (CIU) was defined as a 0.01 increase of $(A^{o}_{385}/A_{385} - 1)$. The CIA is expressed as CIU/mg inhibitor (sample) and calculated as follows:

$$\text{CIU/mg inhibitor} = \frac{[(A^{o}_{385}/A_{385} - 1) \times 100]/\text{mL inhibitor solution}}{\text{mg/ml inhibitor solution}}$$

v. Correction for effect of enzyme concentration

The CIA values, when expressed in terms of CIU, were affected significantly by the amount of enzyme used in the assay, which reflected in the A^{o}_{385} value. Setting the CIA value obtained at $A^{o}_{385} = 0.45$, symbolized by $CIA_{0.45}$, as a reference value, a correction factor c is defined as

$$c = (CIA_{0.45}/CIA - 1),$$

and calculated by the following equation (See DISCUSSION):

$$c = 2.05\,(A^{o}_{385} + 0.13)^{3.44} - 0.315.$$

Correction of CIA at any other A^{o}_{385} to $CIA_{0.45}$ is done by

$$CIA_{0.45} = (1 + c)\,CIA.$$

vi. Expressing CIA in terms of pure BB inhibitor

One ug pure BB inhibitor was shown to have 30.8 CIU. Thus for comparative purposes, CIA can be expressed in terms of pure BB inhibitor per unit sample. If samples contain other inhibitors, we can express CIA in terms of BB inhibitor equivalent, using the same conversion factor.

II. Procedures for Studying the Effect of the Reactant Mixing Sequence on the CIA assay

i. Buffers and solutions

Three preincubation buffers were used: 20 mM acetate buffer, 20 mM Tris buffer and mixture of the two to reach pH values from 2.7 to 9.0. Other buffers and solutions are described in the Methodology section (p. 21).

ii. Assay procedures

All preincubations of the BB inhibitor (0-4 μg) with a-chymotrypsin (16 μg) in the S-last test, or of the inhibitor with BTpNA (0.4 mL stock BTpNA solution) in the E-last test, were carried out in one of the above three preincubation buffers to reach pH values from 2.7 to 9.0, with total volume of 1.5 mL. After a specified time of incubation, 1.6 mL of the assay buffer was added to the premix. This brought the pH of the assay system to 8.1 ± 0.2. Immediately following this step, 0.4 mL of BTpNA solution in the S-last test, or 0.4 mL of enzyme solution (40 μg/mL in 0.001 N HCl) in the E-last test, were added to start the enzymic reaction. The reaction was allowed to proceed for 10 min and stopped by injecting 0.5 mL of 30% acetic acid solution. The yellow color of the reaction mixture was read at 385 nm and the A_{385} value was used as an estimate

of chymotrypsin activity.

iii. Calculating chymotrypsin inhibition

The reader is referred to the Methodology section (p. 22) for a definition of chymotrypsin inhibitor unit and data transformations. In the assay, a series of inhibitor levels are used and $(A^{o}_{385}/A_{385}-1)x\ 100$ is plotted against inhibitor level. A straight line is obtained, the slope of which is taken as the inhibition value which is further corrected for the enzyme concentration effect.

RESULTS AND DISCUSSION

Part 1 TRYPSIN INHIBITION ASSAY

I. General Assay Conditions

i. Enzyme concentration

As shown in Fig. 6, the quantity of porcine trypsin employed in this test should not exceed that corresponding to A_{410}=0.50, if linearity between absorbance and enzyme level is to be maintained. Within this A_{410} range, when two different amounts (6 and 8 μg) of enzyme were used to measure the TIA of the same soy extract, the parallel lines shown in Fig. 7 were obtained. From these lines, the same TIA value, as TUI/mL sample extract, can be derived, indicating that impurity or partial inactivation of the enzyme does not affect the assay. The independence of TIA on enzyme concentration was also addressed in the current method (13,14).

ii. Reaction time

Fig. 8 shows the relationship between A_{410} and reaction time. Linearity was observed up to 13 min of reaction, both in the absence of inhibitors (0.00 mg raw soybean /mL sample solution) and in the presence of inhibitors (0.10 and 0.15 mg raw soybean /mL). The results indicate that the rate of trypsin inhibition, expressed as TUI per mg dry sample per min, was constant when the reaction time remained within the valid assay time range (0-13 min), while the TIA values, expressed as TUI/mg dry sample, increased linearly with time. For this reason, the reaction time for the TIA assay should be standardized to 10

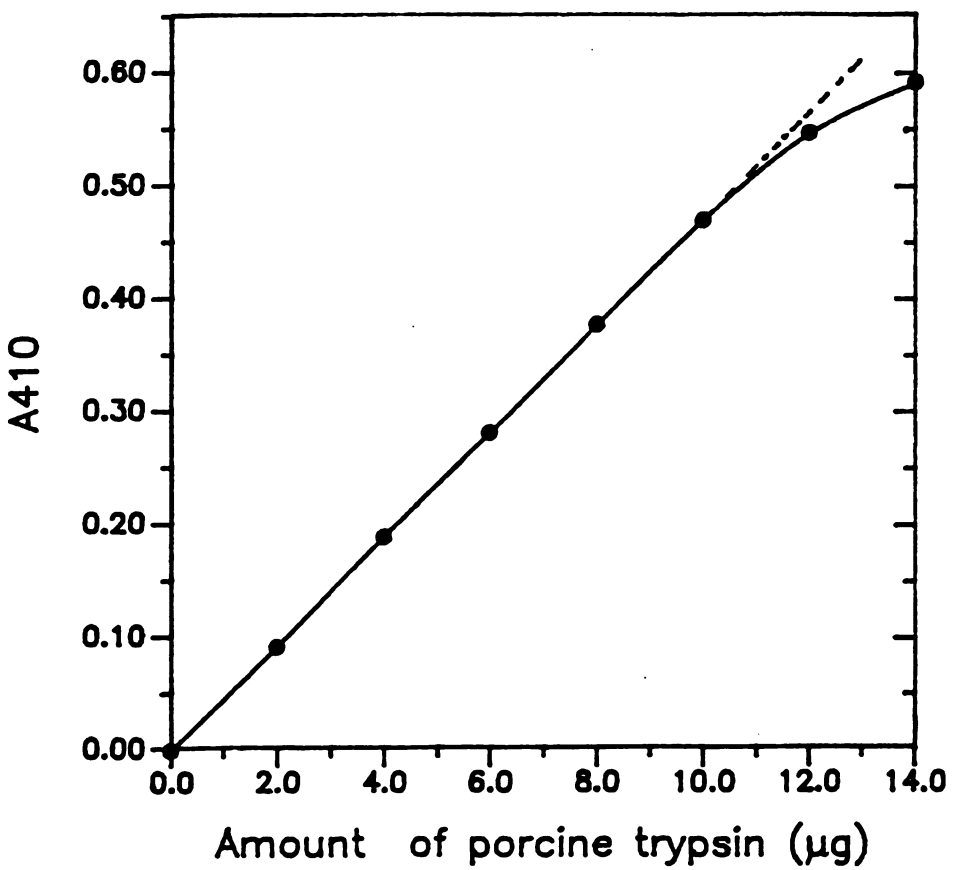

Fig. 6 Relationship between absorbance at 410 nm and amount of porcine trypsin. The reaction time was 10 min.

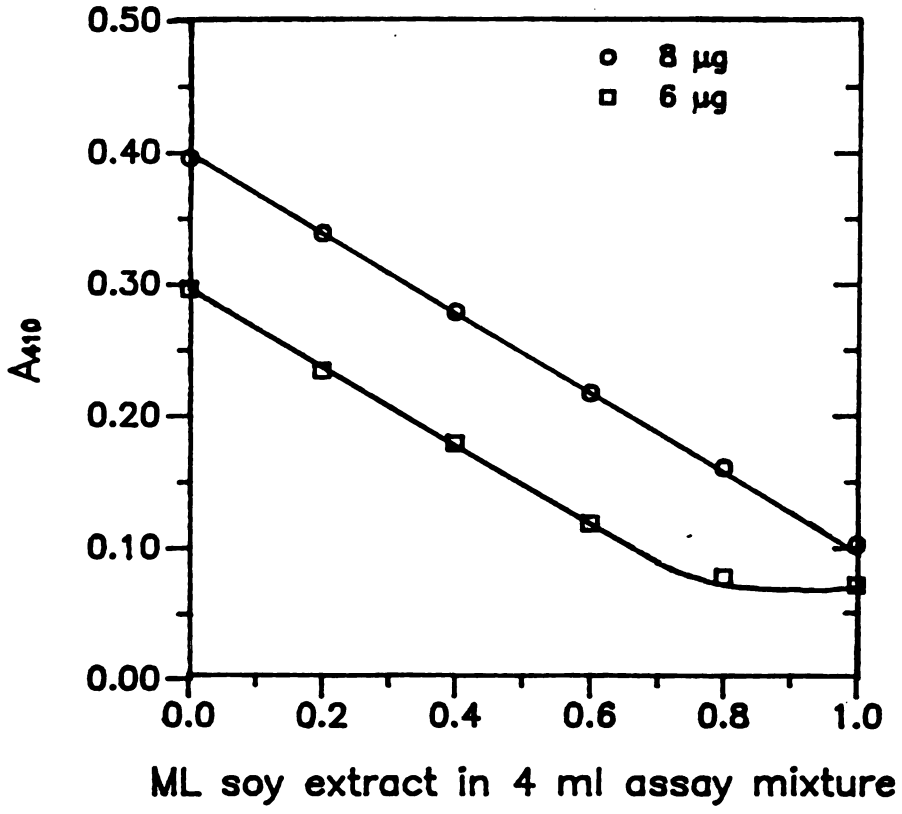

Fig. 7 Effect of porcine trypsin concentration on the assay of soybean TIA.

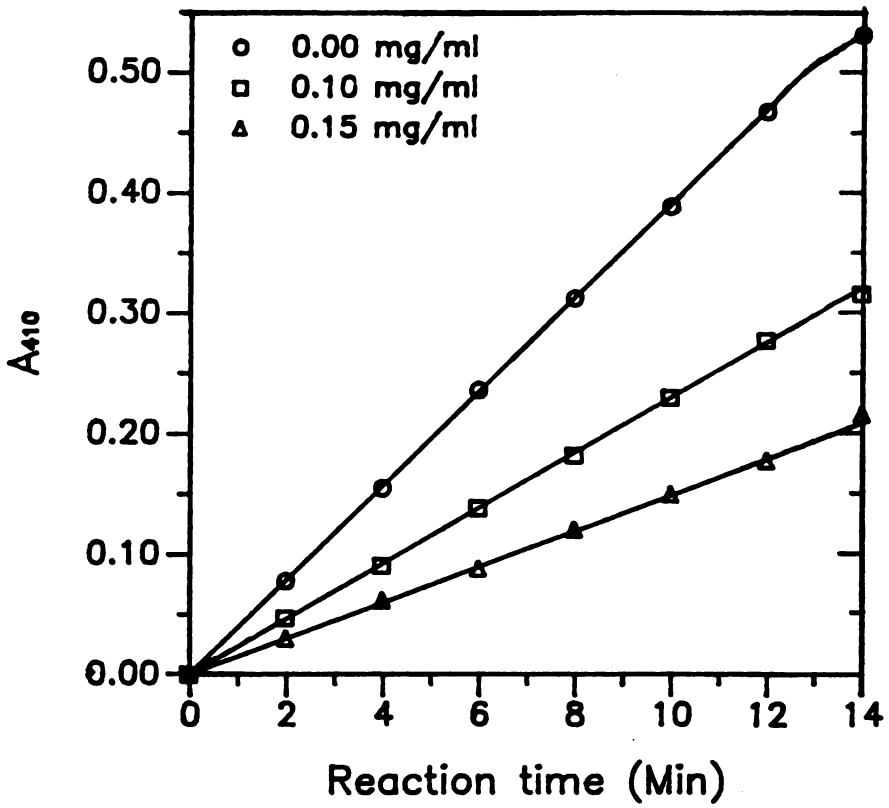

Fig. 8 Relationship between absorbance at 410 nm and reaction time in the absence and in the presence of inhibitors. 8 μg of enzyme preparation was used in the reaction. The inhibitor samples were aqueous extracts of raw soybeans.

min.

iii. Substrate (BAPA) concentration

The apparent Km value for the porcine trypsin-BAPA reaction was found to be 0.96 mM at 37 C in this study. In the modified TIA assay, the BAPA concentration would be 0.46 mM, corresponding to about 1/2 of the Km. Use of excess BAPA concentration is unfeasible due to its poor solubility. As the BAPA concentration affects the trypsin assay, it also affects the TIA assay. Fig. 9 shows that for two different BAPA concentrations, 0.23 and 0.46 mM, the lines connecting A_{410} and amounts of inhibitors are not parallel, a fact which emphasizes the significance of standardizing the BAPA concentration in the TIA measurement. In addition, since BAPA decomposed slowly with time, causing variation of the TIA value, it is recommended that a fresh working BAPA solution be used (65, and this study).

iv. Ca ion concentration

Ca^{++} is known to stabilize trypsin (27). We observed that when Ca^{++} was added at two concentrations, 5 and 10 mM, to the assay buffer, the TIA values were not significantly influenced, but its presence at the 5 mM level is recommended for protection of the enzyme from inactivation. Lehnhardt and Dills (65) observed that the presence of Ca^{++} reduced not only autolytic trypsin inactivation but also the effect of phytate effect on TIA assay.

v. pH of the assay buffer

The optimum pH for hydrolysis of BAPA by porcine trypsin was found to be 8.1 in this study, which is closed to that of bovine trypsin (Erlanger et al. 1961). In order to determine the optimum buffer pH for the TIA assay, the following

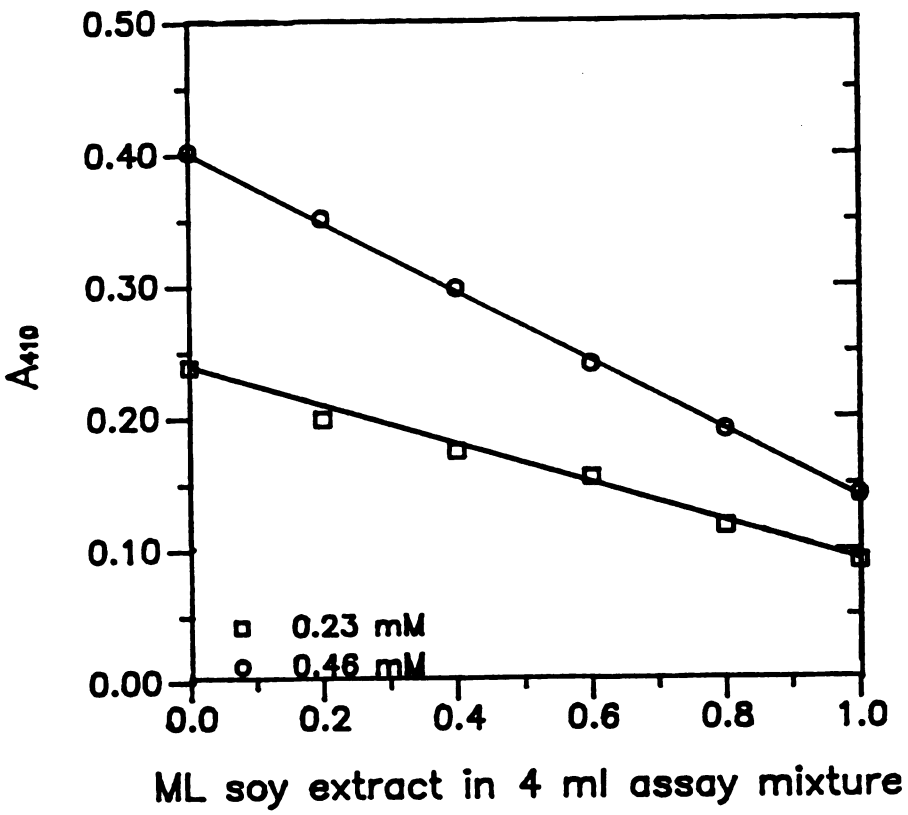

Fig. 9 Effect of substrate (BAPA) concentration on the assay of soybean TIA.

five pH levels were tried: 8.5, 8.1, 7.5, 7.0, and 6.5. The results are summarized in Fig. 10, and indicate that the A_{410} vs. inhibitor quantity lines were not exactly parallel and the line corresponding to pH 8.1 led to the greatest TIA value (highest slope of the line).

II. Effect of the Reactant Mixing Sequence on the TIA assay

In assaying enzyme inhibition, preincubation of inhibitor with enzyme before addition of substrate is commonly practised (13-14,16,19). This is thought to be necessary for obtaining equilibrium data (1). However, while investigating the soybean Kunitz inhibitor, Viswanatha and Liener (72) found that a change in the order of mixing the reactants exerted a considerable influence on the extent of inhibition. In our study, for measuring trypsin inhibition of the soybean BB inhibitor, two procedures were used: the common procedure in which the substrate is added last, after mixing inhibitor with enzyme; being hereafter referred to as "the S-last test", and a new procedure in which enzyme is added last to the mixture of inhibitor and substrate, being hereafter referred to as "the E-last test". The results are presented in Fig. 11 and indicate that the S-last test gave considerably lower inhibition values than the E-last test when the premix pH was 3.5 and preincubation time was as short as 3 min. Under the same preincubation conditions, similar results were reached with the Kunitz inhibitor. The effect of the reactant sequence on trypsin inhibition assay is hereafter referred as "the reactant sequence effect".

i. Effect of the preincubation time on the reactant sequence effect

In the E-last test, when the time of incubating the premix of I (inhibitor) with S (substrate) was varied from 0 to 10 min and the premix pH was constant at

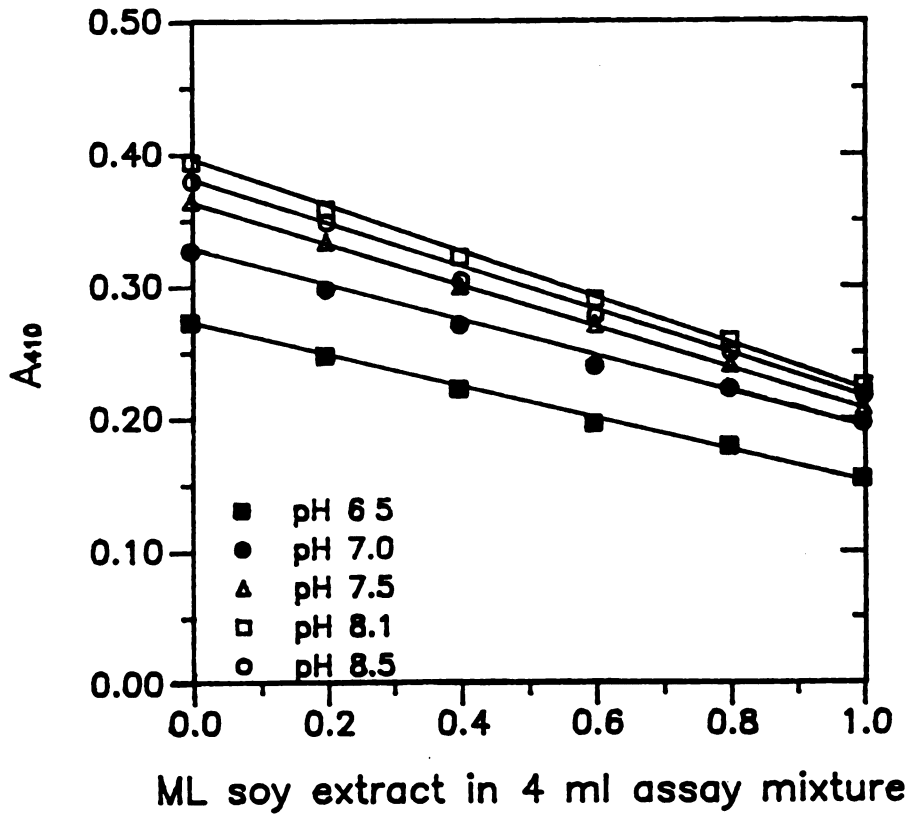

Fig. 10. Effect of assay buffer pH on the assay of soybean TIA.

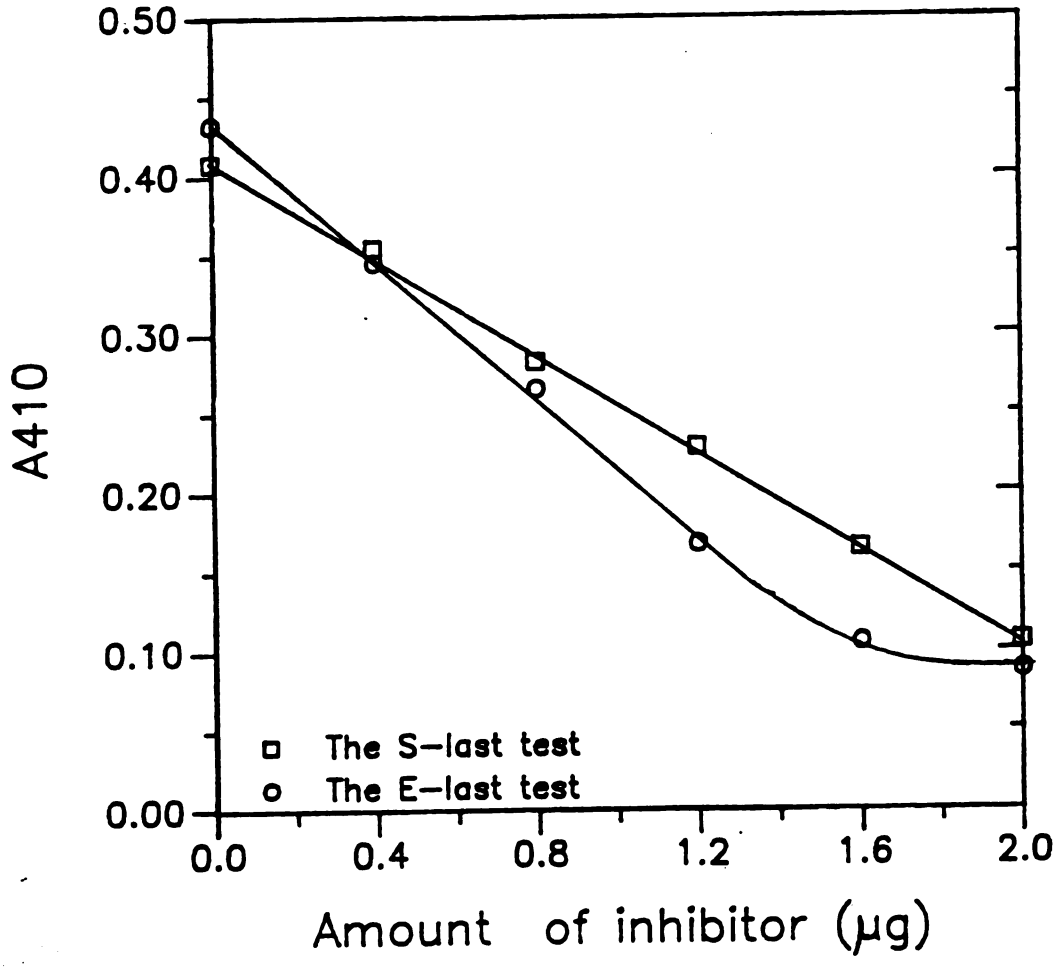

Fig. 11 Effect of the sequence of mixing the reactants on the assay of soybean Bowman-Birk trypsin inhibition. The premix pH was 3.5 and the preincubation time 3 min. Details of the tests are described under Materials and Methods.

3.5, the same trypsin inhibition was obtained (data not shown), indicating that the preincubation time in the E-last test had no effect on the trypsin inhibition assay. However, in the S-last test, when the time of incubating the premix of I with E was changed from 0 to 10 min, while the premix pH was fixed at 3.5, the extent of trypsin inhibition varied, indicating that the preincubation time in the E-last test had an effect on the trypsin inhibition assay.

At any particular preincubation time, the relative difference between the two tests was expressed as [(Ae-As)/Ae X 100%], where As is the trypsin inhibition obtained by the S-last, and Ae is the trypsin inhibition obtained by the E-last test. Since Ae remained constant regardless of the preincubation time, it was regarded as a reference. The data are presented in Fig. 12 with two pure inhibitors and show that, when the premix pH was constant at 3.5, the trypsin inhibitions obtained by the S-last test were always lower than those obtained by the E-last test, for the porcine trypsin-BB inhibitor system and the bovine trypsin-BB inhibitor system. At the beginning of preincubation, the relative difference between the two tests increased with time. After 3-5 min, the curves leveled off.

The data of Fig. 12 also indicate that the extent of this time-dependence feature of the reactant sequence effect varied between porcine and bovine trypsins. The maximum relative difference in BB inhibitor activity between the two tests was about 57% for the porcine trypsin and only 25% for the bovine trypsin.

ii. Effect of the premix pH on the reactant sequence effect

Like the preincubation time, the premix pH was also found to influence the reactant sequence effect of the trypsin inhibition assay. In the E-last test,

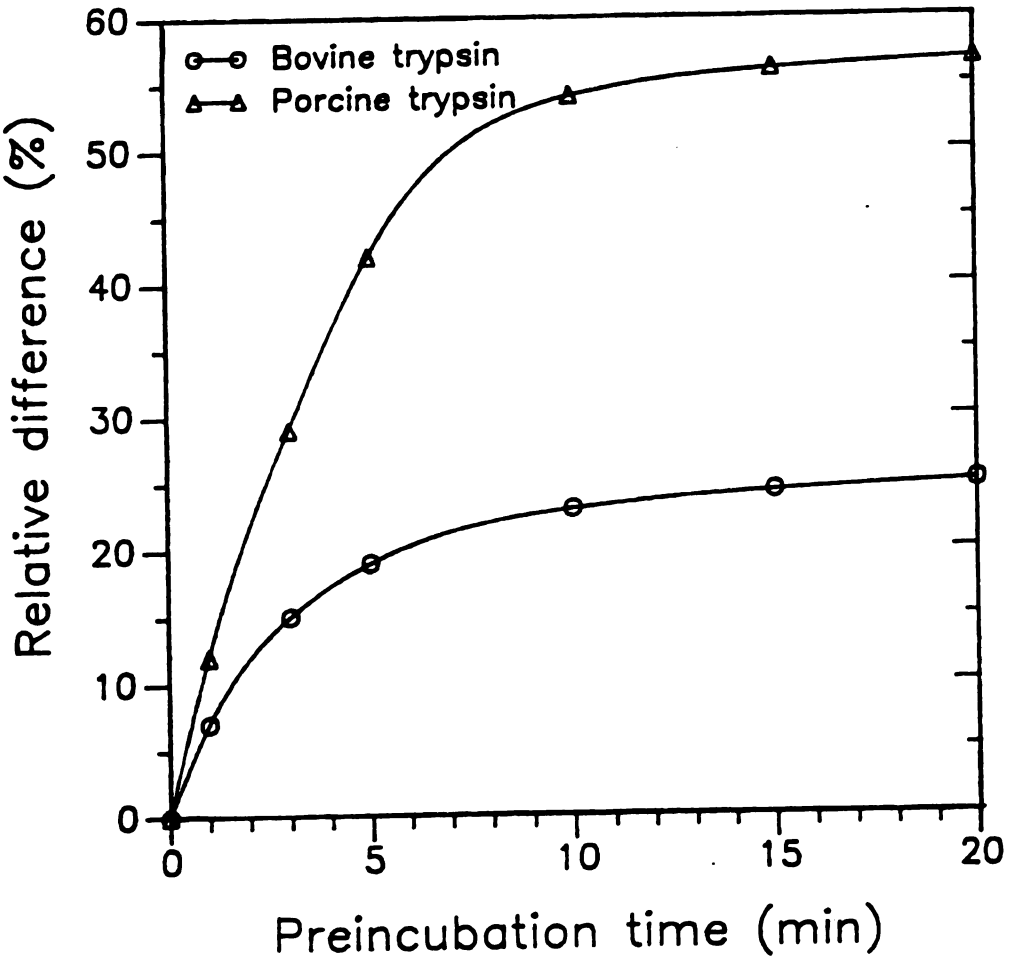

Fig. 12 Relative difference in soybean Bowman-Birk trypsin inhibition values obtained by the S-last and the E-last tests as a function of the preincubation time. The relative difference was expressed as [(Ae-As)/Ae x 100%], where Ae is the inhibition value obtained by the E-last test and As is the inhibition value by the S-last test. The pH of all premixes was 3.5. Details of the assay are described under Materials and Methods.

when the pH of the premix of I with S was varied from 2.7-9.0 and the preincubation time was held at 10 min, the same trypsin inhibition values were obtained, indicating that the premix pH in the E-last test had no effect on the trypsin inhibition assay. While in the S-last, when the pH of the premix of I and E was varied from 2.7 to 9.0 and the time of premix incubation was kept constant at 10 min, different trypsin inhibitions were found. The relative differences in porcine trypsin inhibitions measured by the two tests were plotted against the premix pH (Fig. 13). The results indicate that the trypsin inhibitions obtained through the S-last test were either equal to or lower than those through the E-last test, depending on the premix pH. At the pH = 2.7, the S-last test estimated the same inhibition values as the E-last test. When the pH increased to 3.5, a maximum difference was observed, indicating that the S-last test gave the lowest values. Above pH = 4.0, the difference decreased sharply. Over the neutral pH range, the two tests gave the same results again. At slightly alkaline pH range, a second peak was observed and, at pH around 8.5, the difference between the two tests began to drop again. The two inhibitors, Kunitz and BB inhibitors, showed the same pH-dependence pattern, but the Kunitz inhibitor was affected less by pH and its first peak shifted to the more acidic side.

When bovine trypsin was used to measured the trypsin inhibition of the inhibitors, the patterns of the relative difference between the two tests as a function of pH were similar to that with porcine trypsin, except that the acidic pH peak was less pronounced and the difference between the two inhibitors was negligible (Fig. 14).

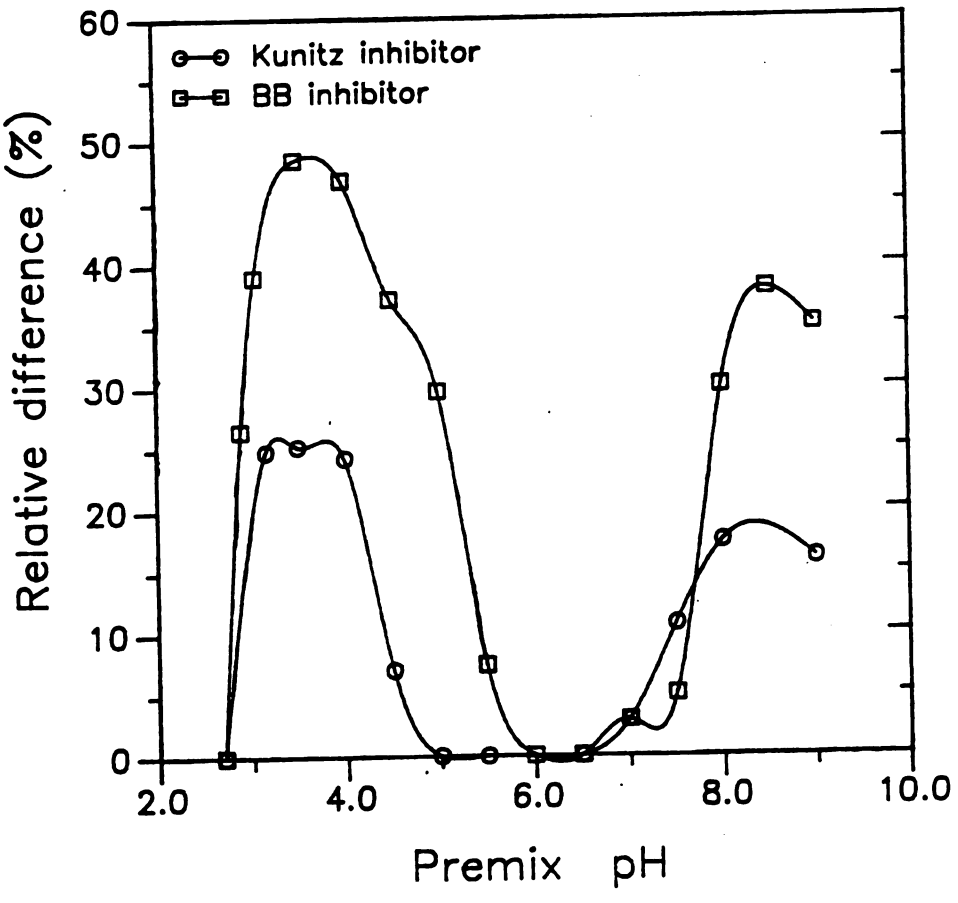

Fig. 13 Relative difference in porcine trypsin inhibition values obtained by the S-last and the E-last tests as a function of the premix pH. The relative difference was expressed as [(Ae-As)/Ae x 100%], where Ae is the inhibition value obtained by the E-last test and As is the inhibition value by the S-last test. The preincubation time was constant at 10 min. Details of the assay are described under Materials and Methods.

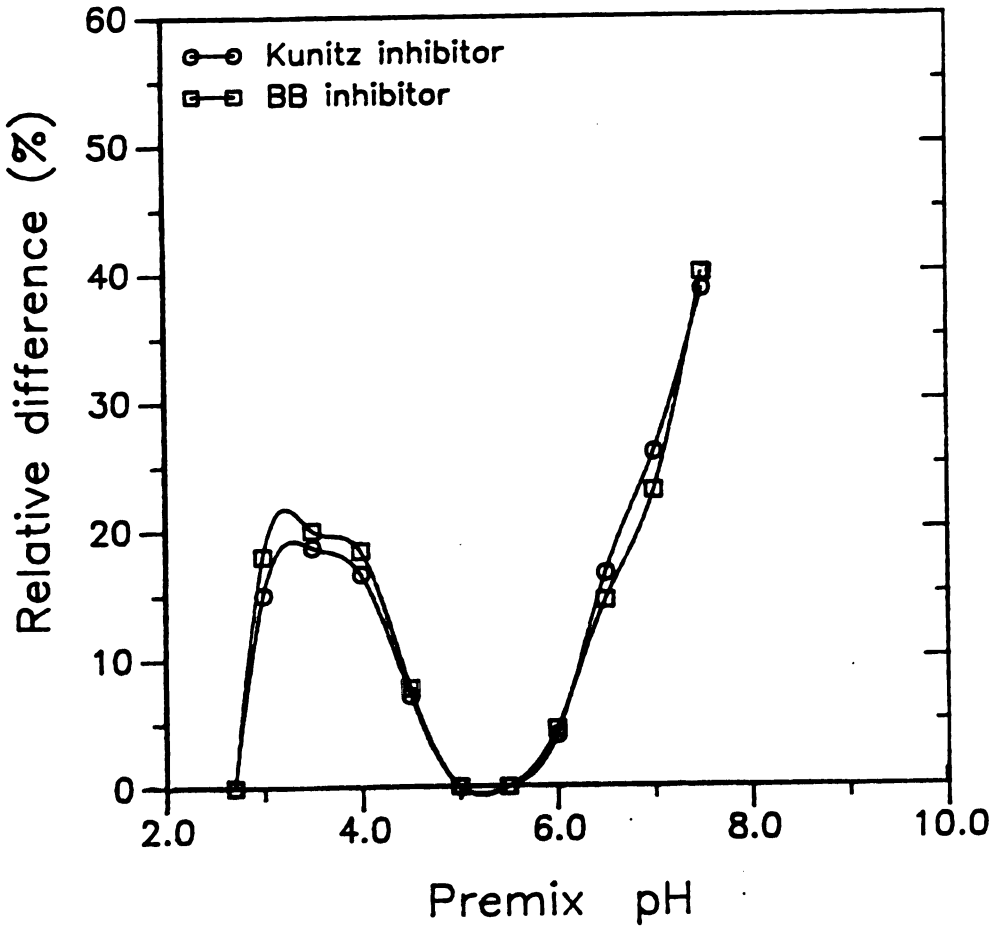

Fig. 14 Relative difference in bovine trypsin inhibition values obtained by the S-last and the E-last tests as a function of the premix pH. The relative difference was expressed as [(Ae-As)/Ae x 100%], where Ae is the inhibition value obtained by the E-last test and As is the inhibition value by the S-last test. The preincubation time was constant at 10 min. Details of the assay are described under Materials and Methods.

iii. Jumping the premix pH

We have shown that the E last test gave the same trypsin inhibition value regardless of premix pH and preincubation time while the S-last test did not. Regarding the premix pH, for the porcine-BB inhibitor system, the trypsin inhibition values obtained by the S-last test were either equal to the reference value (E-last value) when the premix pH was less than 2.7 or near neutral, or lower than the reference value when the pH was 2.7-5.5 or 7.5-9.0. Here, premix pHs which are associated with the manifestation of a reactant sequence effect are considered effective pHs, while those resulting in no sequence effect are considered noneffective pHs. A separate study was conducted to see whether jumping the premix pH in the S-last test from effective to noneffective levels during preincubation can restore the inhibitory capacity of the S-last test to that of the E-last test. The results of Fig. 15 indicate that in the S-last test, 10 min preincubation at pH 3.5 followed by 10 min preincubation at pH 6.5 restored the inhibition capacity to that of the E-last test (same slopes). So did the 10 min preincubation at pH 9.0 followed by 10 min preincubation at pH 6.5 (Fig. 16). However, Fig. 17 shows that 10 min preincubation at pH 3.5 followed by 10 min preincubation at pH 2.5 did not restore the inhibition capacity to that of the E-last test.

Note that jumping the premix pH in the E-last test was not tried since the premix pH has no effect on the trypsin inhibition assay in the E-last test.

iv. TIA assay as related to limited hydrolysis of inhibitors

Two hypotheses are possible to explain the reactant sequence effect observed in this experiment: (a) that an interaction between I and S occurs in the E-last test, resulting in increased trypsin inhibition in this test over the S-last

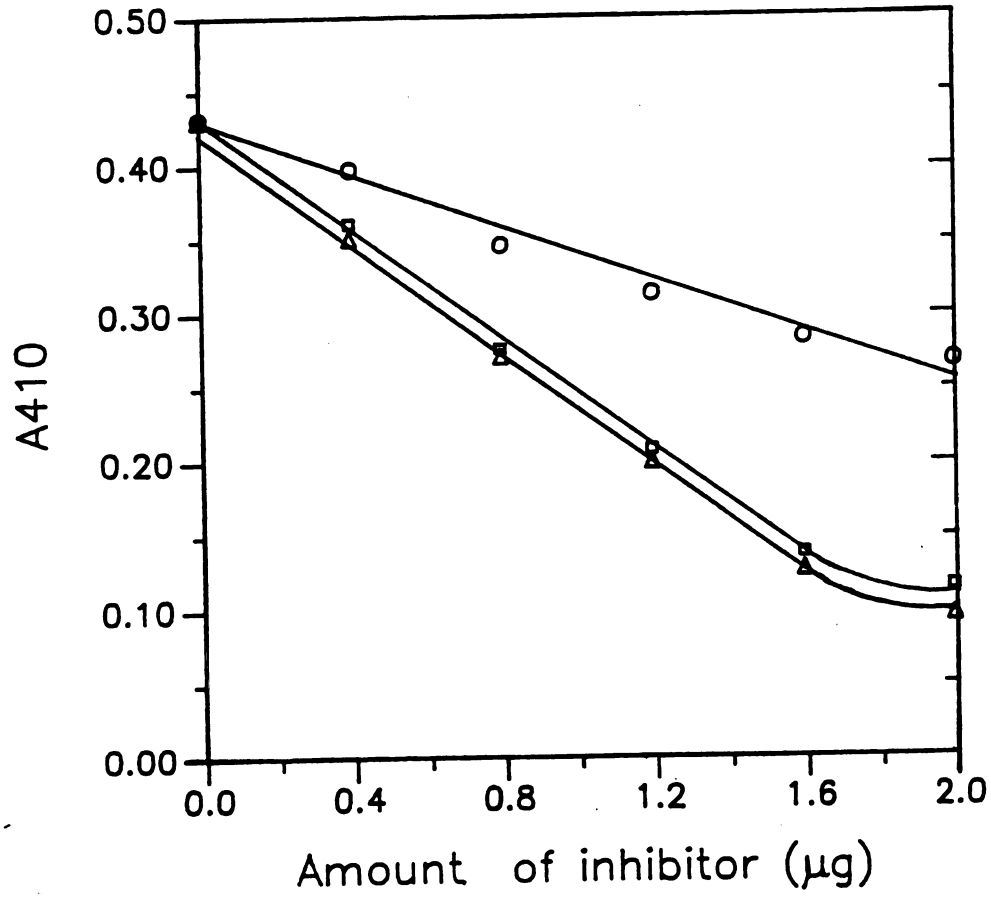

Fig. 15 Jumping the premix pH from 3.5 to 6.5 during assaying porcine trypsin inhibition of soybean BB inhibitor.

⊙——⊙ S-last test, a 20 min preincubation at pH 3.5; ⊡——⊡ S-last test, a 10 min preincubation at pH 3.5 followed by a 10 min premix incubation at pH 6.5. pH jumping was carried out by adding 1 ml 40 mM Tris buffer, pH 8.0 to 1.5 ml of premix (20 mM acetate buffer, pH 3.5) △——△ E-last test, a 20 min preincubation at pH 3.5. All tests were finally run at pH 8.1 ± 0.2 for the ten-minute enzymic reaction.

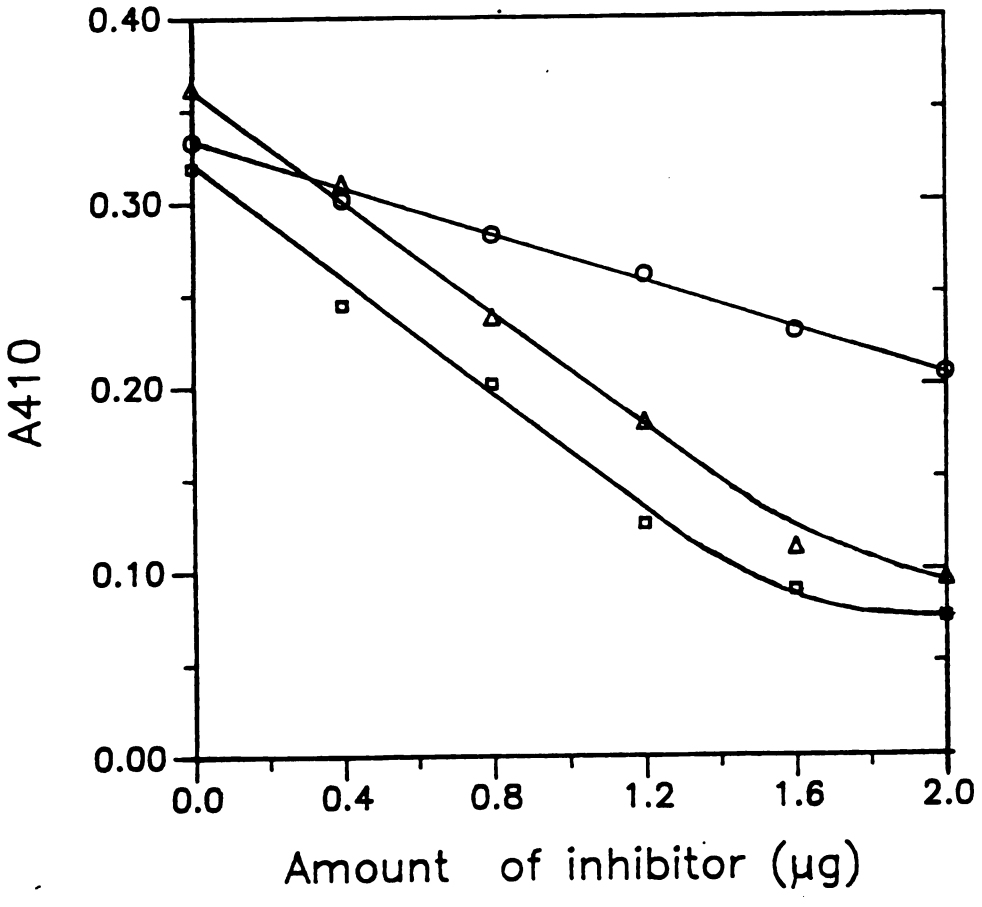

Fig. 16 Jumping the premix pH from 9.0 to 6.5 during assaying porcine trypsin inhibition of soybean BB inhibitor.

⊖——⊖ S-last test, a 20 min preincubation at pH 9.0; ⊡——⊡ S-last test, a 10 min preincubation at pH 9.0 followed by a 10 min premix incubation at pH 6.5. pH jumping was carried out by adding 1 ml 0.03 N HCl, pH 1.9 to the 1.5 ml premix (20 mM Tris buffer, pH 9.0)

△——△ E-last test, a 20 min preincubation at pH 9.0. All tests were finally run at pH 8.1 ± 0.2 for the ten-minute enzymic reaction.

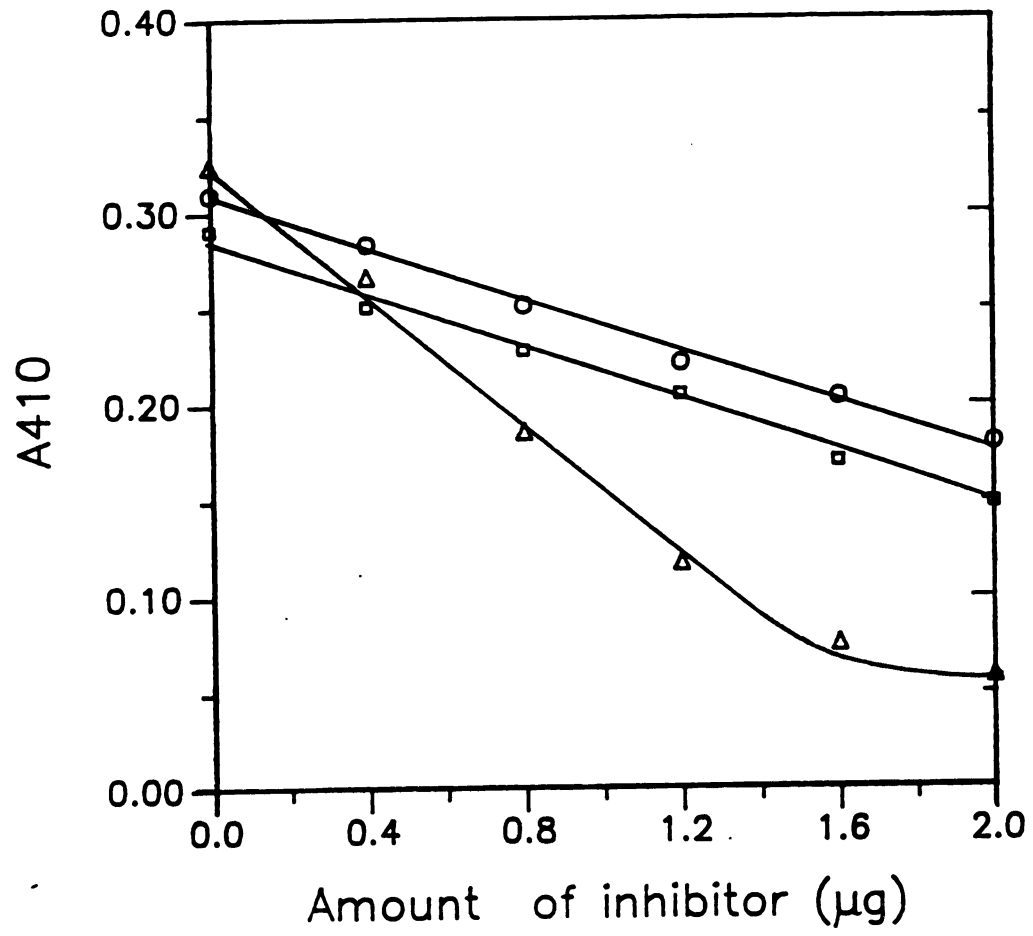

Fig. 17 Jumping the premix pH from 3.5 to 2.5 during assaying porcine trypsin inhibition of soybean BB inhibitor.

⊙——⊙ S-last test, a 20 min preincubation at pH 3.5; ⊡——⊡ S-last test, a 10 min preincubation at pH 3.5 followed by a 10 min premix incubation at pH 2.5. pH jumping was carried out by adding 1 ml 0.02 N HCl, pH 2.3 to 1.5 ml of premix (20 mM acetate buffer, pH 3.5)

△——△ E-last test, a 20 min preincubation at pH 3.5. All tests were finally run at pH 8.1 ± 0.2 for the ten-minute enzymic reaction.

test; and (b) that an interaction between I and E occurs in the S-last test, resulting in decreased trypsin inhibition in this test compared to the E-last test.

Hypothesis (a) is readily rejected by the fact that the E-last test gives the same trypsin inhibition regardless of the premix pH and the preincubation time, under which S and I could interact.

Hypothesis (b) remains the only one to explain the lower inhibition values observed in the S-last test and it happens to be in accordance with the reactive site model of Laskowski, Jr. (21), that trypsin is capable of attacking its own inhibitors as if they are substrates. In the S-last test, where I is premixed with E in a near equimolar ratio, at a relative high temperature (37 C), a conversion of I to I^* would occur during the period of preincubation. Thus, the reactant sequence effect observed in this study is attributed to a limited hydrolysis of I by the enzyme it inhibits into I^*, which has the same reaction activity as I, but lower affinity (association constant) towards trypsin (1,40).

Assuming that $[I]_0$ is the concentration of total virgin inhibitor, the following relationship would exist after preincubation time t:

$$[I]_0 = [I]_t + [I^*]_t \qquad [1]$$

where, $[I^*]_t$ is the concentration of I^* produced during preincubation and $[I]_t$ is the concentration of I remained.

Also assuming that a is the trypsin inhibition per unit concentration of I and b is the trypsin inhibition per unit concentration of I^*, then, in the E-last test we measured the total virgin inhibitor activity, $(a[I]_0)$, while in the S-last test we measured the activities of both I and I^*, $(a[I]_t + b[I^*]_t)$.

If b<a, that is, I^* is less active towards trypsin than I, then

$$(a[I]_t + b[I^*]_t) < (a[I]_0) \ , \qquad [2]$$

indicating that there is a reactant sequence effect.

If b=a, that is, I^* is as active as I towards trypsin, then

$$(a[I]_t + b[I^*]_t) = (a[I]_0), \qquad [3]$$

indicating there is no reactant sequence effect.

Here we should point out that previous studies showed that the hydrolysis rate of inhibitors by trypsin (10^{-2}-10^{-3} sec^{-1}) was much slower than that of common peptides (about 1 sec^{-1}) (Laskowski et al 1974) and that an observable amount of conversion of I into I^* took hours (41,47-48). Yet, results of this study showed that even though the preincubation lasted minutes, a difference in inhibition values between the two tests was observable, indicating significant conversion of I to I^*. The discrepancy may be due to differences in reaction systems. For example, in previous studies, the enzyme was used in a catalytic amounts (molar ratio of enzyme to inhibitor, 1:50 or 1:100) at 25 C, 20 C or 4 C, while in this study, almost stoichiometric amounts of enzyme and inhibitor were used at 37 C.

Evidence of the limited proteolytic cleavage of the inhibitors by trypsin has been demonstrated a) by the appearance of two bands on an analytical disc gel electrophoresis of a preparation of inhibitor preincubated with trypsin (41,48), and b) by the fact that Sephadex chromatography of pure RCM (reduced carboxymethylated) virgin inhibitor produced one component of high molecular weight, while that of pure RCM modified inhibitor produced two components of smaller molecular weights (21,47).

One of interesting findings of the inhibitor research was that the hydrolysis of the reactive site peptide bond does not proceed to completion. Instead, an equilibrium between I and I^* is established (29,41,48,73). In Fig. 12, the fact that the relative difference between the two tests approached a maximum probably indicates that the systems were near equilibrium.

The rate constant k_{cat} for the hydrolysis of I into I^* was found pH-dependent (41,43,73). It would therefore be expected that the trypsin inhibition assayed by the S-last test is dependent on the premix pH. This was the case in this study.

Since hydrolysis of inhibitor by trypsin is very similar to that of a normal peptide bond except for a slow reaction rate (29,41,73), if the peptide bond hydrolysis does not perturb the pK values of any preexistent ionizable groups on the inhibitor, the pH dependence of K_{hyd} for conversion of I to I^* can be expressed as

$$K_{hyd} = K^o_{hyd} (1 + [H^+]/K_1 + K_2/[H^+]) \qquad [4]$$

where K^o_{hyd} is the minimal value of K_{hyd} corresponding to the hydrolysis to fully ionized products, and K_1 and K_2 are the ionization constant of the newly formed COOH and NH_4 terminals (74).

As stated before, the Y-axis values in Figs. 13 and 14 represent the following equation:

$$Y = \{a[I]_o-(a[I]_t + b[I^*]_t)/(a[I]_o). \qquad [5]$$

Substituting $[I]_t$ from [1],

$$Y = \{((a-b)/a).([I^*]_t/[I]_o\} \qquad [6]$$

Thus, the relative difference in Y-axis values of Figs. 13 and 14 did not exactly represent the K_{hyd} value, which is expressed as

$$K_{hyd} = [I^*]_e/[I]_e \qquad [7]$$

where $[I]_e$ and $[I^*]_e$ are concentrations of virgin and modified inhibitors at equilibrium, respectively. However, when t reaches equilibrium time, eq. [6] become

$$Y = \{((a-b)/a).([I^*]_e/[I]_o\}. \qquad [8]$$

From eqs. [1] [7] and [8], we know that Y is a monotonically increasing function of K_{hyd}.

$$Y = \{(a-b)/a\}.\{K_{hyd}/(1 + K_{hyd})\} \quad [0 < (a-b)/a < 1] \quad [9]$$

Therefore, the pH dependence patterns of the reactant sequence effect shown in Figs. 13 and 14 should follow eq. [6]; that is, Y should rises sharply at both low and high pH levels. The exception noticed in the porcine trypsin-Kunitz inhibitor system might be due to the finding of Mattis and Laskowski (42) that the pH dependence of K_{hyd} for hydrolysis of the Arg^{63}-Ile reactive site peptide bond of soybean Kunitz inhibitor was complicated by His^{71} perturbation.

The less pronounced acidic peak observed in the bovine trypsin-inhibitor system (Fig. 14), as compared with the porcine trypsin-inhibitor system (Fig. 13), might due to (i) slower conversion of I to I^* by bovine than porcine trypsin (lower K_{cat}), or (ii) smaller difference between the inhibitions of I^* and I on bovine trypsin than between inhibitions on porcine trypsin (smaller value of a-b), or (iii) combination of both (i) and (ii). The sharp rise observed starting at pH 7.0 with the bovine trypsin is mainly due to instability of bovine trypsin in alkaline media (27). As a result, the A_{410} readings in the absence of inhibitor were close to or even lower than those of the readings in the presence of inhibitor in the S-last test, because binding of the inhibitor to the enzyme protected the latter from inactivation.

Studying the interaction of soybean Kunitz inhibitor with trypsin, Ozawa and Laskowski, Jr. (21) found that a single Arg-Ile bond was split catalytically and Finkenstadt and Laskowski (75) demonstrated that the split bond could be resynthesized in the presence of an equimolar amount of enzyme under certain conditions. Similarly, studies on the interaction of soybean BB inhibitor with trypsin (46) showed a close parallel with the trypsin-soybean Kunitz inhibitor case. Their conclusion was that, under certain conditions, for the conversion of I to I^*, a catalytic amount of trypsin is needed while in the resynthesis of I

from I^*, equimolar quantity of the enzyme is required, since trypsin serves both as a catalyst and as supplier of the driving force for the resynthesis. The recovery of trypsin inhibition capacity in the S-last test to that in the E-last test as shown in Figs. 15 and 16 would indicate resynthesis of I from I^*, granted that near equimolar amounts of trypsin and inhibitors were used. It appears that, at certain pH ranges (2.7-5.5 and 7.5-9.0), hydrolysis of I prevails over its resynthesis while at another pH range (near neutral), resynthesis of I is the dominant process. The pH dependence of the trypsin inhibition obtained by the S-last test is actually determined by the relative rate between the conversion of I to I^* and the resynthesis I from I^* by trypsin.

It was not surprising that the 10 min premix incubation at pH 3.5 followed by 10 min premix incubation at pH 2.5 did not restore the inhibition capacity, as shown in Fig. 17, since at pH below 2.7, the EI complex completely dissociates (8). Thus, at this pH range, I^* could not be converted back to I and vice versa.

Because of the low concentration (about 10^{-6} M) of the inhibitors used in this assay system, direct evidence by electrophoresis or by chromatography for the limited hydrolysis of the inhibitors is difficult. Lyophilization and dialysis of the premixes would change the pH and shift the I^*/I equilibrium.

In summary, this study has shown that the S-last test gives trypsin inhibition values which depend on the premix pH and preincubation time. Since the E-last test does not depend on these conditions, it is preferable to the S-last test for assaying trypsin inhibitors of protein nature.

As most protease inhibitors are also of protein nature, studies are needed to verify if their assays are affected by the reactant sequence or not. In part 2, we will discuss the effect of the reactant sequence on the chymotrypsin inhibition assay.

III. Modification of the Current Method for Determining TIA in Soybeans

After extensive investigation on the effects of general assay conditions and the reactant mixing sequence on the assay of two pure soybean trypsin inhibitors, the current method (13,14) for determining the antitryptic activity of soybean products is significantly modified. Details are discussed below.

i. Extracting the inhibitors

Four solvents were compared for their ability to extract the greatest amount of trypsin inhibitors from both raw and cooked soybean samples and for ease of sample cleanup: 0.01N NaOH solution (pH about 10.0), 0.001 N HCl solution (pH about 2.5), the assay buffer (pH 8.2) and distilled water (pH about 6.5). The ratio of dry sample to all solvents was 0.5 g/50 mL. The sample was extracted with each solvent for three time intervals: 30, 60 and 120 min. The results are summarized in Table 2 and indicate that distilled water, the assay buffer and NaOH solution are equally efficient extractants and better than the HCl solution. The NaOH extract was not destabilized by subsequent addition of the assay buffer and therefore could not be filtered. The values shown in Table 2 for this extract have been obtained by filtration after the enzyme reaction. Water is preferable over the assay buffer, as aqueous extracts are more readily destabilized by mixing with an equal volume of the assay buffer. After filtration, a clear and colorless solution is obtained, which is ready for further dilution. Since shaking for periods greater than 30 min did not increase the amount of extracted inhibitors when water was used as extractant, a 30 min shaking is considered adequate. In the current method of Smith et al.(13), a triple choice is given: 2 min homogenization, 3 hr stirring and overnight

Table 2 Extraction of trypsin inhibitors from raw and cooked soybeans by various solvents and different shaking times *

Extractants	Shaking time (min)		
	30	60	120
Raw soybeans			
0.01N NaOH solution (pH 10.0)	-	-	172.2^{a}
0.001N HCl solution (pH 2.5)	162.1^{a}_{x}	170.4^{a}_{y}	169.4^{a}_{y}
The assay buffer (pH 8.2)	169.4^{b}_{x}	168.8^{a}_{x}	172.6^{a}_{x}
Distilled water (pH 6.5)	171.0^{b}_{x}	170.0^{a}_{x}	171.3^{a}_{x}
30 min boiled soybeans			
0.01N NaOH solution (pH 10.0)	-	-	-
0.001N HCl solution (pH 2.5)	18.7^{a}_{x}	19.6^{a}_{xy}	20.1^{a}_{y}
The assay buffer (pH 8.2)	23.1^{b}_{x}	23.7^{b}_{x}	24.2^{b}_{x}
Distilled water (pH 6.5)	24.1^{b}_{x}	23.6^{b}_{x}	24.3^{b}_{x}

*. Means of duplicate measurements as TUI/mg dry sample. The data were statistically analysed using analysis of variance in a factorial design. Separation of means was conducted using the Least Significant Difference at the 5% level of probability.

a-b Column means bearing different superscripts differ significantly.

x-y Row means bearing different subscripts differ significantly.

soaking.

ii. Sample cleanup before the reaction

In the current method, a dilute NaOH solution is used for extracting soybean samples. The extract is a rather stable suspension and it is used as is in running the enzymatic reaction. The reaction mixture is filtered after addition of acetic acid and measured photometrically. In the proposed modification, the soy sample is extracted with water and the extract is destabilized with the assay buffer and filtered before further dilution for the enzymic reaction. Trials were made to test whether filtering before or after the color reaction gave the same TIA values. Fig. 18 shows that the two procedures produced the same inhibition value (same slope of lines connecting A_{410} to quantity of sample inhibitors). The lower color readings obtained from the samples filtered after the enzyme reaction are probably due to sorption of p-nitroaniline by the filter paper. Sample cleanup before the enzyme reaction not only gave the higher color reading but also made it possible to reduce the volume of the reaction mixture (see Section vi).

Two clarifying agents, the assay buffer, pH 8.2, and 20 mM acetate buffer, pH 3.5, as well as two filter papers, Whatman No. 2 and No. 5 were compared for the extract cleanup. The results are summarized in Table 3 and indicate that combining the assay buffer with No. 2 filter paper results in the highest TIA value for the soy sample.

iii. Choosing a proper sample dilution

It has been shown that when trypsin activity is plotted against levels of inhibitor, the activity deviates from linearity at high levels of inhibitors (13-14,16). Because of this characteristic, both the AACC (64) and the current

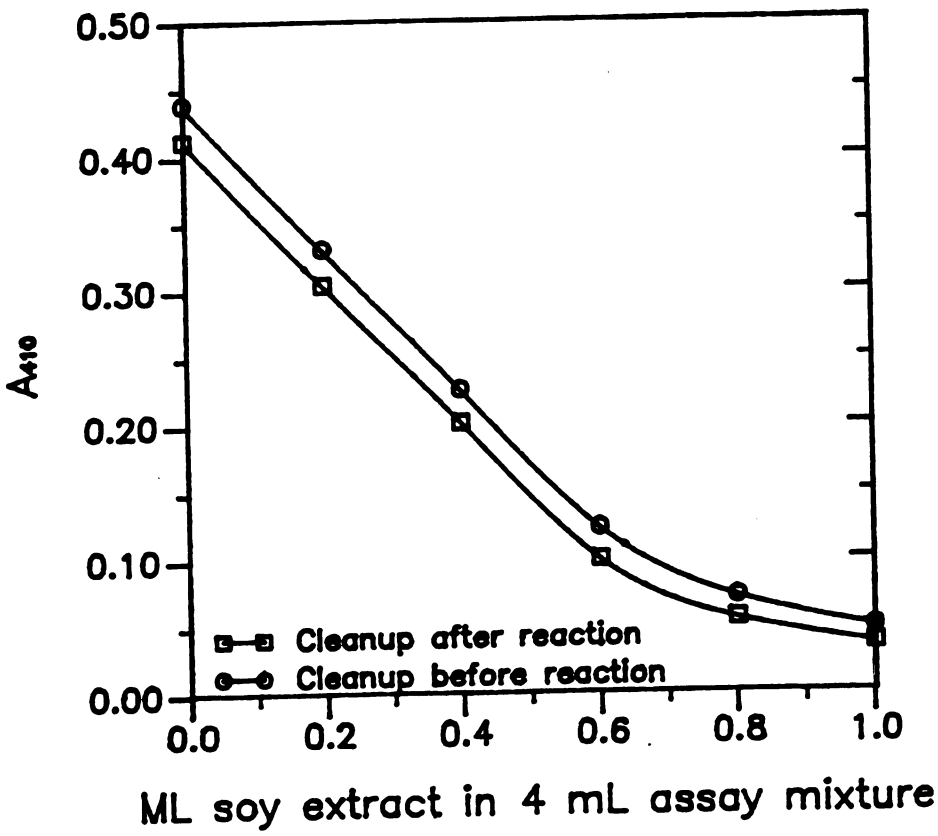

Fig. 18 Effect of cleanup of soy extracts before or after the color reaction on the TIA assay

Table 3 Comparison between two clarifiers and two filter papers in the cleanup of a raw soybean extract for the TIA assay (TIA as TUI/mg dry sample)*

Clarifiers	Filter papers	
	No. 2	No. 5
The assay buffer, pH 8.2	173.5^{a}_{x}	167.6^{a}_{y}
20 mM Acetate buffer, pH 3.5	160.4^{b}_{x}	152.1^{b}_{y}

* Means of duplicate measurements on raw Corsoy cv. soybeans. The data were statistically analysed using analysis of variance in a factorial design. Separation of means was conducted using the Least Significant Difference at the 5% level of probability.

a-b Column means bearing different superscripts differ significantly.

x-y Row means bearing different subscripts differ significantly.

methods (13,14) call for use of a sample dilution which results in 40-60% trypsin inhibition. By using the modified procedure, we observed a similar curve (Fig. 18). It was further shown that the location of the curving varied with the source of enzyme and the kind of inhibitor samples. Table 4 summarizes these results which indicate that except for the porcine trypsin-BB inhibitor system, the other systems displayed inhibition curves leveling off at about 75% inhibition. The leveling-off of the trypsin inhibition curve is attributed to a partial dissociation of the trypsin-inhibitor complex (76).

These curving loci differed from those reported previously. E. g., when assaying raw soy extract with bovine trypsin, Kakade et al. (16) observed a curving locus at 55% of trypsin inhibition; Hamerstrand et al. (14), at 60%; in our study, the curving locus was at 75%. These differences might be due to assay system variables , such as Ca^{++} and buffer concentrations, soy sample cleanup, etc.

Theoretically, any raw sample dilution which results in less than 75% trypsin inhibition should produce the same TIA value. However, in practice, this was not observed. When 55 TIA measurements were performed on separate or common extracts from the same raw soybean sample, using various dilutions to represent widely different levels of trypsin inhibition, the results shown in Fig. 19 were obtained. TIA values corresponding to less than 30% trypsin inhibition are broadly scattered, probably because even small experimental errors are greatly enlarged when large dilution factors enter the calculations. The decline of TIA value above 75% inhibition is expected as it is determined by the characteristic inhibition curve.

With 55 independent measurements, the relative standard deviation (RSD) was ±3.5% when the dilution was within the range of 30-70% trypsin inhibition, while RSD became ±3% when the dilution range was 40-60% inhibition. The

Table 4. Curving loci in the line connecting trypsin activity and inhibitor concentration (refer to Fig. 18).

Samples	Curving loci as ranges of % inhibition*	
	Bovine trypsin	Porcine trypsin
Pure Kunitz inhibitor	84 - 87	75 - 78
Pure BB inhibitor	84 - 87	64 - 68
Raw soybean extract	74 - 78	74 - 76
Cooked soybean extract	84 - 86	83 - 86

* Triplicate measurements.

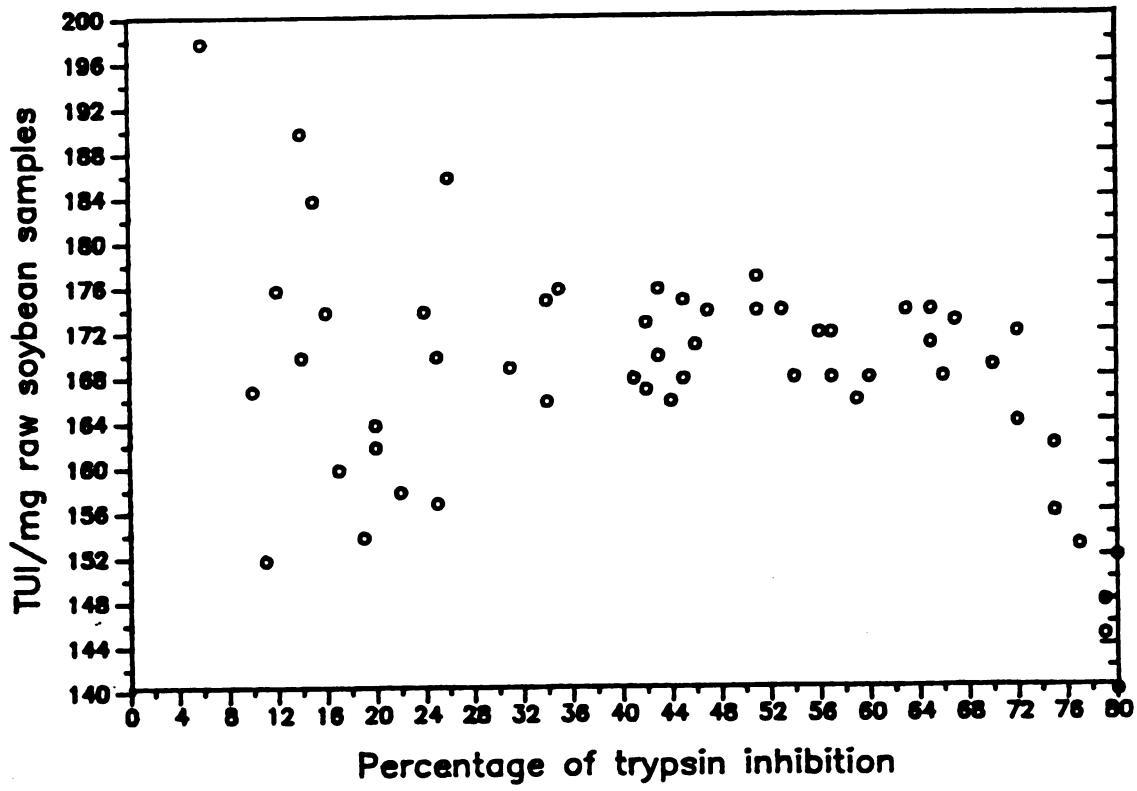

Fig. 19 Effect of degree of trypsin inhibition obtained by various dilutions of a raw soybean extract on the estimate of antitryptic activity in soybeans.

convenience of sample dilution corresponding to a 30-70% trypsin inhibition outweighs the benefit of the extra 0.5 RSD value accompanying a dilution to a 40-60% inhibition; therefore, the dilution to a 30-70% inhibition is recommended.

iv. Using porcine instead of bovine trypsin

Bovine trypsin is used for assaying trypsin inhibitors both in the AACC and the current methods, although it is unstable in alkaline solution (Buck et al 1962a). We observed that 10 min incubation at 37C, with pH as low as 7.5, resulted in a sharp decrease of bovine trypsin activity. Since TIA is commonly assayed at pH 8.1, which is the optimum for trypsin activity against BAPA (62), enzyme inactivation would be expected during the assay. On the other hand, porcine trypsin, like human trypsin, is relatively stable at alkaline pH (28) and should be more suitable for assaying TIA. Moreover, when the TIA of a soy extract was assayed with both enzymes, it was found that porcine trypsin was inhibited more than bovine trypsin (Fig. 20). In several comparative tests, the TUI/mL of soy extract tested with bovine trypsin was about 2/3 of that tested with porcine trypsin. Therefore, using porcine trypsin not only avoids autolytic enzyme inactivation during the assay but also increases the sensitivity of the measurement.

v. Using the E-last test

The reactant sequence effect was observed previously on the assay of the activity of two pure soybean trypsin inhibitors (Kunitz and Bowman-Birk). The same effect was also observed when a raw soybean extract was assayed for TIA (Fig. 21).

In the E-last test, when the time of incubating a premix of soybean

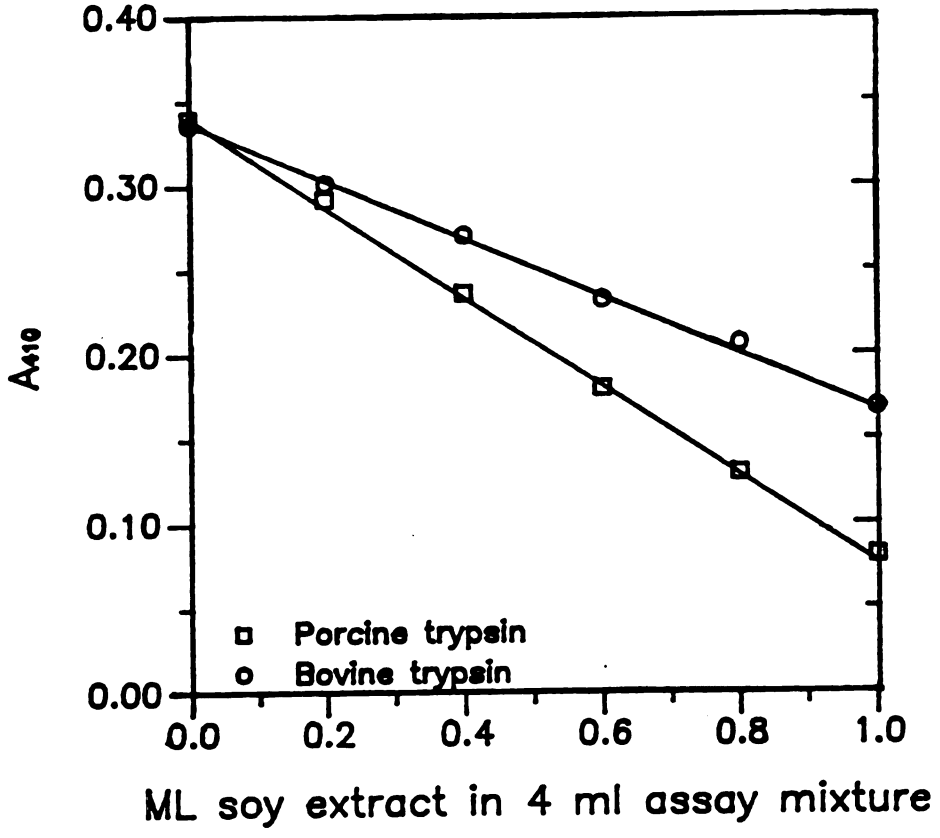

Fig. 20 Comparison of porcine and bovine trypsins for assaying TIA in soybeans

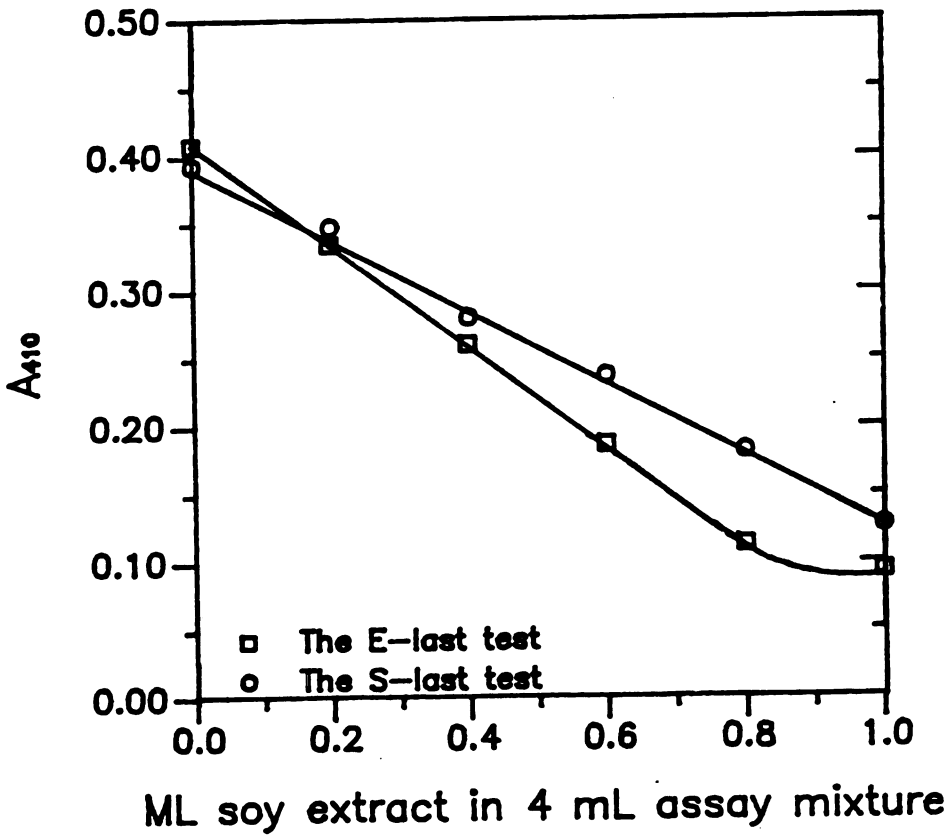

Fig. 21 Effect of the sequence of mixing the reactants on the assay of antitryptic activity in soybeans. In the S-last test, 0.5 ml of porcine trypsin solution prepared with 20 mM acetate buffer, pH 3.5, was premixed with 1.0 ml of sample solution prepared with the acetate buffer. Three min later, 2.0 ml of BAPA solution was added and the reaction was allowed to proceed for 10 min. In the E-last test, the enzyme was added 3 min after mixing the substrate with the sample solution.

extract with BAPA or the pH of this premix was varied, the same inhibition value was obtained, indicating that the preincubation time and premix pH had no effect on the TIA assay.

In the S-last test, when the time of incubating a premix of soybean extract with either porcine or bovine trypsin was varied, while the pH of this premix was fixed, the inhibition values obtained were different. The data are presented in Fig. 22, in which the relative difference between the S-last and the E-last tests was expressed as [(Ae-As)/Ae X 100%], where As is the TIA obtained by the S-last test and Ae is the TIA by the E-last test (since Ae remained constant regardless of the preincubation time, it was regarded as a reference). The results of Fig. 22 indicate: a) when the premix pH was 3.5, the TIA values obtained by the S-last test were always lower than those by the E-last test and the relative difference of the two tests was a function of preincubation time; in the first few min, the difference increased almost linearly with time and after about 5 min, the curve leveled off, b) the difference in TIA between the E-last and the S-last tests was greater for porcine trypsin than bovine trypsin.

Also in the S-last test, when the premix pH was varied, while the time of incubating this premix was fixed at 10 min, different TIA values were obtained (Fig. 23). The results indicate that, like preincubation time, the premix pH had an effect on the TIA assay in the S-last test. As the pH increased from 2.7 to 9.0, the S-last test estimated TIA values either equal to or lower than the E-last test. There were two peaks corresponding to the largest difference between the two tests, one on the acidic side and one on the alkaline side. For the bovine trypsin, the alkaline peak was incomplete, as this enzyme is unstable above pH 7.5. Again, the difference in TIA between the E-last and S-last tests is greater for the porcine than the bovine trypsin.

Under certain conditions, the lower inhibition observed in the S-last test

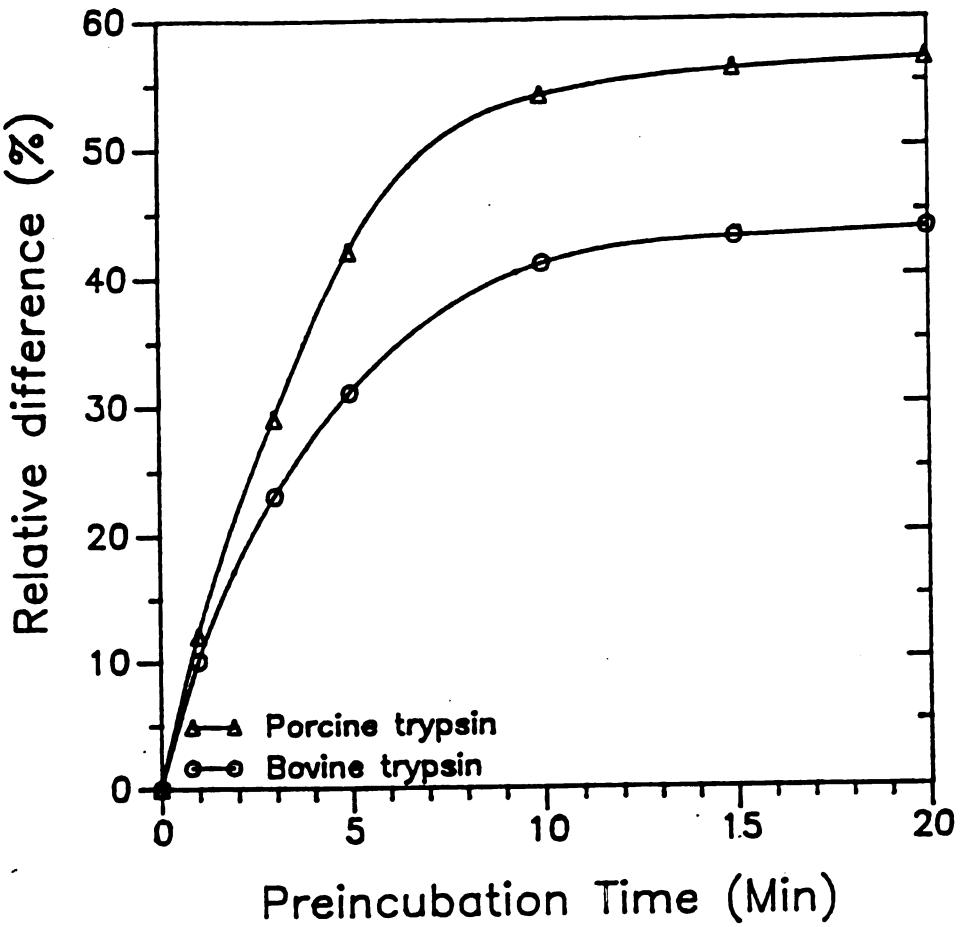

Fig. 22 Relative difference in TIA obtained by the S-last and the E-last tests as a function of the preincubation time. The relative difference is expressed as (Ae-As)/Ae X 100%, where Ae is the TIA obtained by the E-last test and As is the TIA by the S-last test. The premix pH was kept constant at 3.5, while the preincubation time varied from 0 to 20 min.

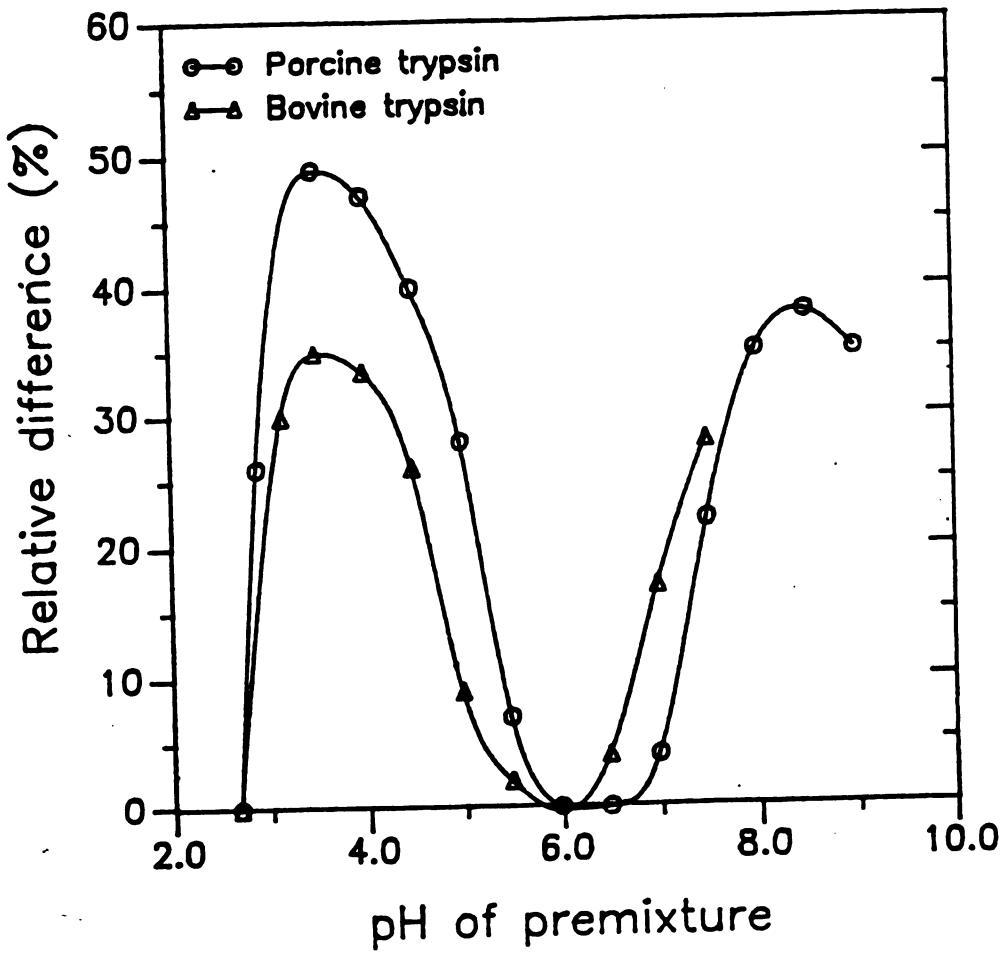

Fig. 23 Relative difference in TIA obtained by the S-last and the E-last tests as a function of the premix pH. The relative difference is expressed as (Ae-As)/Ae X 100%, where Ae is the TIA obtained by the E-last test and As is the TIA by the S-last test. The preincubation time was kept constant at 10 min, while the premix pH varied from 2.7 to 9.0.

was attributed to a limited hydrolysis of the inhibitor by the trypsin it inhibits, in accordance with the reactive site model (21). It is interesting to note that, although aqueous soy extract contains both Kunitz and BB inhibitors (2), the pattern of the reactant sequence effect on its TIA assay was different from that of either of the two pure inhibitors. This was true particularly with bovine trypsin. For example, the changes in the relative difference between the E-last and the S-last tests as functions of preincubation time and premix pH were more pronounced for the bovine trypsin-soy extract combination (Figs. 22 and 23) than for the trypsin-Kunitz inhibitor or the trypsin-BB inhibitor combinations studied previously (Figs. 12, 13 and 14).

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

vi. Reducing the volume of the reaction mixture

Two different assay (reaction mixture) volumes, 4 mL and 8 mL, were compared for estimating the TIA of a soy extract. In the 4 mL assay, the procedure used was the same with that described in the METHODS section. In the 8 mL assay, the same procedure was used except for doubling the volume of each reactant solution. The results of Fig. 24 were obtained. As the concentration of all reactants in the two assay systems is the same, twice the amount of soy extract

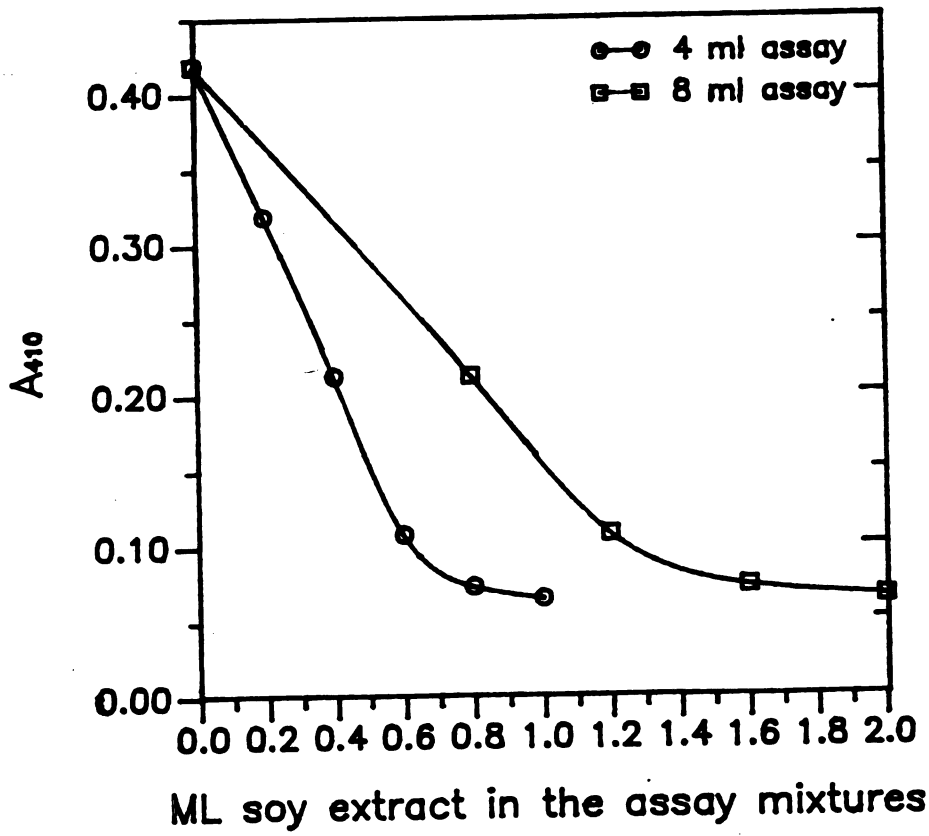

Fig. 24 Effect of the reaction mixture volume on the measurement of antitryptic activity in soybeans. In the 4 ml assay, 1 ml of sample solution was used; in the 8 ml assay, 2 ml of sample solution was used and all other reagents were doubled.

present in the 8 mL assay will be needed to cause the same level of trypsin inhibition as in the 4 mL assay. Thus, when TIA is expressed as TUI/mL of soy extract, the number expressing the inhibition will be twice as large when the 4 mL assay is used as when the 8 mL assay. E.g., from Fig. 24, for 0.4 mL soy extract, the TUI/mL derived from the 4 mL assay is

$$(0.42-0.22)/0.4 \times 100 = 50,$$

while for the 8 mL assay is

$$(0.42-0.32)/0.4 \times 100 = 25.$$

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

vii. Expressing TIA

Kakade et al (16,20) arbitrarily defined a trypsin unit (TU) as causing an increase of 0.01 absorbance at 410 nm per 10 min and TIA was measured as trypsin units inhibited (TUI) or trypsin inhibitor units (TIU) per mg sample. The advantage of this expression is its independence of the purity of trypsin used in the assay. However, for comparative purposes, Kakade et al. (16) also expressed the TIA in terms of the absolute amount of pure trypsin inhibited. This was done by referring to a standard curve relating absorbance or (TU) to trypsin concentration. It was calculated that 1 ug pure bovine trypsin has 1.9 TU. Hamerstrand et al. (14) attempted to express TIA in terms of mg trypsin inhibitor per g sample, calculated on the assumption that 1 μg trypsin is equivalent to 1 μg trypsin inhibitor, while Smith et al. (13) stated that the expression in mg trypsin inhibitor has no advantage over that in mg trypsin inhibited. Since the actual molar concentration of enzyme or inhibitor is difficult to determine and the amount of inhibitor protein does not represent

its activity, in order to standardize the reporting of the inhibitor activity, in the modified method, we used the standard enzyme unit defined by the Commission on Enzyme of the International Union of Biochemistry. One International Unit of enzyme is the amount that catalyzes the formation of 1 μ mole of product per min under defined conditions. As the molar absorption coefficient (a_m) of p-nitroaniline at 410nm was found to be 7760 in this study, one TU is equivalent to 0.000516 IU under the assay conditions specified. We therefore express TIA in terms of both TUI (trypsin units inhibited) and IUI (International Units Inhibited).

viii. Applying the modified method to some legume products

The TIA in some commercial soy products and legume seeds was measured according to both the current and the modified procedures. The results are presented in Table 5. Comparison of the two methods indicates that the modified procedure estimates a) much higher values when the TIA is expressed as TUI/mg sample, and b) higher values when the TIA is expressed as IUI/g sample. The modified method also reduces the relative standard deviation of the estimates. In summary, the proposed modification for measuring TIA in soybean products has a theoretical basis (reactant sequence effect as related to limited hydrolysis of inhibitors) and a practical significance. It can eventually be used for measuring TIA in many other proteinaceous food products.

Table 5 TIA in some commercial soy products and legume seeds assayed by both the current and the modified methods [a]

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

[a] Mean of duplicate measurements ± S.D.

[b] TUI, Trypsin Units Inhibited, where one TU is defined as 0.01 A_{410} per 10 min reaction, under the assay conditions of the current method (pH 8.1 at 37C, with 10 mL assay volume and bovine trypsin).

[c] IUI, International Units Inhibited, where one TU is equivalent to 0.00129 IU under the assay conditions in (b).

[d] One TU is defined as 0.01 of A_{410} per 10 min reaction, under the assay conditions of the modified method (pH 8.1 at 37C, with 4 mL assay volume and porcine trypsin).

[e] One TU is equivalent to 0.000516 IU under the assay conditions in (d).

Part 2 CHYMOTRYPSIN INHIBITION ASSAY

I. Methodology

i. Enzyme activity vs enzyme concentration

Bundy (15) showed that the rate of liberation of p-nitroaniline as a function of chymotrypsin concentration was linear over a certain range. We observed a similar relationship. The results of Fig. 25 indicate that A_{385} was proportional to chymotrypsin concentration up to 35 μg in a 4 mL-reaction mixture. At zero enzyme concentration, A_{385} was nonequal to zero with water as a blank. In the proposed method, 16 μg of enzyme was used, which was in the linear range of Fig. 25.

ii. Enzyme activity vs reaction time

Fig. 26 shows the A_{385} as a function of the reaction time both in the absence and the presence of the soybean BB inhibitor. The results indicate that the enzyme activity was proportional to the reaction time over the period studied. In the assay, 10 min reaction time was adopted. Again since water was used as a blank, A_{385} was nonequal to zero at zero reaction time.

iii. Chymotrypsin inhibition curve

The decrease of A_{385} due to increasing inhibitor concentration is shown in Fig. 27. Unlike the trypsin inhibition curve reported in Part 1, the chymotrypsin inhibition curve was nonlinear over the entire inhibitor concentration range. This unique characteristic makes it difficult to define a chymotrypsin inhibitor unit (CIU).

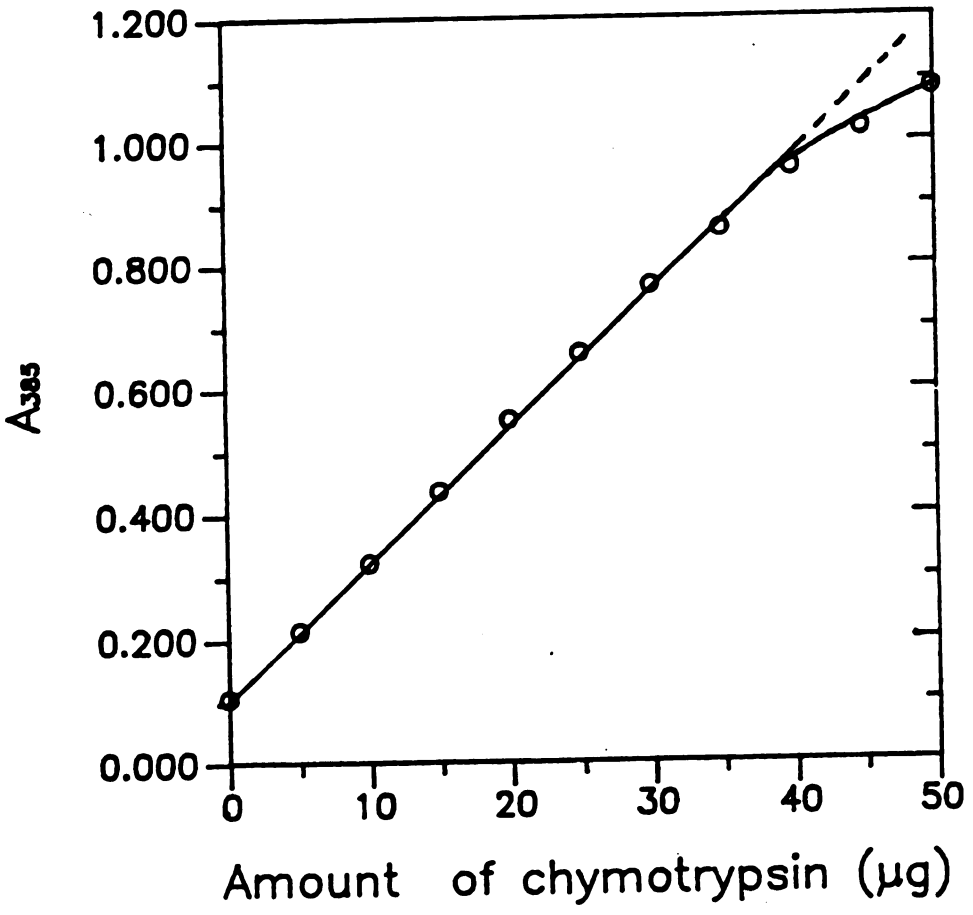

Fig. 25 Relationship between chymotrypsin activity (A_{385}) and amounts of enzyme. The reaction time was 10 min, BTpNA 0.24 mg, and the assay volume 4ml.

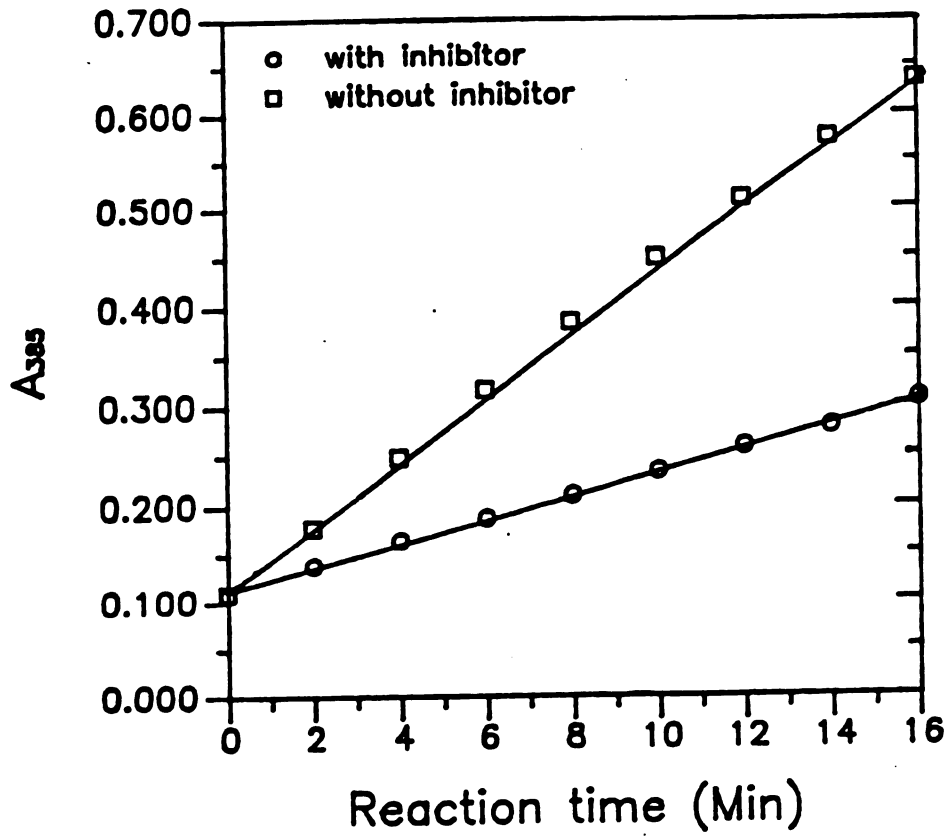

Fig. 26 Relationship between chymotrypsin activity (A_{385}) and the reaction time in the absence of and in the presence of soybean BB inhibitor. The assay volume was 4 ml and chymotrypsin 16 μg.

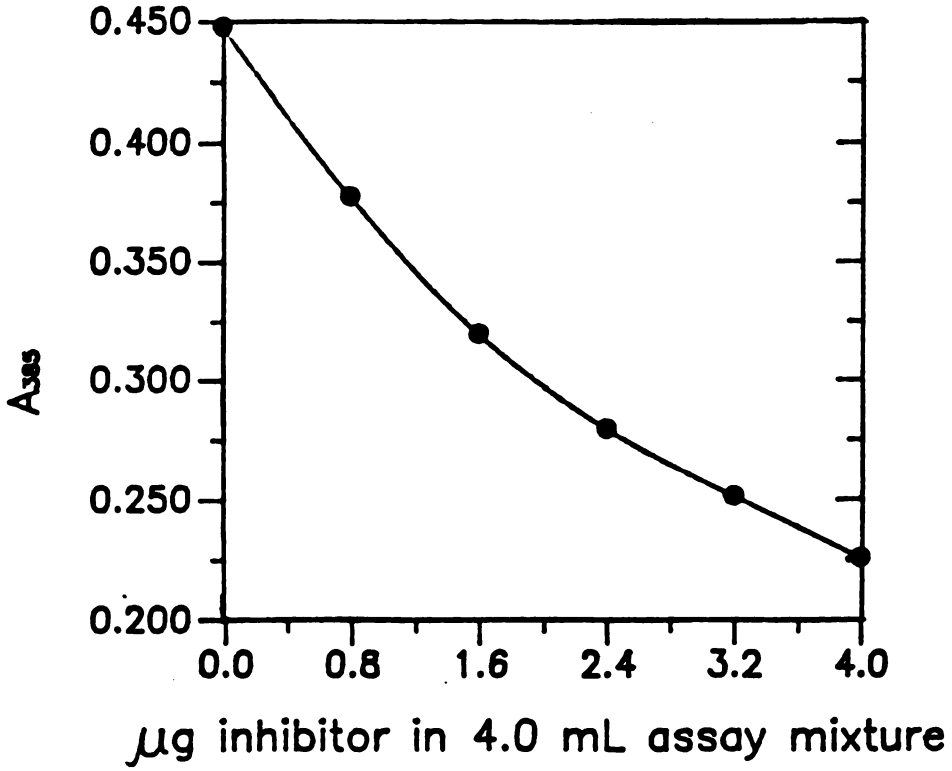

Fig. 27 Chymotrypsin activity (A_{385}) as a function of soybean BB inhibitor concentration.

In the method of Kakade et al. (17), the CIA is expressed in a similar way to trypsin inhibitor activity (Fig. 5), that is, as the number of chymotrypsin units (CU) inhibited, one CU being defined as the 0.01 increase of A_{275} under their assay conditions. Since the CIA, expressed on a per mL basis, was found to decrease as the level of inhibitor solution increased, they calculated the "true" CIA by extrapolating to zero volume of inhibitor solution. However, due to the characteristic inhibition curve observed in our study, this extrapolation, when applied to our data, caused serious uncertainty in estimating CIA. Consequently, it can not be adopted for our data treatment.

iv. Linearization of the inhibition curve

Efforts were made to linearize the inhibition curve of Fig. 27 through fitting into various mathematical functions. It was found that when water was used as a blank, the curve fitted best the reverse ratio function, $y = 1/(a + bx)$, where, y represents A_{385}, and x represents inhibitor concentration [I]. As a result, linearization of the curve was accomplished by reversing A_{385} (Fig. 28). Linear regression of $1/A_{385}$ with [I] gave a coefficient of 0.998 + 0.002, with n=20 and alpha =0.02.

v. Defining chymotrypsin inhibitor units (CIU)

In Fig. 28, the value of $1/A_{385}$ varied considerably with the amount of enzyme used in the assay and it was not zero when [I]=0. Obviously, further data transformation was needed before defining CIU. To do this, values of Y-axis in Fig. 27 were transformed according to equation

$$Y = A^{o}_{385}(1/A_{385} - 1/A^{o}_{385}) \times 100$$
$$= (A^{o}_{385}/A_{385} - 1] \times 100,$$

where A^{o}_{385} is the chymotrypsin activity at [I]=0. Under the assay conditions

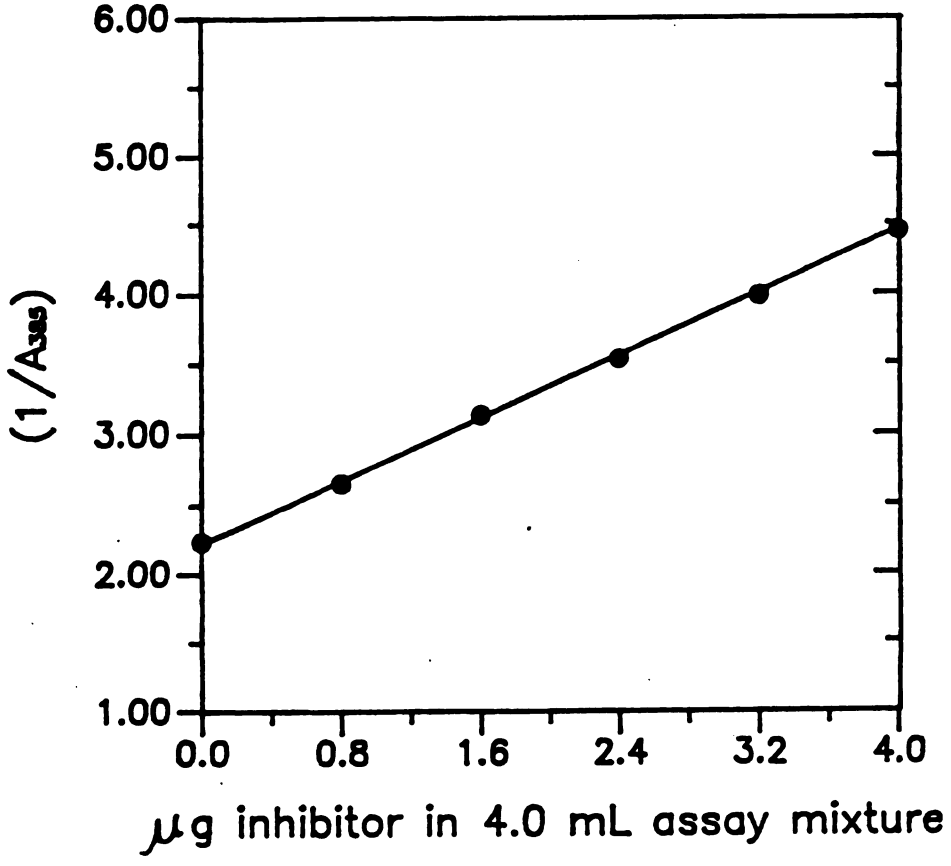

Fig. 28 Reversed values of chymotrypsin activity ($1/A_{385}$) as a function of soybean BB inhibitor concentration. The data are transformed from Fig. 27.

specified in this study, one chymotrypsin inhibitor unit (CIU) was arbitrarily defined as a 0.01 increase of (A^{o}_{385}/A_{385} -1). Fig. 29 shows the relationship between CIA (in terms of CIU) and inhibitor concentration. Note that at [I]=0, CIA=0 and as [I] increases, CIA increases linearly over the inhibitor concentration studied.

vi. Effect of enzyme concentration on the CIA assay

Under the definition of CIU, when the same amount of inhibitor was assayed for its CIA value with various enzyme concentrations, which reflect in A^{o}_{385}, different CIA values were obtained. The CIA value as a function of A^{o}_{385} is shown in Fig. 30. In general, as A^{o}_{385} increased, the CIA value decreased. Linear regression over the A^{o}_{385} range of 0.3-0.6 gives the following equation:

$$CIA = - 55.3\ A^{o}_{385} + 56.0, \qquad [1]$$

with a r=0.974 (n=30).

In order to standardize the assay conditions, we set the CIA value obtained at A^{o}_{385}=0.45 as a reference value and is symbolized as $CIA_{0.45}$, and all CIAs at any other A^{o}_{385} should be corrected against A^{o}_{385}=0.45. A correction factor, c, is defined as:

$$c = (CIA_{0.45}/CIA - 1), \qquad [2]$$

To find out the relationship between c and A^{o}_{385}, use equation [1] to calculate serial values of CIA at different A^{o}_{385} values, including $CIA_{0.45}$ at A^{o}_{385}=0.45, and then use equation [2] to calculate serial values of c vs A^{o}_{385}. Plot c vs A^{o}_{385} in Fig. 31. The curve fits the function $y = ax^{b}$. According to this model, linearization of Fig. 31 into Fig. 32, with a r=0.999, gives the following experimental equation suitable for calculating the correction factor c at any A^{o}_{385} between 0.3 and 0.6.

$$c = 2.05\ (A^{o}_{385} + 0.13)^{3.44} - 0.315. \qquad [3]$$

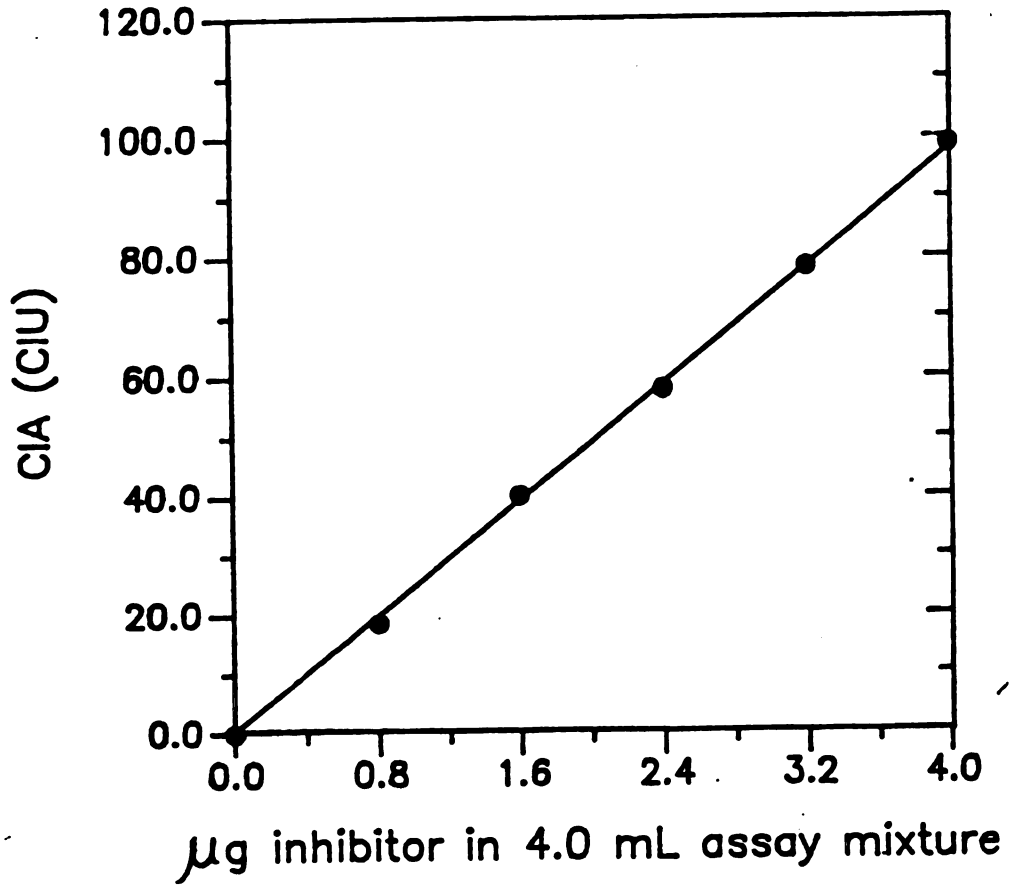

Fig. 29 Chymotrypsin inhibitor activity (CIA) as a function of soybean BB inhibitor. One chymotrypsin inhibitor unit (CIU) is defined as an 0.01 increase of (A°_{385}/A_{385} - 1). The data are transformed from Fig. 27.

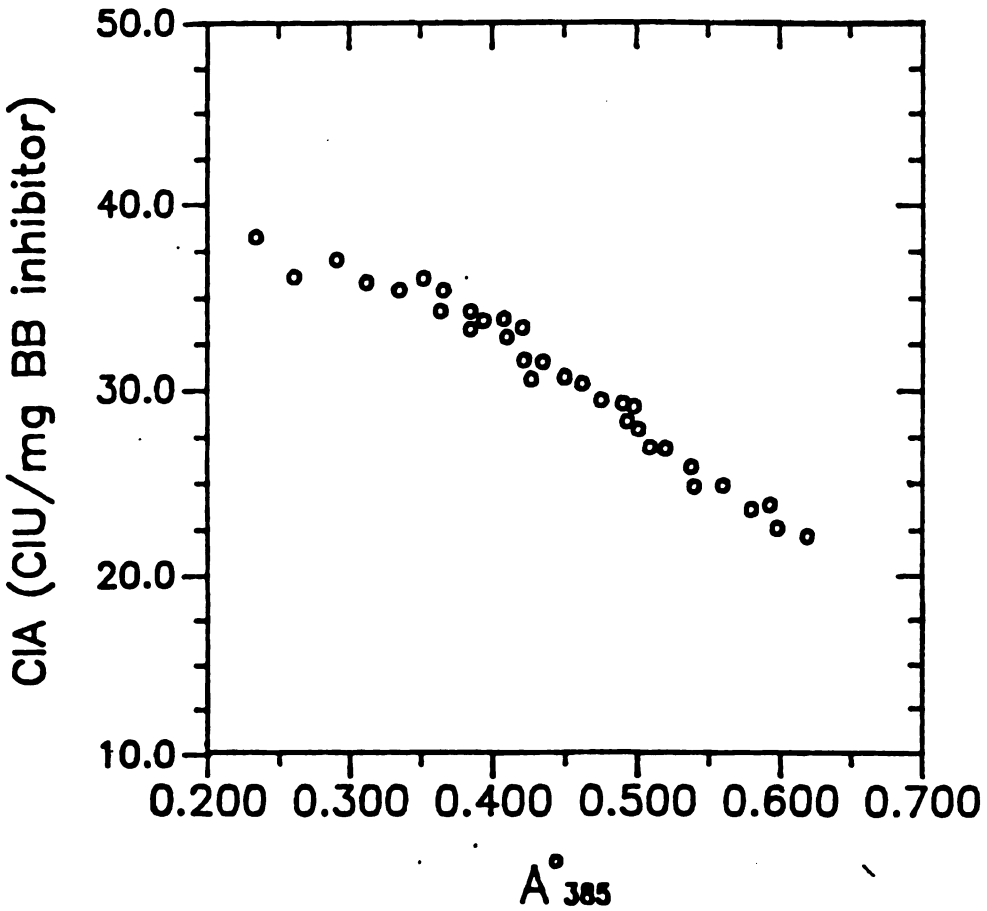

Fig. 30 Effect of A°_{385}, corresponding the amount of chymotrypsin, on the CIA assay.

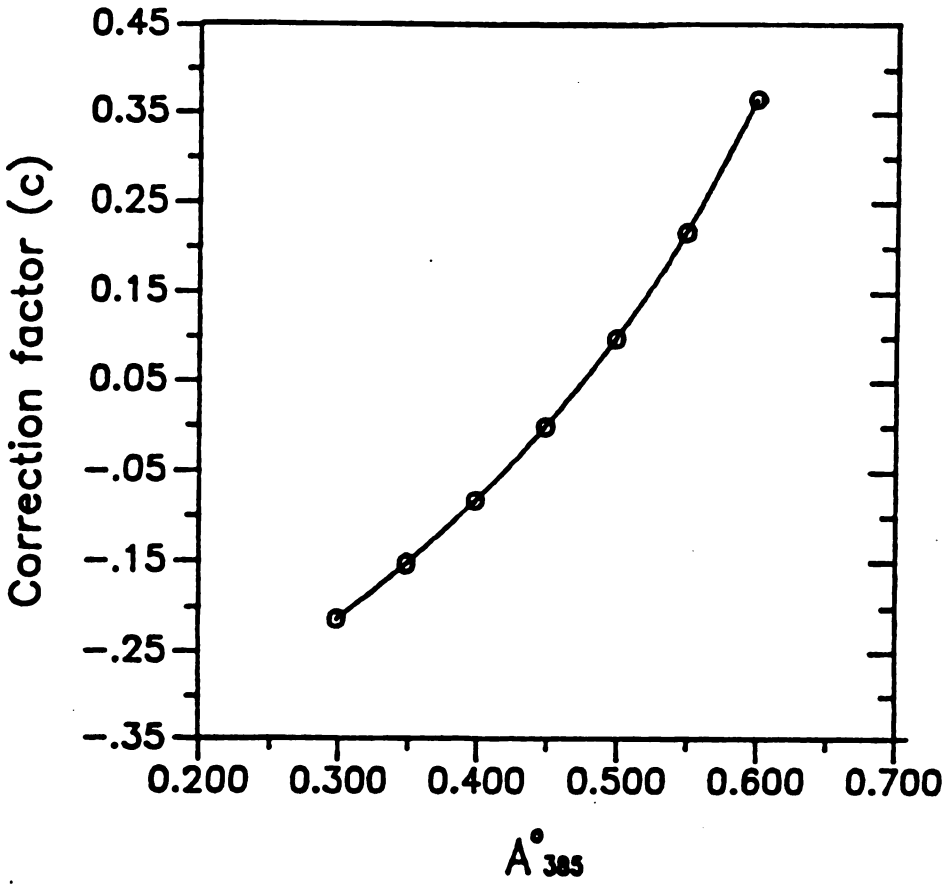

Fig. 31 The correction factor c as a function of A°_{385} for CIA assay.

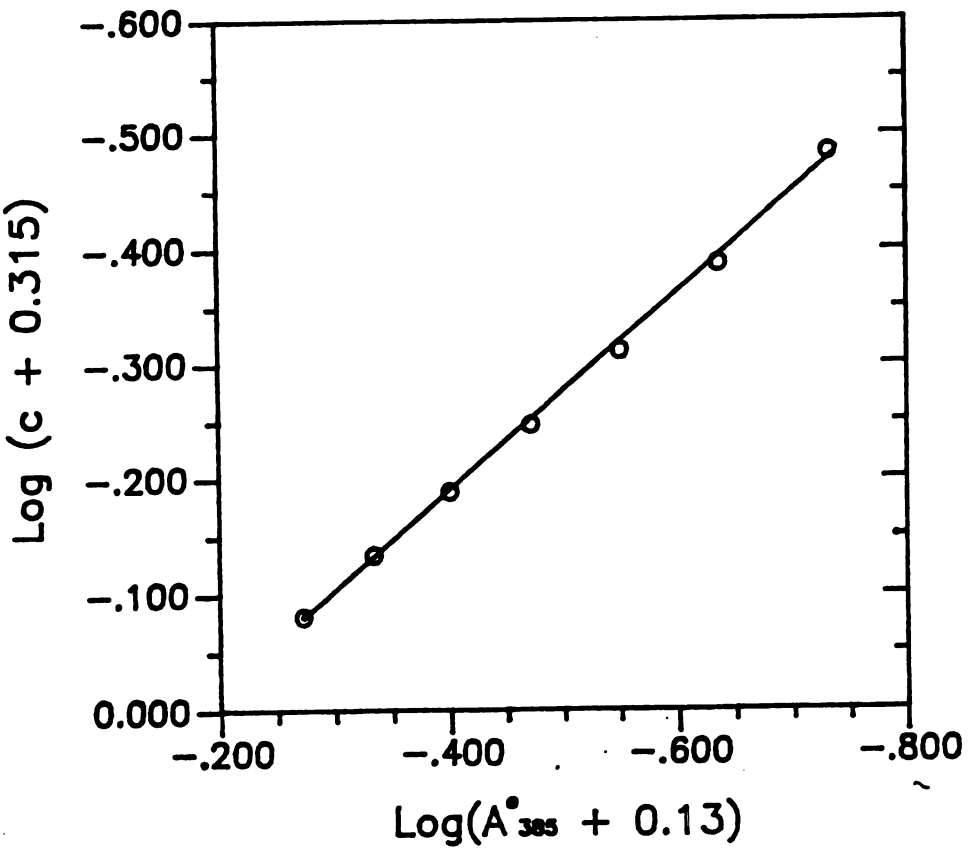

Fig. 32 Linearization of the correction factor c as a function of A°_{385}. The data were transformed from Fig. 31.

For correction of the enzyme concentration effect, we first use eq. [3] to find c at a given A^{o}_{385} and then eq. [2] to find $CIA_{0.45}$ at a given CIA:

$$CIA_{0.45} = (1+c)\ CIA. \qquad [4]$$

E.g. a sample has a CIA value of 112 CIU per mg at A^{o}_{385}=.39. Thus,

$$c = 2.05\ (0.39 + 0.13)^{3.44} - 0.315 = -0.099,$$

while, $CIA_{0.45} = (1 - 0.099) \times 112 = 101$ CIU/mg sample.

vii. Inhibitor dilution effect

When different inhibitor concentrations, representing widely different percentage chymotrypsin inhibitions, were used for the CIA measurement, the values as CIU/mg inhibitor varied (Fig. 33). Inhibitions lower than 35 % resulted in a lower estimate of CIA. This might be due to the characteristic inhibition curve of Fig. 27, which deviated from the reverse ratio function $y = 1/(bx + a)$ at the lower inhibitor concentration range. On the other hand, inhibitions higher than 65% gave scattered results, probably because of the data transformation: any small error would be greatly enlarged when being reversed. Consequently, dilutions resulting in 35-65% inhibition are considered proper for the assay. On Fig. 33, the data in this range had a relative standard deviation of ±4.8% with n=22.

viii. Presence of acetone

The presence of acetone in the assay mixture presents a problem. On one hand, because of the low water solubility of BTpNA, presence of acetone is required. On the other hand, acetone is a competitive inhibitor to hydrolysis of the substrate by chymotrypsin (15). However, as long as the acetone concentration is standardized, its presence has no effect on the CIA assay. In the assay, 10 volume percent acetone was used. It is considered a minimum limit,

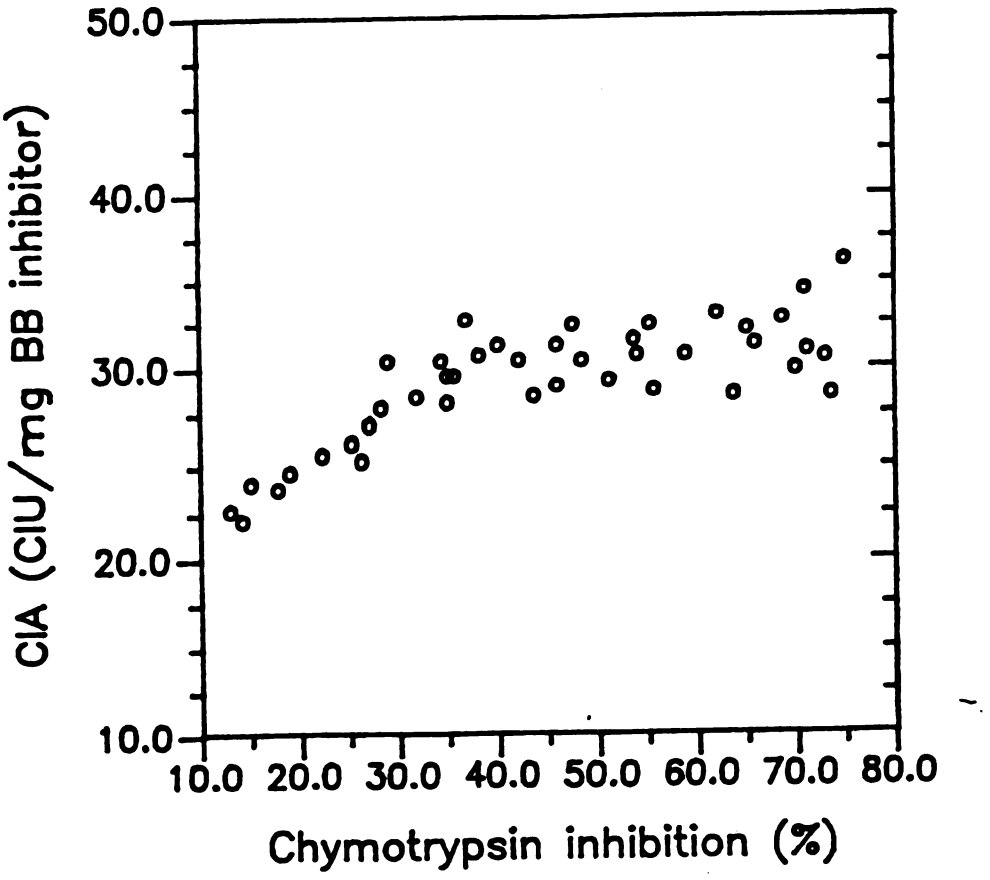

Fig. 33 Effect of percentage of chymotrypsin inhibition, corresponding various dilutions of the soybean BB inhibitor solution, on the CIA assay.

since below this level, BTpNA could not be dissolved completely. ix. Application of the new method to some legume products

In our laboratory, the proposed procedure has been adopted for the routine CIA assay of soybeans, soybean products and other legume seeds, since the aqueous extracts of these samples gave a similar type of inhibition curve to pure soybean BB inhibitor. The aqueous sample extraction was carried out according to the procedure for trypsin inhibition assay in Part 1. The results are presented in Table 6 and they are given in terms of both CIU/mg sample and mg BB inhibitor equivalent/g sample. In summary, the proposed CIA assay, although involves mathematical data transformation, is relatively simple and reliable.

II. Effect of the Sequence of Mixing Reactants on the CIA Assay

For measuring the CIA of the soybean BB inhibitor, two tests were used: the E-last test as in the proposed method described above and the S-last test, as in the method of Kakade et al. (17). The results showed that the two tests gave different inhibition curves (Fig. 34). For calculating CIA, the data of Fig. 34 are transformed into the data of Fig. 35, which clearly indicate that the S-last test gives considerably lower inhibition values than the E-last test, when the premix pH is 4.0 and the preincubation time is as short as 3 min. As in the TIA assay, the effect of the reactant sequence on the CIA assay is hereafter referred to as "the reactant sequence effect".

i. Effect of the preincubation time on the reactant sequence effect

In the E-last test, when the time of incubating the premix was varied from 0 to 40 min and the premix pH was constant at 4.0, the same inhibition value was

Table 6 CIA in some commercial soy products and legume seeds assayed by the new method *

Samples	Chymotrypsin inhibitor activity	
	CIU/mg	mg BB inhi. Equi./g
Raw soybeans	88.8 ± 4.4	2.88 ± 0.14
Boiled soybeans	21.0 ± 1.5	0.68 ± 0.05
Soy isolate I	35.7 ± 2.1	1.16 ± 0.07
Soy isolate II	27.8 ± 1.6	0.90 ± 0.05
Toasted soy flour	11.8 ± 0.7	0.38 ± 0.02
Cowpeas	17.9 ± 0.6	0.58 ± 0.02
Navy beans	111.0 ± 4.7	3.60 ± 0.15
Pinto Beans	101.1 ± 4.4	3.28 ± 0.14
Pure BB inhibitor	30800.0 ± 100	

* Mean of duplicate measurements ± SD.

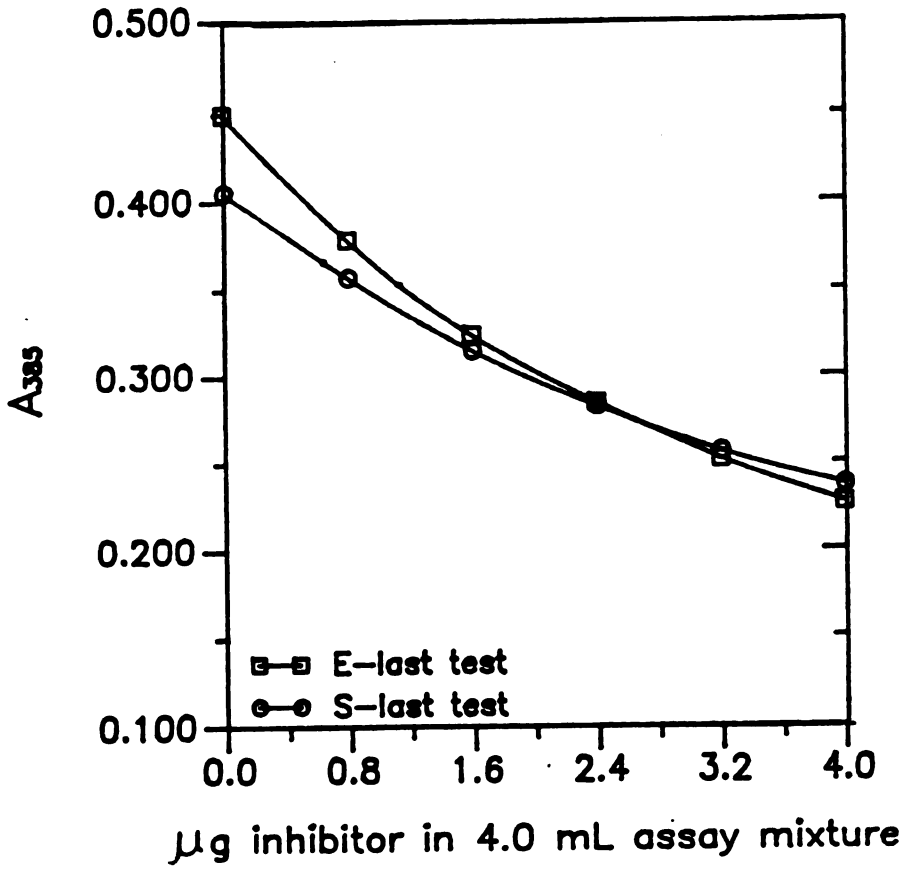

Fig. 34 Relationship of A_{385} vs inhibitor concentration in the S-last and the E-last tests. The premix pH was 4.0 and the preincubation time 3 min. Details of the tests are described under Materials and Methods.

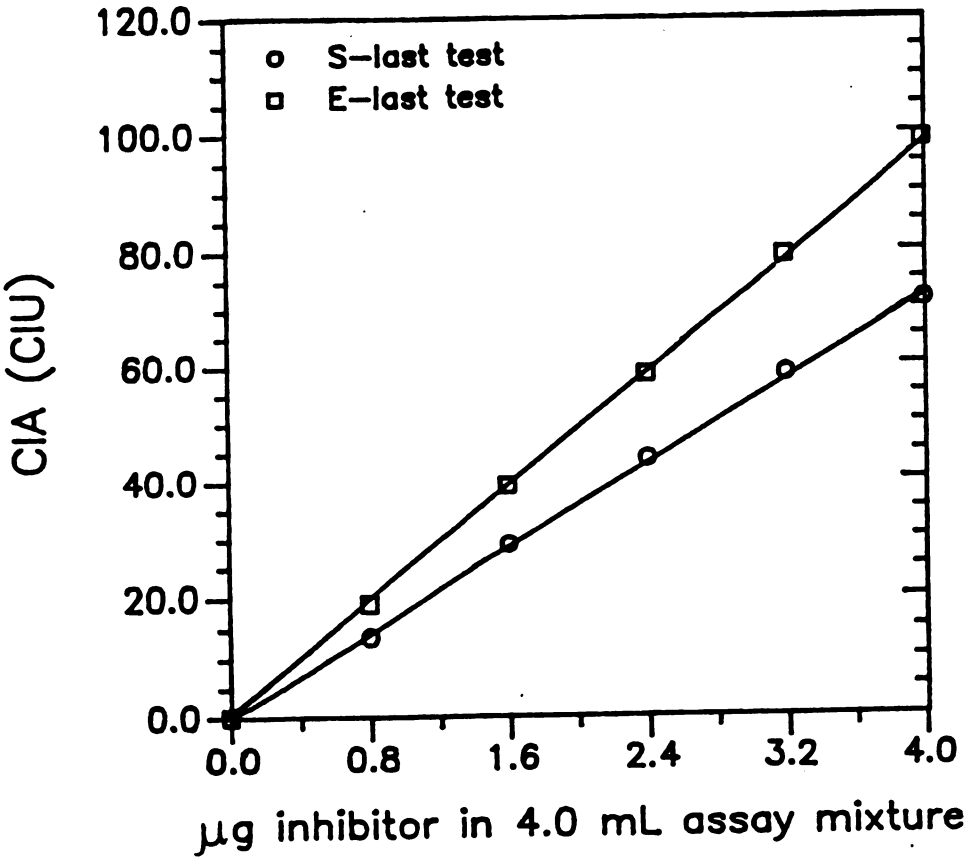

Fig. 35 Relationship of CIA vs inhibitor concentration in the S-last and the E-last tests. One chymotrypsin inhibitor unit (CIU) is defined as an 0.01 increase of (A°_{385}/A_{385} - 1). The data were transformed from Fig. 34.

obtained (data not shown), indicating that the preincubation time in the E-last test had no effect on the CIA assay. However, in the S-last test, when the time of incubating the premix was changed from 0 to 40 min, while the premix pH was fixed at 4.0, different inhibitions were obtained, indicating that the preincubation time in the E-last test had an effect on the CIA assay.

At any particular preincubation time, the relative difference between the two tests is expressed as [(CIAe-CIAs)/CIAe X 100%], where CIAs is the CIA obtained by the S-last, CIAe is the CIA obtained by the E-last test. Since CIAe remained constant regardless of the preincubation time, it was regarded as a reference. The data presented in Fig. 36 show that, when the premix pH was constant at 4.0, the CIAs obtained by the S-last test were always lower than those obtained by the E-last test. At the beginning of preincubation, the relative difference between the two tests increased almost linearly with time. After 15 min, the curves leveled off. The maximum value was about 83%.

ii. Effect of the premix pH on the reactant sequence effect

Like the preincubation time, the premix pH was also found to influence the reactant sequence effect of the CIA assay. In the E-last test, when the pH of the premix was varied from 2.7-9.0 and the preincubation time was held at 10 min, the same inhibition values were obtained, indicating that the premix pH in the E-last test had no effect on the CIA assay. In the S-last test, when the pH of the premix was varied from 2.7 to 9.0 and the time of premix incubation was kept constant at 10 min, different inhibition values were found. The relative differences in CIAs measured by the two tests were plotted against the premix pH (Fig. 37). The results indicate that the CIA obtained through the S-last test were either equal to or lower than that through the E-last test, depending on the premix pH. There are two peaks corresponding to the maximum

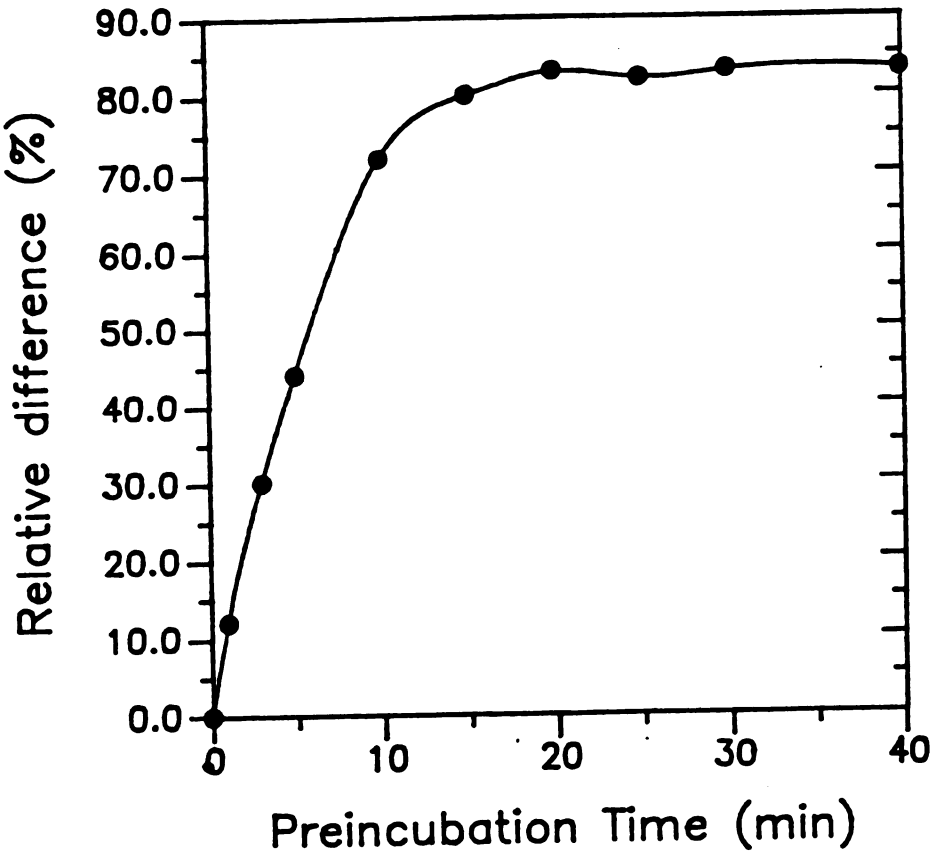

Fig. 36 Relative difference in CIA of soybean BB inhibitor, assayed by the S-last and the E-last tests as a function of the preincubation time. The relative difference is defined as (CIAe-CIAs)/CIAe x 100%, where CIAs is the CIA of the S-last test and CIAe is the CIA of the E-last test. The premix pH was 4.0. Details of the assay are described under Materials and Methods.

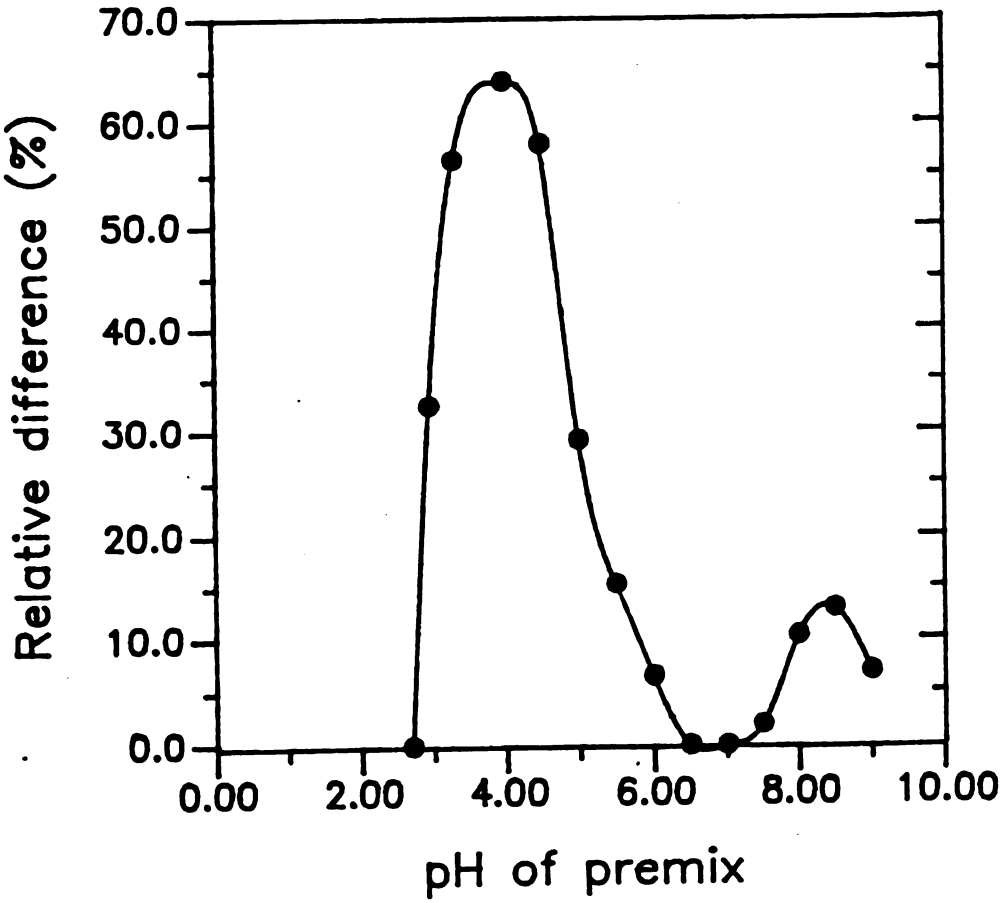

Fig. 37 Relative difference in CIA of soybean BB inhibitor, assayed by the S-last and the E-last tests as a function of the premix pH. The relative difference is defined as (CIAe-CIAs)/CIAe x 100%, where CIAs is the CIA of the S-last test and CIAe is the CIA of the E-last test. The preincubation time was 10 min. Details of the assay are described under Materials and Methods.

difference between the two tests, the larger peak on the acidic side (pH about 4.0) and the smaller peak on the alkaline side (pH about 8.5). At the pH = 2.7 or between 6.5-7.5, the S-last test estimated the same inhibition values as the E-last test.

iii. Abrupt change of the premix pH

In terms of the premix pH effect on the reactant sequence effect, the CIA value of the BB inhibitor obtained by the S-last test was either equal to the reference value (E-last value) when the premix pH was less than 2.7 or near neutral, or lower than the reference value when the pH was 2.7-6.5 or 7.5-9.0. Here, premix pHs which result in the sequence effect are considered effective pHs, while those resulting in no sequence effect are considered noneffective pHs. A separate study was conducted to see whether an abrupt change of the premix pH from effective to noneffective during preincubation in the S-last test can restore the chymotrypsin-inhibiting capacity of S-last test to that of the E-last test. The results are presented in Table 7.

Comparison of the two S-last tests, test No. 1 and test No. 2 showed that additional 10-min preincubation at pH 7.0 resulted in a small gain in the chymotrypsin inhibition. Comparison of the two S-last tests, test No. 1 and test No. 3, showed that abrupt change of premix pH from 4.0 to 7.0 during a 20 min preincubation resulted in a large gain in the inhibition value.

iv. The CIA assay as related to limited hydrolysis of inhibitors

In Part 1, the effect of the reactant mixing sequence on the assay of trypsin inhibitory activity has been attributed to the limited hydrolysis of inhibitor, in accordance with the reactive model of Laskowski, Jr. (21). Since under the same conditions, chymotrypsin is also capable of attacking its own

Table 7 Effect of an abrupt change of premix pH from 4.0 to 7.0 on assaying the CIA of soybean BB inhibitor [a]

No. of test	Test conditions	CIU/mg [b]	$\frac{CIAe-CIAs}{CIAe} \times 100$
S-last tests			
1	a 10-min preincubation at pH 4.0 plus a 10-min preincub. at pH 7.0 [c]	10.0 ± 0.4	59.4
2	a 10-min preincubation at pH 4.0	8.8 ± 0.4	64.3
3	a 20-min preincubation at pH 4.0	4.4 ± 0.2	82.1
4	a 10-min preincubation at pH 7.0	24.3 ± 1.2	1.2
E-last test [e]			
1	a 20-min preincubation at pH 4.0	24.6 ± 1.0	00.0

[a] All tests were finally run at pH 8.1 ± 0.2 for the 10-min enzymatic reaction.

[b] Mean of duplicate measurements ± SD.

[c] The pH abrupt change was carried out by adding 1.0 mL 40 mM Tris buffer, pH 8.2, to 1.5 mL of premix (20 mM acetate buffer, pH 4.0).

[e] Abruptly changing the premix pH in the E-last test was not done since the premix pH had no effect on the trypsin inhibition assay in the E-last test.

inhibitors (45,46,77), the same explanation could be applied to the reactant sequence effect on the CIA assay observed in this Part (Fig. 35).

In the S-last test, where I is premixed with E in a near equimolar ratio, at a relative high temperature (37 C), a conversion of I to I^* would occur during the period of preincubation, while in the E-last test, I is premixed with S and no conversion of I to I^* would take place.

Assume that $[I]_0$ is the concentration of total virgin inhibitor, at preincubation time t,

$$[I]_0 = [I]_t + [I^*]_t \qquad [5]$$

where, $[I^*]_t$ is the concentration of the chymotrypsin-modified inhibitor produced during preincubation and $[I]_t$ is the concentration of remaining I.

Also assume that **a** is the CIA per unit concentration of I and **b** is the CIA per unit concentration of I^*.

Thus, in the E-last test, we measured the CIA of total virgin inhibitor ($a[I]_0$), while in the S-last test, we measured the CIAs of both I and I^* ($a[I]_t + b[I^*]_t$).

Since the chymotrypsin-modified inhibitor is known to be almost inactive towards chymotrypsin (46), that is, $b = 0$, then

$$(a[I]_t + b[I^*]_t) = a[I]_t < a[I]_0, \qquad [6]$$

indicating that there is a pronounced reactant sequence effect.

Regarding preincubation time, the maximum difference between the S-last and the E-last tests for the chymotrypsin-BB inhibitor system (Fig. 36) is larger than the corresponding difference for trypsin-BB inhibitor system (Fig. 12). This might be attributed to the difference in the inhibition capacity between trypsin and chymotrypsin modified BB inhibitors towards their own enzyme. Whereas the trypsin-modified inhibitor acts only more slowly than the virgin inhibitor towards trypsin, the chymotrypsin-modified inhibitor is almost

inactive towards chymotrypsin (46). The Y-axis value in Fig. 36 represent the following equation:

$$Y = \{a[I]_o - (a[I]_t + b[I^*]_t)\}/a[I]_o \qquad [7]$$

When b=0, substituting eq. [5] into eq. [7] gives

$$Y = [I^*]_t/[I]_o \qquad [8]$$

The maximum relative difference of about 83% shown in Fig. 36 indicates an 83% conversion of I to I^*. This finding is in accordance with the observation by Frattali and Steiner (46), that an 80% conversion is possible. However, it took 48 hrs to reach this conversion in that study, while in our study it took about 20 min. This discrepancy may be due to differences in reaction systems. In their study, the chymotrypsin was used in a catalytic amount (molar ratio of enzyme to inhibitor was 1:100) at 25 C, while in our study, a near stoichiometric amount of the enzyme was used at 37 C.

The effect of the premix pH on the reactant sequence effect can be explained by the fact that both the rate constant k_{cat} and the equilibrium constant K_{hyd} for the hydrolysis of I into I^* are pH-dependent (41,42). Here, K_{hyd} is defined as

$$K_{hyd} = [I^*]_e/[I]_e \qquad [9]$$

where $[I]_e$ and $[I^*]_e$ are the concentrations of virgin and modified inhibitors at equilibrium, respectively . When t reaches equilibrium time, the Y-axis values in Fig. 37 becomes

$$Y = [I^*]_e/[I]_o \qquad [10]$$

Since from eqs. [5], [9] and [10] we know that, when $K_{hyd} > 0$, Y is a monotonically increasing function of K_{hyd},

$$Y = K_{hyd}/(1+K_{hyd}), \qquad [11]$$

the pH dependence pattern of the reactant sequence effect shown in Fig. 37 should follow the eq. [4] shown in Part 1, that is, Y should rise at both low

and high pH levels. The smaller alkaline peak might be due to the fact that K_{cat} of hydrolysis at alkaline medium is smaller than that at acidic medium (29), resulting in less conversion of I to I^* in 10 minute preincubation.

Under certain conditions, the modified inhibitor can be cleaved and reformed (21,75). Studying the interactions of the BB inhibitor with both trypsin and chymotrypsin, Frattali and Steiner (46) found that, for both cases, conversion of I to I^* occurred at pH 4.0, at room temperature, with catalytic amount of enzyme, while regeneration of I from I^* took place upon prolonged exposure at pH 8.0, at 4C in a near stoichiometric amount of enzyme. They also stated that the regeneration of trypsin-modified inhibitor was faster than that of the chymotrypsin-modified inhibitor. In Part 1, we showed that pH jumping resulted in almost complete recovery of trypsin inhibitor activity (Figs. 15 and 16) while in Part 2, we observed only a partial recovery of chymotrypsin inhibitor activity, following pH jumping (Table 7). This difference in degree of recovery may be attributed to the difference in regeneration rates for the two cases.

Finally, the reaction between trypsin and soybean Kunitz inhibitor is known to be instantaneous. The half life of the reaction is about 4 sec with a second-order velocity constant of 2×10^7 L/Mole/sec (78). Although data for interactions betwen other proteinases and their inhibitors are unavailable, thc reactant sequence effect on inhibition assays observed in this Part as well as in previous Part could be used as a basis to propose that binding between a protein inhibitor with a serine protease is instantaneous. This hypothesis may be regarded as a complement to the standard mechanism of Laskowski, Jr. (21).

CONCLUSIONS

The modified method for measuring trypsin inhibition in soybean products has a theoretical basis (the effect of the reactant mixing sequence on the assay is taken into consideration) and a practical significance. It can eventually be used for measuring trypsin inhibitor activity in many other proteinaceous food products. The proposed method for chymotrypsin inhibition assay, although involves mathematical data transformation, is relatively simple and reliable.

Regarding the effect of the reactant mixing sequence on the inhibition assays, it was found that the inhibition value of the S-last test (adding substrate last to the reaction mixture) was either equal to or lower than that of the E-last test (adding enzyme last to the reaction mixture), depending on the premix pH and preincubation time, while the values of the E-last test were constant regardless of the premix pH and the preincubation time.

These observations are in accordance with the reactive site model for proteinaceous inhibitors of serine proteases (21). The inhibitors bind to the enzyme as if they were substrates, but very tightly, and are cleaved very slowly at a peptide bond referred to as the reactive site. For assaying the aforementioned type of inhibitors, the common practice of sufficiently preincubating inhibitor with enzyme for obtaining an equilibrium data in an inhibition assay is no longer valid, and the new procedure (E-last test) is preferable to the common procedure (S-last test). In addition, the observations suggest an instantaneous binding between the aforementioned type of inhibitors and the enzyme, which may be regarded as a complement to the standard mechanism.

RECOMMENDATIONS FOR FUTURE STUDIES

As most protease inhibitors are also of protein nature, studies are needed to verify if their assays are affected by the reactant sequence or not.

Because of the low concentration (about 10^{-6} M) of the inhibitors used in this study, direct evidence by electrophoresis or by chromatography for the limited hydrolysis of the inhibitors is difficult. However, if relatively larger amounts (10^{-3} M) of inhibitor and enzyme are tested under the condition similar to the inhibition assay (37 C and stoichiometric ratio of enzyme to inhibitor), either electrophoresis or chroatography can then be used to verify if the limited hydrolysis can occur in minutes or not.

And finally, the procedure for measuring the reactant sequence effect on inhibition assay may eventually be developed as an analytical tool for kinetic study of inhibitor hydrolysis.

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