1992

Comparison of two synthetic substrates and two trypsin sources for measuring trypsin inhibitor activity in soy bean, winged bean, and green gram

Pratibha L. Samar
San Jose State University

Follow this and additional works at: https://scholarworks.sjsu.edu/etd_theses

Recommended Citation

Samar, Pratibha L., "Comparison of two synthetic substrates and two trypsin sources for measuring trypsin inhibitor activity in soy bean, winged bean, and green gram" (1992). *Master's Theses*. 503.
DOI: <https://doi.org/10.31979/etd.qpqb-usb4>
https://scholarworks.sjsu.edu/etd_theses/503

This Thesis is brought to you for free and open access by the Master's Theses and Graduate Research at SJSU ScholarWorks. It has been accepted for inclusion in Master's Theses by an authorized administrator of SJSU ScholarWorks. For more information, please contact scholarworks@sjsu.edu.

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 1351074

**Comparison of two synthetic substrates and two trypsin sources
for measuring trypsin inhibitor activity in soy bean, winged
bean, and green gram**

Samar, Pratibha Lodha, M.S.

San Jose State University, 1992

U·M·I

300 N. Zeeb Rd.
Ann Arbor, MI 48106

COMPARISON OF TWO SYNTHETIC SUBSTRATES AND TWO TRYPSIN
SOURCES FOR MEASURING TRYPSIN INHIBITOR ACTIVITY IN
SOY BEAN, WINGED BEAN, AND GREEN GRAM

A Thesis Presented to
The Faculty
of
The Department of Nutrition and Food Science
San Jose State University

In Partial Fulfillment of the Requirements for the
Degree of Master of Science
in Nutritional Science

by
Pratibha L. Samar
December, 1992

APPROVED FOR THE DEPARTMENT OF
NUTRITION AND FOOD SCIENCE

Dr. Panfilo Belo

Dr. Elveda Smith

Dr. Miriam Saltmarch

APPROVED FOR THE UNIVERSITY

ABSTRACT

COMPARISON OF TWO SYNTHETIC SUBSTRATES AND TWO TRYPSIN SOURCES FOR MEASURING TRYPSIN INHIBITOR ACTIVITY IN SOY BEAN, WINGED BEAN, AND GREEN GRAM

by Pratibha L. Samar

The objective of this study was to compare two synthetic substrates (BAPA and TAME) and two trypsin sources (bovine and porcine) in the determination of trypsin inhibitor (TI) activity in soy bean, winged bean, and green gram. The TI activities in aqueous extracts prepared from defatted beans were determined by original BAPA, modified BAPA, and TAME methods and compared using a 3 x 3 x 2 factorial analysis of variance.

The TI activity varied significantly with substrate, source of trypsin, and type of legume used. Modified BAPA gave significantly higher TI activity ($p \leq .05$) followed by original BAPA and TAME. The TI activity obtained with bovine trypsin was significantly higher ($p \leq .05$) than porcine trypsin when BAPA and modified BAPA methods were used. Similar results were obtained for soy bean and green gram when TAME was used. Regardless of the substrate or the enzyme used, the TI activity was significantly higher in soy bean ($p \leq .05$) followed by winged bean and green gram.

ACKNOWLEDGEMENTS

I would like to thank Dr. Panfilo Belo, my graduate advisor, for his time and advice throughout all phases of this research which helped me in achieving this academic goal. I am thankful to Dr. Elveda Smith and Dr. Miriam Saltmarch, my thesis committee members, for their helpful suggestions and comments on my thesis. I also wish to thank San Jose State University Research Foundation for giving me research grant to buy chemical reagents and other supplies.

Dedication

This thesis is dedicated to my husband, Vipin Samar, whose encouragement, support and endless patience made it possible for me to finish my graduate studies in Food Science.

PREFACE

This thesis is written in a publication style. The second chapter is written in journal style format in accordance with January, 1992 guidelines and will be submitted to the Journal of Agricultural and Food Chemistry. Chapters 1 and 3 are written according to the guidelines outlined in the Publication Manual of the American Psychological Association, 3rd. edition, 1989.

Table of Contents

	Page
List of Tables.	ix
List of Figures.	x
CHAPTER	
1 INTRODUCTION AND REVIEW OF LITERATURE	1
Introduction	1
Objectives	3
Significance of the Study	3
Review of Literature.	4
Nutritional Aspects of Trypsin Inhibitor	4
Dietary Intake of Trypsin Inhibitor	8
Types of Trypsin Inhibitors	10
Trypsin Inhibitors in Soy Bean	11
Trypsin Inhibitors in Winged Bean	12
Trypsin Inhibitors in Green Gram	13
Quantitation of Trypsin Inhibitor	13
Natural Substrate	14
Synthetic Substrate	14
Trypsin Sources	17
2 JOURNAL ARTICLE	20
AUTHORS' TITLE PAGE	21
ABSTRACT	22
INTRODUCTION	23
MATERIALS AND METHODS	25

Sample and Sample Preparation	25
Proximate Analysis	25
Extraction of Trypsin Inhibitor	25
Determination of Trypsin Inhibitor Activity by Original BAPA Method.....	26
Determination of Trypsin Inhibitor Activity by TAME Method	27
Determination of Trypsin Inhibitor Activity by Modified BAPA Method	28
Analysis of Data	28
RESULTS AND DISCUSSIONS.....	29
Determination of Trypsin Inhibitor by Original BAPA Method	29
Determination of Trypsin Inhibitor by TAME Method.....	30
Determination of Trypsin Inhibitor by Modified BAPA Method	30
Comparison of Original BAPA and TAME Methods	31
Comparison of Original and Modified BAPA Methods	32
Comparison of Bovine and Porcine Trypsin.....	32
CONCLUSION	33
LITERATURE CITED	36
3 SUMMARY AND RECOMMENDATIONS	40
Summary	40
Recommendations	42
REFERENCES	43

List of Tables

Table	Page
1. Trypsin Inhibitor Content of Some Common Legumes	5
2. Average Daily Protein and Trypsin Inhibitor Intake	
From the British Household Diet	9
I. Proximate Composition of Soy Bean, Winged Bean and	
Green Gram on Wet Basis	34
II. Trypsin Inhibitor Activity (TIU/mg Protein) in Soy Bean,	
Winged Bean, and Green Gram	35

List of Figures

Figure	Page
1. Regulation of Secretion of Trypsin by Pancreas.	7

Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

As the discrepancy between the world's supply of protein and the growth of the global population continues to widen, ways to bridge this gap have become a matter requiring immediate action. Based on available statistics, it is estimated that, by the end of the century, the population of the world will equal six billion and that a two fold increase in plant protein and a four fold increase in animal protein will be needed to feed this population (Liener & Kakade, 1980). The importance of using plant proteins in countries in which protein-calorie malnutrition (PCM) exists is well recognized. The increasing cost of animal proteins has prompted food technologists, nutritionists, and biochemists to consider plant proteins as a major source of dietary protein (Salunkhe, 1982).

Soy bean (Glycine max L.), winged bean (Psophocarpus tetragonolobus [L.] DC), and green gram (Vigna radiata [L.] wilczek) are among the legumes which have potential as protein rich food sources. They are cheap and within the purchasing capabilities of an average family in underdeveloped countries (Liener & Kakade, 1980). However, the nutritive value of the legume seeds is limited by the presence of antinutritional factors such as phytates, alkaloids, phytohemagglutinins, saponins, tannins, cyanogenic factors, and trypsin inhibitors. Among these, trypsin inhibitor (TI) has been studied extensively because it is present in most legumes (Liener & Kakade, 1980). The antinutritional effect of TI is due to its combination with trypsin to form an inactive complex which interferes with protein digestion. In addition to TI, trypsin

inhibition in raw beans is also caused by polyphenols (e.g., tannins) (Elias et al., 1979; Fernandez et al., 1982; Tan et al., 1984). Trypsin inhibitor causes pancreatic hypertrophy in chicks, mice, and rats (Chernick et al., 1948; Kakade et al., 1973; Rackis, 1965). In addition, Chan and DeLumen (1982a) reported that the TI in winged bean causes spleen hypertrophy.

Due to increased use of plant proteins in developing countries to alleviate PCM, it is important to measure TI in legumes. Although both protein and non-protein TI have been identified in legumes, most assay methods for TI are based only on the determination of the protein fraction (Hafez & Mohamed, 1983a; 1983b). Spectrophotometric methods currently used for TI assay are based on the in-vitro reduction in hydrolysis rate of the trypsin substrate. Both the natural trypsin substrate, casein (Kunitz, 1947), and the synthetic trypsin substrates, benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA) (Erlanger et al., 1961) and p-toluene-sulfonyl-L-arginine methyl ester (TAME) (Hummel, 1959), are utilized in measuring TI activity in legumes. Among these, BAPA has been used extensively and is reported to be not only more reliable but also more convenient when compared to casein (Kakade et al., 1969).

The benzoyl-DL-arginine-p-nitroanilide hydrochloride method has undergone various modifications (Hamerstand et al., 1981; Kakade et al., 1974; Liu & Markakis, 1989; Smith et al., 1980) to increase simplicity, accuracy, and reproducibility. Although the BAPA method has been widely used, it is time consuming and requires a 10 min incubation period at 37 °C before terminating the reaction prior to the measurement of absorbance. Compared to BAPA, TAME is simpler since it is conducted at room temperature and does not require termination of the reaction. There

are several studies (Chan & DeLumen, 1982b; Delumen & Belo, 1981; Delumen & Salamat, 1980; Sathe & Salunkhe, 1981) in which the TAME method has been used, and there is a need to compare BAPA and TAME methods. The trypsin inhibitor activity is measured using either bovine or porcine trypsin. Although porcine trypsin is more stable in alkaline conditions, bovine trypsin is used extensively in the determination of TI activity (Buck et al., 1962).

Objective

This study was undertaken to compare two synthetic substrates, BAPA and TAME, and two trypsin sources, bovine and porcine, in the determination of trypsin inhibitor activity in soy bean, winged bean, and green gram. An attempt to modify the original BAPA method to make it suitable for continuous spectrophotometric assay for trypsin activity at room temperature, rather than at 37 °C, was also undertaken.

Significance of the Study

The use of plant proteins to alleviate PCM is a priority in underdeveloped countries. To alleviate malnutrition, the development of low cost food using indigenous sources of vegetable proteins has been emphasized (Salunkhe, 1982). Soy bean and other legume products are versatile sources of medium quality and low cost proteins. However, the nutritive value of these legumes is limited by the presence of significant antinutritional factors. Among these factors, TI has been studied extensively since it exerts antinutritional effects and causes interference with protein digestion (Chan & DeLumen, 1982a; Chernick et al., 1948; Kakade et al., 1973; Rackis, 1965).

Since TI content is an important parameter in evaluating the quality

of proteinaceous food, its measurement is important. Because various substrates are used for the measurement of TI, wide variations in TI levels have been reported. As a result, a direct comparison of the TI content in legumes is difficult. Currently, the BAPA method is widely used. However, when compared to TAME, the process is tedious and time consuming. There is a need for a simple, fast, and reliable method to measure TI. The comparison of two substrates, BAPA and TAME, and two enzymes, bovine and porcine, will contribute information towards establishing an appropriate method for measuring TI activity.

Review of Literature

Trypsin inhibitors that are found in a wide variety of food used for human consumption inhibit proteolytic activity of trypsin. The trypsin inhibitor content of commonly used legumes is shown in Table 1. Among these legumes, soy, lima, and winged beans have high TI content, whereas green gram and lentils have low TI content. Trypsin inhibitors have attracted the attention of scientists in many disciplines: 1) nutritionists, because of the possible effect on the nutritive value of plant proteins; 2) protein chemists, because the reactions of these inhibitors with enzymes provide a simple model for studying protein-protein interaction; and 3) members of the medical profession, because the unique pharmacological properties of these inhibitors have clinical application in the field of medicine (Liener & Kakade, 1980).

Nutritional Aspects of Trypsin Inhibitor

One of the earlier studies on nutritional quality of soybeans was conducted by Osborne and Mendel in 1917. They found that rats fed raw soy bean failed to grow at the normal rate; however, cooking soy beans

Table 1

Trypsin Inhibitor Content of Some Common Legumes

Legumes	Trypsin Inhibitor Activity (TIU/mg sample)
Lima bean	46.81
Soy bean	41.55
Winged bean	41.40
Navy bean	18.23
Northern bean	18.08
Chickpea	18.80
Cowpea	12.20
Green gram	9.96
Fieldpea	7.61
Lentils	5.12

Note. From "Antinutritive Factors in Eleven Legumes and Their Air-classified Protein and Starch Fractions" by K. Elkowicz and F. W. Sosulski, 1982, *Journal of Food Science*, 47, p. 1302.

under moist conditions improved the ability of soy beans to support normal growth. Other researchers demonstrated that prolonged ingestion of a diet containing soy bean led to pancreatic hypertrophy in several animals (Chan & DeLumen, 1982a; Chernick et al., 1948; Kakade et al., 1973; Rackis, 1965). The low nutritional value of raw soy protein and its effect on the pancreas are attributed to several factors: 1) protein derived from soy beans (as well as other legumes) is deficient in sulfur containing amino acids, methionine and cysteine (Rackis & Gumbmann, 1981); 2) the raw soy proteins with no inhibitory activity are refractory to enzymatic attack unless denatured by heat (Kakade et al., 1973); and 3) soy bean TI interferes with the digestion and utilization of soy proteins and eventually leads to overstimulation of the pancreas (Liener, 1979).

The mechanism of action of TI involves its interference with proteolysis of dietary protein by pancreatic enzymes, notably trypsin (Figure 1). When protein reaches the duodenum, it stimulates the secretion of digestive proteases, trypsin and chymotrypsin, from the pancreas. Normally, the secretory activity of the pancreas decreases as the concentration of active proteolytic activity in the gut increases. However, the model used to explain this alteration in pancreatic activity is based on the feedback mechanism mediated by the hormone, cholecystokinin (CCK), which is released from the intestinal wall (Green & Lyman, 1972). Cholecystokinin stimulates the pancreas to secrete digestive enzymes when food is present with a low concentration of proteases. When TI is present in gut, an inactive complex is formed due to the combination of trypsin and trypsin inhibitor. This inactive complex effectively lowers the concentration of active proteases and causes continual release of CCK.

Figure 1. Regulation of secretion of trypsin by pancreas.

From "Significance for Humans of Biologically Active Factors in Soy Bean and other Food Legumes" by I. E. Liener, 1979, *Journal of American Oil Chemists' Society*, 56, p. 122

The prolonged stimulation of the pancreas due to continual release of trypsin causes pancreatic hypertrophy (Green & Lyman, 1972; 1973).

According to Liener (1979), the increased secretion of pancreatic enzymes may exacerbate the already existing deficiency of sulfur containing amino acids (methionine and cysteine) in legume proteins. The enzyme inhibitor complex does not slow enzyme secretion from the pancreas. The hypersecretory activity of the pancreas increases the demand for synthesis of pancreatic enzymes which, in turn, increases the demand for sulfur containing amino acids. Thus, the hypersecretory activity of the pancreas creates a situation in which there is an increased need for sulfur containing amino acids in a diet already deficient in methionine and cysteine.

Dietary Intake of Trypsin Inhibitors

According to Rackis et al. (1985), there might be a significant increase in TI intake with increased use of plant protein. However, little data on dietary intake of TI are available. Doell et al. (1981) reported an average daily intake of 295 mg trypsin inhibitor activity (TIA) in the British diet (Table 2). The total protein consumed was about 70 gm/day, which is approximately equivalent to 4.0 mg TIA per gm protein consumed. Soy-based infant formulae supply 40-60 mg TIA/day (Rackis et al., 1985). A shift to a pure vegan or lacto-ovo vegetarian diet would increase the per capita consumption of TI (Liener & Kakade, 1980).

Since TI is inactivated by moist heat treatment, it is assumed that the residual levels of inhibitors in heat-treated products are of limited nutritional concern. However, due to the necessity to achieve a balance between the amount of heat required to destroy all the TI and to minimize

Table 2

Average Daily Protein and Trypsin Inhibitor Intake From the British Household Diet

Food	Protein (gm/day)	TI Activity (mg/day)	TI Activity (mg/gm protein)
Milk and milk products	17.9	56.8	3.2
Meat	19.5	19.8	1.0
Fish	2.9	1.5	0.5
Eggs	3.7	93.6	25.3
Vegetables	3.3	80.3	12.6
Fruits and nuts	0.7	11.8	16.9
Bread and cereals	18.7	25.3	1.4
Other foods	1.0	5.5	5.5

Note. From "Trypsin Inhibitor Activity of Conventional Foods Which are Part of the British Diet and Some Soya Products" by B. H. Doell, C. J. Ebdon, and C. A. Smith, 1981, *Qualitas Planatarum Plant Foods for Human Nutrition*, 31, p. 148.

the loss in functional properties of protein, most commercially available products retain 5 - 20% of the original TI content (Rackis et al., 1985). These levels are nutritionally significant in long term animal feeding studies (McGuinness et al., 1982; Rackis & Gumbmann, 1981). In a study conducted by Rackis and Gumbmann (1981), rats fed processed soy protein products stopped growing after 146 days; whereas rats fed a casein containing diet continued to grow until 300 days which is normal growth period.

Since there is an increasing use of whole legumes, legume flours, concentrates, and isolates, three population groups which may be exposed to low levels of TI are: 1) infants ingesting soy-based formulae due to an allergy to cow's milk, 2) pure vegetarians who rely on plant proteins rather than animal proteins, and 3) patients with hyperlipidemia who use a soy protein diet to reduce blood cholesterol (Liener, 1986).

Types of Trypsin Inhibitors

The proteolytic enzymes found in nature are classified on the basis of the nature of their active sites and the reaction mechanism involved (Richardson, 1977). These groups include serine proteinases (e.g., trypsin, chymotrypsin, thrombin, plasmin, and elastase), the sulphydryl proteinases (papain, bromelin, and ficin), the metalloproteinases (carboxypeptidases A and B and aminopeptidases), and the acidic proteinases (pepsin and rennin).

Much of the early work on the proteinase inhibitors in plants was concentrated almost exclusively on the inhibitors of trypsin, a serine protease in the digestive tract (Richardson, 1977). Trypsin inhibition in raw legumes is due to a heat labile factor which is present mainly in

cotyledons (true TI) and the heat resistant factors (tannins and polyphenols) which are present in seed coat (Elias et al., 1979; Fernandez et al., 1982). Although methods commonly used to measure TI do not differentiate between true TI and polyphenols, TI activity has been attributed to true TI (Fernandez et al., 1982).

While TI found in plants are both protein and non-protein TI (Hafez & Mohamed, 1983a; 1983b), only protein TI has been studied extensively. Protein trypsin inhibitors found in plants are typically polypeptides and proteins composed of L-amino acids linked through peptide bonds; these contain high percentages of half cystine residues which form disulfide cross links. It is not uncommon for inhibitors to have one half cystine per eight to ten amino acid residues. Plant inhibitors are typically low or devoid of methionine, histidine, and tryptophan but are often rich in aspartic and glutamic acids, serine, and lysine residues. Plant proteinase inhibitors vary in size from 4000 to 60,000 MW. They are generally stable molecules and are often resistant to heat and extremes in pH. This stability is attributed, in part, to the high degree of cross-linking through disulfide bridges (Ryan, 1981).

Trypsin Inhibitors in Soy Bean

Proteinase inhibitors found in soy bean are classified as the Kunitz Inhibitor (KSTI) and the Bowman-Birk Inhibitor (BBI). Kunitz proteinase inhibitor, purified by Kunitz from soy bean (Kunitz, 1945; 1946), is a globular protein containing 181 amino acids (Odani & Ikenaka, 1972), has a molecular weight of 20,100, and has an isoelectric pH of 4.65 (Kunitz, 1946). Kunitz proteinase inhibitor, a strong inhibitor of trypsin and a weak inhibitor of chymotrypsin, contains two disulfide bonds and is

generally reported as being heat labile (Kunitz, 1947). At the center of the active site are the two amino acids, lysine and isoleucine (Freed & Ryan, 1980).

In 1944, BBI was discovered by Bowman. Birk (1961) further characterized the inhibitor which was later named Bowman-Birk inhibitor. It is a small protein containing 71 amino acids with a molecular weight of 8000 and an isoelectric pH of 4.2. Bowman-Birk inhibitor is rich in cystine residues, contains seven disulfide bonds, and is generally reported as being a heat stable proteinase inhibitor (Birk, 1961; Bowman, 1944). It is a double headed inhibitor with independent sites for bovine trypsin and chymotrypsin. The study by Odani and Ikenaka (1972) showed that lysine and serine are present at the antitryptic site of BBI.

Trypsin Inhibitors in Winged Bean

Three trypsin inhibitors have been isolated from winged bean, winged bean trypsin inhibitor - 1 (WBTI-1), winged bean trypsin inhibitor - 2 (WBTI-2), and winged bean trypsin inhibitor - 3 (WBTI-3). Of these inhibitors, WBTI-2 and WBTI-3 constitute 60% of the TI fraction. They are basic proteins with molecular weights of 20,500. Winged bean trypsin inhibitor - 1, the minor inhibitor comprising about 10% of TI fraction, is an acidic protein with molecular weight of 10,500 (Chan & De Lumen, 1982b; Kortt, 1979). Amino acid analysis has indicated that WBTI-2 and WBTI-3 are composed of 184 and 180 amino acid residues, respectively. Winged bean trypsin inhibitor-2 has one histidine, one tryptophan, and three methionine residues. In contrast, WBTI-3 has no histidine or methionine but has three tryptophan residues. Both proteins contain four cystine residues and are rich in aspartic and glutamic acids, glycine, valine, and

leucine. Both WBTI-2 and WBTI-3 strongly inhibit trypsin but only weakly inhibit chymotrypsin (Kortt, 1979).

Trypsin Inhibitors in Green Gram

The presence of TI in green gram was first reported by Sohonie and Bhandarkar (1955). Chu and associates (Chu & Chi, 1965; Chu et al., 1965) subsequently isolated two inhibitors A and B from green gram in crystalline form. Estimates of the molecular weight of inhibitor A ranges between 8000 and 9000. Inhibitors A and B contain 72 amino acid residues. There is one tyrosine and phenylalanine along with a high number of polar side chains, particularly serine and acidic residues. There are eight half cystine residues which participate in disulfide bond formation in the protein molecule.

Chrispeels and Baumgartner (1978) isolated two protease inhibitor fractions from the cotyledons of green gram. One of these has a molecular weight of 12000 and is inhibitory toward trypsin and the seed endopeptidase. The other inhibitor is smaller, has a molecular weight of 2000, and exhibits inhibitor activity only toward the seed endopeptidase. None of these inhibitors has been further characterized.

Quantitation of Trypsin Inhibitor

Currently used methods for the determination of TI in legumes are based upon the reduction in the hydrolysis rate of trypsin substrate resulting from the addition of TI extract. Both natural (casein) and synthetic (amides and esters of amino acids) substrates are used in the determination of TI because trypsin hydrolyzes protein and amides and esters of amino acid derivatives (Kunitz, 1947; Schwert et al., 1948).

Natural Substrate

In the past, casein was used as a substrate to measure TI activity. The most commonly used method employing casein was described by Kunitz (1947) and involved spectrophotometric determination of the breakdown products produced by a given concentration of trypsin both in the presence and absence of the inhibitor. During the hydrolysis of casein by trypsin, products soluble in trichloroacetic acid (TCA) are formed, and the tyrosine and tryptophan contents of these are determined at a wavelength of 280 nm.

Synthetic Substrate

Amides have been widely used to measure TI activity. In 1961, Erlanger et al. introduced the use of a synthetic substrate, BAPA. They compared two substrates, L-lysine p-nitroanilide dihydrobromide (LPA) and benzoyl DL-arginine p-nitroanilide hydrochloride and found that DL-BAPA was hydrolyzed more rapidly. It was shown that DL-BAPA can be used in systems containing as little as 1.0 µg trypsin/ml. Based on this, it was reported to be a more sensitive amide substrate of trypsin. In addition, the hydrolysis can be followed conveniently by colorimetric procedures at 410 nm because one of the products, p-nitroaniline, is yellow in color. Erlanger et al. (1961) also compared D-BAPA and L-BAPA and found that not only was D-BAPA hydrolyzed slowly compared to L-BAPA but also was a potent competitive inhibitor.

In 1969, Kakade et al. recommended the use of DL-BAPA as a substrate for the TI activity assay after comparing it with casein. The rate of hydrolysis of casein by trypsin did not follow zero order kinetics under the conditions defined by Kunitz (1947) and often did not yield

reproducible results; the tryptic hydrolysis of BAPA followed a zero order reaction, and a linear relationship was observed between the quantity of p-nitroaniline released and the concentration of the active enzyme. The amount of trypsin inhibited with BAPA was higher (about 25 to 30%) than the value obtained with casein (Erlanger et al., 1961). In their review of TI, Laskowski and Laskowski (1954) noted that the inhibitor activity of the soy bean TI was higher with synthetic substrates, TAME and α -benzoyl-L-arginine ethyle ester (BAEE), compared to natural substrates, casein and hemoglobin. Fritz et al. (1968) reported that the proteinase inhibitors from mammalian tissues exhibit a similar phenomenon which can be attributed to an interference with the formation of the enzyme inhibitor complex. They hypothesized that this interference occurs as a result of the steric hindrance caused by the larger molecules of the natural substrates. On this basis, Kakade et al. (1969) theorized that, if this effect were true for soy bean inhibitors, the BAPA method should provide a more accurate means of assessing the true inhibitor content of soybeans. On the basis of this consideration and the simplicity of the procedure, the BAPA method was adopted for the routine determination of the TI activity (Kakade et al., 1969).

A collaborative study organized by the American Association of the Cereal Chemists (AACC) and the American Oil Chemists Society (AOCS) revealed additional problems with the accuracy and reproducibility of the BAPA procedure when analyzing raw and heat treated soy flour (Rackis et al., 1974). This study resulted in a modified assay (Kakade et al., 1974) which improved both the accuracy and reproducibility. The modified assay was accepted as the official procedure for the TI analysis by AACC (1983).

A semi-automated application of the modified method (Kakade et al., 1974) was developed by Egberg et al. (1975).

The method proposed by Kakade et al. (1974) was further modified in the same way by two different research groups (Hamerstand et al., 1981; Smith et al., 1980) who used a single inhibitor level rather than serial inhibitor levels. This modification bypassed the cumbersome data interpretation involving either extrapolation to zero or an averaging over a range of inhibition levels. The reasons for the modifications resulted from two observations: 1) the pattern of the enzymatic activity and inhibitor concentration were diverse and 2) the extrapolation method of data interpretation uses data that were not in the region in which zero order kinetics is followed.

In 1982, Lehnhardt and Dills (Charpentier & Lemmel, 1984) reported an additional change in the procedure of Kakade et al. (1974) and allowed for analysis of a single diluted extract that inhibited 10 to 90% of the trypsin under assay conditions. They showed that 1.0 μg of pure trypsin produced 2.16 TU under the conditions of the assay and this factor was substituted for the 1.9 TU factor used previously (Hamerstand et al., 1981; Kakade et al., 1969; 1974; Smith et al., 1980). This method was automated by Charpentier and Lemmel (1984). Liu and Markakis (1989) proposed further modifications: 1) that water rather than dilute alkali be used for extracting the inhibitors, 2) that the aqueous extract be destabilized with tris buffer before rather than after the reaction, 3) that porcine rather than bovine trypsin be used, 4) that the enzyme and not the substrate be added last to the reaction mixture, and 5) that the volume of the reaction mixture be reduced from 10.0 to 4.0 ml. These

modifications increased the sensitivity in the measurement of TI content.

Esters of amino acids derivatives are also used for the determination of trypsin activity. Of these, α -benzoyl-L-arginine methyl ester and α -p-toluenesulfonyl-L-arginine methyl ester are most active and are hydrolyzed about 60 times faster than the corresponding amides. Schwert et al. (1948) proposed a method based upon the change that occurs in ultra violet absorbance during the hydrolysis of amino acids esters by these enzymes. The change between the absorbance of the ester and that of the corresponding acid during hydrolysis of the ester was measured. The substrate BAEE together with α -benzoyl-L-arginine (BA) as a blank were used for trypsin assay (Schwert et al., 1948). Although this method was until then one of the most satisfactory methods proposed for assay of proteolytic enzymes, it had limitations: 1) two solutions of acid and ester were required and 2) BAEE was slowly hydrolyzed by chymotrypsin rendering this substrate unsuitable for the assay of traces of trypsin in the presence of a relatively large amount of chymotrypsin (Hummel, 1959).

In 1959, Hummel proposed a method based on the use of TAME as a substrate because TAME is more rapidly hydrolyzable by trypsin than BAEE and is not hydrolyzed by chymotrypsin. Therefore, TAME is suitable for the assay of traces of trypsin in the presence of large amounts of chymotrypsin.

Trypsin Sources

Bovine and porcine trypsins are two commonly used enzymes in the measurement of TI (Liener, 1979). Most proteinase inhibitors, such as Kunitz soy bean TI, specifically inhibit bovine trypsin at a molar ratio of 1:1. However, Travis and Roberts (1969) found that soy bean TI did not

inhibit human trypsin based upon the measurement of esterolytic activity of human trypsin with synthetic substrates. In another study, Feeney et al. (1969) determined that the Kunitz soy bean TI, which had been repeatedly demonstrated to be an inhibitor of bovine trypsin, had little inhibitory activity towards human trypsin. Therefore, it was concluded that inhibition of bovine trypsin by naturally occurring inhibitors is no index of the inhibitory activity against human trypsin. The Birk soy bean TI, however, exhibited approximately equal inhibitory activity against both human and bovine trypsins. It was concluded that the activity of a particular inhibitor may be so specific that the inhibitor fails to inhibit enzymes from different species (Feeney et al., 1969).

In 1973, Mallory and Travis isolated two forms of trypsin from human pancreas: 1) the cationic form which constitutes the major component and 2) the anionic form which accounts for less than 33% of the total TI activity of pancreatic juice. The minor anionic form is inhibited completely by soy bean TI at a molar ratio of 1:1, whereas the cationic form is only weakly inhibited (20%).

In further support of the probability that soy bean inhibitors are less affective against human trypsin, Liener (1979) reported a relationship between the size of the pancreas of various species of animals and the nature of the response of the pancreas to TI. Animals with pancreas weighing more than 0.3% of body weight became hypertrophic when fed raw soybeans or the TI. On this basis, it was predicted that the pancreas of humans would be insensitive to the effects of soy bean inhibitor.

Although bovine trypsin is unstable in alkaline solution, it is the most commonly used trypsin for TI determination. Compared to bovine,

the structure of porcine trypsin is intrinsically more resistant to self digestion (Buck et al., 1962; Liu & Markakis, 1989). Incubation for 10 min at 37 °C and pH 7.5 results in a sharp decrease of bovine trypsin activity whereas porcine trypsin is stable under similar conditions. Since TI activity is commonly assayed at pH 8.1, the optimum for trypsin activity (Erlanger et al., 1961), enzyme inactivation occurs during the assay. On this basis, use of porcine trypsin for determination of TI content is recommended (Liu & Markakis, 1989).

Chapter 2

JOURNAL ARTICLE

COMPARISON OF TWO SYNTHETIC SUBSTRATES AND TWO TRYPSIN
SOURCES FOR MEASURING TRYPSIN INHIBITOR ACTIVITY IN
SOY BEAN, WINGED BEAN, AND GREEN GRAM

Pratibha L. Samar, Panfilo S. Belo^{*}, Elveda Smith,
and Miriam Saltmarch

Department of Nutrition and Food Science
San Jose State University
1 Washington Square
San Jose, CA 95192.

^{*} Address questions and comments to: Dr. Panfilo Belo, Department of Nutrition and Food Science, San Jose State University, 1 Washington Square, San Jose, CA 95192, (408) 924-3108.

ABSTRACT

The objective of this study was to compare two synthetic substrates (BAPA and TAME) and two trypsin sources (bovine and porcine) in the determination of trypsin inhibitor (TI) activity in soy bean, winged bean, and green gram. The TI activities in aqueous extracts prepared from defatted beans were determined by original BAPA, modified BAPA, and TAME methods and compared using a 3 x 3 x 2 factorial analysis of variance. The TI activity varied significantly with substrate, source of trypsin, and type of legume used. Modified BAPA gave significantly higher TI activity ($p \leq 0.05$) followed by original BAPA and TAME. The TI activity obtained with bovine trypsin was significantly higher ($p \leq 0.05$) than porcine trypsin when BAPA and modified BAPA methods were used. Similar results were obtained for soy bean and green gram when TAME was used. Regardless of the substrate or the enzyme used, the TI activity was significantly higher in soy bean ($p \leq 0.05$) followed by winged bean and green gram.

INTRODUCTION

As the discrepancy between the world's supply of protein and the growth of the global population continues to widen, ways to bridge this gap have become a matter requiring immediate action. Based on available statistics, it is estimated that, by the end of the century, the population of the world will equal six billion and that a two fold increase in plant protein and a four fold increase in animal protein will be needed to feed this population (Liener and Kakade, 1980). The importance of using plant proteins in countries in which protein-calorie malnutrition (PCM) exists is well recognized. The increasing cost of animal proteins has prompted food technologists, nutritionists, and biochemists to consider plant proteins as a major source of dietary protein (Salunkhe, 1982).

Soy bean (Glycine max L.), winged bean (Psophocarpus tetragonolobus [L.] DC), and green gram (Vigna radiata [L.] wilczek) are among the legumes which have potential as protein rich food sources. They are cheap and within the purchasing capabilities of an average family in underdeveloped countries (Liener and Kakade, 1980). However, the nutritive value of the legume seeds is limited by the presence of antinutritional factors such as phytates, alkaloids, phytohemagglutinins, saponins, tannins, cyanogenic factors, and trypsin inhibitors. Among these, trypsin inhibitor (TI) has been studied extensively because it is present in most legumes (Liener and Kakade, 1980). The antinutritional effect of TI is due to its combination with trypsin to form an inactive complex which interferes with protein digestion. In addition to TI, trypsin inhibition in raw beans is also caused by polyphenols (Elias et al., 1979; Fernandez et al., 1982; Tan et al., 1984). Trypsin inhibitor causes

pancreatic hypertrophy in chicks, mice, and rats (Chernick et al., 1948; Kakade et al., 1973; Rackis, 1965). In addition, Chan and DeLumen (1982a) reported that the TI in winged bean causes spleen hypertrophy.

Due to increased use of plant proteins in developing countries to alleviate PCM, it is important to measure TI in legumes. Although both protein and non-protein TI have been identified in legumes, most assay methods for TI are based only on the determination of the protein fraction (Hafez & Mohamed, 1983a, b). Spectrophotometric methods currently used for TI assay are based on the in-vitro reduction in hydrolysis rate of the trypsin substrate. Both the natural trypsin substrate, casein (Kunitz, 1947), and the synthetic trypsin substrates, benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPA) (Erlanger et al., 1961) and p-toluene-sulfonyl-L-arginine methyl ester (TAME) (Hummel, 1959), are utilized in measuring TI activity in legumes. Among these, BAPA has been used extensively and is reported to be not only more reliable but also more convenient when compared to casein (Kakade et al., 1969).

The BAPA method has undergone various modifications (Hamerstand et al., 1981; Kakade et al., 1974; Liu and Markakis, 1989; Smith et al., 1980) to increase simplicity, accuracy, and reproducibility. Although the BAPA method has been widely used, it is time consuming requiring a 10 min incubation period at 37 °C before terminating the reaction prior to the measurement of absorbance. Compared to BAPA, TAME is simpler since it is conducted at room temperature and does not require termination of the reaction. There are several studies (Chan and DeLumen, 1982b; Delumen and Belo, 1981; Delumen and Salamat, 1980; Sathe and Salunkhe, 1981) in which the TAME method has been used, and there is a need to compare

BAPA and TAME methods. The trypsin inhibitor activity is measured using either bovine or porcine trypsin. Although porcine trypsin is more stable in alkaline conditions, bovine trypsin is used extensively in the determination of TI activity (Buck et al., 1962).

This study was undertaken to compare two synthetic substrates, BAPA and TAME, and two trypsin sources, bovine and porcine, in the determination of trypsin inhibitor activity in soy bean, winged bean, and green gram. An attempt to modify the original BAPA method to make it suitable for continuous spectrophotometric assay for trypsin activity at room temperature, rather than at 37 °C, was also undertaken.

MATERIALS AND METHODS

Sample and Sample Preparation. Soy bean and green gram were obtained from a local grocery store; winged bean (chimbu variety) was provided by Dr. De Lumen, University of California at Berkeley. Soy bean, winged bean, and green gram were ground in a Straub model 4E grinding mill and passed through a 60 mesh screen. The samples were defatted in soxhlet apparatus for 6-7 hr using petroleum ether as an extracting solvent. The residue obtained was dried in the hood to remove residual solvent. Defatted powder was used in subsequent analyses and for TI activity assay.

Proximate Analysis. Analyses of crude protein, crude fat, ash, moisture, and total carbohydrates (by the difference) were performed on full fat flour according to the methods of the Association of Official Analytical Chemists (AOAC, 1984).

Extraction of Trypsin Inhibitor. The procedure used for extraction of TI was that described by Liu and Markakis (1989). A 0.5 gm

defatted sample was extracted with 50 ml distilled water for 30 min on a mechanical shaker (GCA/Precision Scientific, Model 25) at 200 rpm. Ten ml of the sample suspension was stabilized by adding an equal volume of the tris buffer (0.05 M, pH 8.2) followed by vigorously shaking for 2-3 min before filtering through a Whatman No. 2 paper. The protein level of filtrate was determined using bovine protein as a standard (Lowry et al., 1951). Freshly prepared aqueous extracts were used for TI activity determination.

Determination of Trypsin Inhibitor Activity by Original BAPA

Method. The method used for TI determination was that of Liu and Markakis (1989). The assay buffer was 0.05 M tris buffer at pH 8.2 and contained 0.01 M CaCl_2 . A stock trypsin solution was prepared by dissolving 10 mg crystalline bovine (type II, Sigma Chemical Co.) or porcine trypsin (type IX, Sigma Chemical Co.) in 50 ml of 0.001 M HCl solution, pH approximately 2.5 containing 0.0025 M CaCl_2 . The solution was held at 5 °C. To prepare a working trypsin solution, 2 ml of the stock solution was diluted to a total volume of 25 ml using the 0.001 M HCl solution. A stock BAPA (Sigma Chemical Co.) solution was prepared by dissolving 400 mg of BAPA in 10 ml of dimethylsulfoxide. This solution is stable at room temperature. A working BAPA solution was prepared by diluting 0.25 ml of stock BAPA solution to a total volume of 25 ml using the assay buffer warmed to 37 °C. Fresh working BAPA solution was prepared for each assay. Samples were prepared by diluting aqueous extracts of defatted beans with water until there was 30 to 70% trypsin inhibition (Liu and Markakis, 1989).

To measure TI activity, 1.0 ml of sample was added to each test tube

containing 2.0 ml of BAPA followed by 0.5 ml of trypsin. After 10 min the reaction was stopped by adding 0.5 ml of 30% acetic acid solution. The samples were also prepared without inhibitors using 1.0 ml of water. The reagent blank was prepared by using BAPA (2.0 ml), water (1.5 ml), and acetic acid (0.5 ml). The reaction was conducted at 37 °C, and the absorbance was measured at 410 nm. The trypsin inhibitor activity is expressed as trypsin inhibitory unit (TIU) per mg protein in which one TIU of trypsin inhibitors is defined as the amount of inhibitor that inhibits 1.0 µg of pure trypsin (Tan et al., 1984).

Determination of Trypsin Inhibitor Activity by TAME Method.

The method used for TI determination was from Worthington Biochemical Corporation (1972). The assay buffer was a 0.046 M tris buffer at pH 8.1 and contained 0.0115 M CaCl₂. A stock trypsin solution was prepared by dissolving 25 mg of bovine or porcine trypsin in 25 ml of 0.001 M HCl; after which the solution was stored at 5 °C. To prepare a working trypsin solution, 0.38 ml of the stock solution was diluted to a total volume of 25 ml using the 0.001 M HCl solution. A working TAME solution was prepared by dissolving 37.9 mg of p-toluene-sulfonyl-L-arginine methyl ester hydrochloride in 10.0 ml distilled water.

The rate of hydrolysis of TAME was measured by the increase in the absorbance at 247 nm with a Shimadzu UV visible recording spectrophotometer both in the presence and absence of the inhibitor. A mixture of 0.1 ml of 0.001 M HCl, 0.3 ml substrate, and 2.6 ml buffer was pipetted in a cuvette. In a second cuvette, 0.1 ml sample, 2.5 ml buffer, and 0.1 ml diluted enzyme were added. After 5 min of incubation at room temperature, 0.3 ml of substrate was added. The contents were mixed,

and the absorbance was recorded at 30 sec intervals for 3 min at 247 nm. The reaction was also conducted in the absence of the inhibitor. The trypsin inhibitor activity is expressed as trypsin inhibitory unit per mg protein in which one inhibitory unit of trypsin inhibitors (TIU) is defined as the amount of inhibitor that inhibits 1.0 µg of pure trypsin (Tan et al., 1984).

Determination of Trypsin Inhibitor Activity by Modified BAPA Method. The original BAPA method was modified as follows:

1) the assay used a total volume of 3.0 ml instead of 4.0 ml while maintaining the same enzyme and substrate concentration, 2) the reaction was conducted at room temperature for 3 min rather than at 37 °C for 10 min, and 3) the change in absorbance was monitored using a continuous recording spectrophotometer.

The procedure used was as follows: 1.5 ml each of BAPA and H₂O were added in a 1 cm cuvette. The contents were mixed, and the spectrophotometer was zeroed at 410 nm. In another cuvette, 1.5 ml BAPA, 1.1 ml sample, and at zero time, 0.4 ml of diluted trypsin were added. The contents were mixed, and absorbance was recorded at 30 sec intervals for 3 min at 410 nm. The reaction was also conducted in the absence of inhibitor by replacing the sample with water. The experiment was conducted at room temperature, and the TI activity was calculated as per in the original BAPA method.

Analysis of Data. Determinations were performed in triplicate, and data were analyzed using 3 x 3 x 2 factorial analysis of variance.

RESULTS AND DISCUSSIONS

The results of proximate analysis of full-fat flour prepared from soy bean, winged bean, and green gram are presented in Table I. As expected, soy bean showed the highest protein and oil contents followed by winged bean and green gram. The proximate composition of soy bean, winged bean, and green gram is within the range of values reported in the literature (Adsule et al., 1986; Kadam and Salunkhe, 1984; Smith and Circle, 1978).

Determination of Trypsin Inhibitor by Original BAPA

Method. The trypsin inhibitor content of soy bean, winged bean, and green gram determined by original BAPA method is shown in Table II. The trypsin inhibitor activity of soy bean was 310.82 TIU/mg protein (or 77.71 TIU/mg sample) using bovine trypsin which is within the range of 36.74 - 110.9 TIU/mg sample reported by Kakade et al. (1974). The trypsin inhibitor content using porcine trypsin was 247.09 TIU/mg of protein (or 66.77 TIU/mg sample) which is lower than the average TI content of 171.0 TIU/mg sample reported by Liu and Markakis (1989). Using bovine trypsin, the TI content of winged bean was 203.88 TIU/mg protein (or 53.01 TIU/mg sample). This value is higher than the range of 15.20 - 32.40 TIU/mg sample reported in the literature (Tan and Wong, 1982) which may be attributed to the difference in the variety of winged bean. The trypsin inhibitor content in winged bean, when determined with porcine, was 158.63 TIU/mg protein (or 41.24 TIU/mg sample). The trypsin inhibitor activity in green gram was 48.28 TIU/mg protein (or 5.31 TIU/mg sample) and 35.44 TIU/mg protein (or 3.90 TIU/mg sample) using bovine and porcine trypsin, respectively. Values for bovine are within the

range reported (Elkowicz and Sosulski 1982; Jaya and Venkataraman, 1980). There are no data available for TI levels in green gram and winged bean using porcine.

Determination of Trypsin Inhibitor by TAME Method. The trypsin inhibitor activity levels in soy bean, winged bean, and green gram determined by TAME method are presented in Table II. Soy bean contained 22.97 TIU/mg protein (or 5.74 TIU/mg sample) and 18.16 TIU/mg protein (or 4.54 TIU/mg sample) with bovine and porcine trypsin, respectively. There are no known data available for measuring the TI level for soy bean using TAME. The trypsin inhibitor content in winged bean was 17.35 TIU/mg protein (or 4.51 TIU/mg sample) using bovine trypsin and 17.53 TIU/mg protein (or 4.55 TIU/mg sample) using porcine trypsin. The trypsin inhibitor levels using bovine are within the range of 3.69 - 9.75 TIU/mg sample reported in the literature (Chan and DeLumen, 1982a; DeLumen and Salamat, 1980). No known studies are available on TI levels in winged bean with porcine trypsin. Green gram contained 4.06 TIU/mg protein (or 0.45 TIU/mg sample) and 3.49 (or 0.38 TIU/mg sample) TIU/mg protein with bovine and porcine trypsin, respectively. There are no known data available for measuring the TI level of green gram using TAME with either of these enzymes.

Determination of Trypsin Inhibitor by Modified BAPA

Method. With the modified BAPA method, soy bean contained 445.56 TIU/mg protein (or 115.85 TIU/mg sample) and 266.83 TIU/mg protein (or 66.71 TIU/mg sample) using bovine and porcine trypsin, respectively. Winged bean contained 243.79 TIU/mg protein (or 63.38 TIU/mg sample) and 175.19 TIU/mg protein (or 45.55 TIU/mg sample) when analyzed with

bovine and porcine trypsin, respectively. Green gram had 58.93 TIU/mg protein (or 6.48 TIU/mg sample) and 47.88 TIU/mg protein (or 5.27 TIU/mg sample) with bovine and porcine trypsin, respectively.

Comparison of Original BAPA and TAME Methods. The trypsin inhibitor levels in soy bean, winged bean, and green gram determined by original BAPA were significantly higher ($p \leq 0.05$) compared to TAME which suggests that TI levels are affected by the nature of the substrate. Regardless of the substrate used, soy bean exhibited the highest TI activity followed by winged bean and green gram.

Although BAPA is used extensively, it appears that the TAME method has some advantages. It is conducted at room temperature with continuous recording spectrophotometer. On the other hand, the BAPA method is more time consuming since it is conducted in an incubator maintained at 37°C and requires 10 min for the completion of the reaction. In addition, after 10 min, the reaction is terminated by adding acetic acid which increases the chances for experimental error. In the TAME method, this extra step is avoided.

It was found that BAPA was sensitive to unclean surfaces. Formation of a precipitate was often observed which was not seen with TAME. BAPA is also more expensive compared to TAME (\$14.60/gm vs. \$5.95/gm) and involves the use of an organic solvent. For stock solution, BAPA is dissolved in dimethylsulfoxide which may affect dissociation of the enzyme inhibitor complex (Fritz et al., 1974). Since TAME is readily soluble in water, this risk is avoided. The BAPA which is commonly used in assays is a mixture of D and L isomers. The D isomer is a competitive inhibitor and is hydrolyzed slowly (Erlanger et al., 1961). Thus, it is

suggested that the values obtained with BAPA may not be a true representation of the amount of TI present in legumes.

Comparison of Original and Modified BAPA Methods. The trypsin inhibitor levels determined by modified BAPA were significantly higher ($p \leq 0.05$) compared to the original BAPA. The difference between the original and modified BAPA methods varied with the source of enzyme and the type of legume. In soy bean, TI levels with modified BAPA increased by 30% and 7% using bovine and porcine, respectively. In winged bean, the TI level determined with bovine was 16% higher, whereas with porcine they increased by only 9%. In green gram, values were 18% and 26% higher with bovine and porcine, respectively.

The difference in the TI activity obtained with these two methods may be attributed to the temperature differences. At the higher temperature, enzymatic reaction between trypsin and the substrate is faster, and fewer reactive sites of trypsin are available for TI to form a complex resulting in low TI activity.

Although the modified BAPA method developed in this study gave significantly higher TI values compared to the original BAPA, it is simpler and faster. Therefore, it would be applicable to a large number of samples. However, a continuous recording spectrophotometer is required.

Comparison of Bovine and Porcine Trypsin. The trypsin inhibitor activity obtained with bovine was significantly higher ($p \leq 0.05$) than porcine trypsin in all three beans when the original and modified BAPA methods were used. Similar results were obtained in soy bean and green gram when TAME was used. This suggests that TI levels are affected by the source of enzyme. However, no significant difference

between porcine and bovine was found in winged bean using TAME. It is theorized that the trypsin source does not have an effect on winged bean TI when TAME is used as a substrate for the determination of TI activity. Regardless of the type of trypsin, the TI level was consistently higher in soy bean, followed by winged bean and green gram.

CONCLUSION

Levels of trypsin inhibitors varied with substrate, source of trypsin, and type of legume used. The modified BAPA method gave significantly higher TI activity ($p \leq 0.05$) followed by the original BAPA and TAME methods which suggests that the TI levels are affected by the nature of the substrate. The TI activity obtained by bovine was significantly higher ($p \leq 0.05$) than porcine trypsin when the BAPA and modified BAPA methods were used. Similar results were obtained in soy bean and green gram when TAME was used as a substrate. Thus, it is concluded that the TI levels are also affected by the source of enzyme. However, no significant difference between porcine and bovine was found in winged bean using TAME, suggesting that there is no impact of the trypsin source on winged bean TI when TAME is used as a substrate. Regardless of the substrate or the enzyme used, the TI activity was significantly higher ($p \leq 0.05$) in soy bean followed by winged bean and green gram. The modified BAPA method developed in this study was simpler and faster than the original BAPA, making it applicable to analysis of a large number of samples. Although the original BAPA method is used extensively, it appears that TAME may be better based on time, simplicity, and cost of analysis.

Table I. Proximate Composition of Soy bean, Winged Bean and Green Gram on Wet Basis^a

composition %	soy bean	winged bean	green gram
moisture	6.65 $\pm$ 0.35	5.75 $\pm$ 0.10	8.16 $\pm$ 0.20
crude protein	35.10 $\pm$ 0.15	30.10 $\pm$ 0.10	24.87 $\pm$ 0.15
crude fat	25.90 $\pm$ 0.14	22.75 $\pm$ 0.21	1.27 $\pm$ 0.02
ash	4.68 $\pm$ 0.10	4.39 $\pm$ 0.08	3.00 $\pm$ 0.10
total carbohydrates ^b	27.67	37.08	62.70

^a mean $\pm$ standard deviation of three determinations

^b calculated by difference

Table II. Trypsin Inhibitor Activity (TIU/mg Protein)^a in Soy bean, Winged Bean, and Green Gram

method	soy bean	winged bean	green gram
original BAPA			
bovine	310.82 ± 6.9 ^{bx}	203.88 ± 4.40 ^{by}	48.28 ± 4.97 ^{bz}
porcine	247.09 ± 14.1 ^{cx}	158.63 ± 7.12 ^{cy}	35.44 ± 2.36 ^{cz}
modified BAPA			
bovine	445.58 ± 17.5 ^{bx}	243.79 ± 17.4 ^{by}	58.93 ± 1.77 ^{bz}
porcine	266.83 ± 10.8 ^{cx}	175.19 ± 6.85 ^{cy}	47.88 ± 2.25 ^{cz}
TAME			
bovine	22.97 ± 0.62 ^{bx}	17.35 ± 0.54 ^{by}	4.06 ± 0.17 ^{bz}
porcine	18.16 ± 0.20 ^{cx}	17.53 ± 0.57 ^{by}	3.49 ± 0.2 ^{cz}

^a mean ± standard deviation of three determinations

Within rows, values followed by b and c differ significantly ($p \leq 0.05$)

Within column, values followed by x, y, and z differ significantly ($p \leq 0.05$)

LITERATURE CITED

- Adsule, R. N.; Kadam, S. S.; Salunkhe, D. K. Chemistry And Technology of Green Gram (*Vigna Radiata* [L] Wilczek). *CRC Cri. Rev. in Food Sci. Nutr.* 1986, 25, 73-105.
- AOAC. *Official Methods of Analysis*, 13th ed. Association of the Official Analytical Chemists: Washington, DC, 1984.
- Buck, F. F.; Vithsyathil, A. J.; Bier, M.; Nord, F. F. On the Mechanism of Enzyme Action LXXIII. Studies on Trypsins from Beef, Sheep and Pig Pancreas. *Arch. Biochem. Biophys.* 1962, 97, 417-424.
- Chan, J.; DeLumen, B. O. Biological Effects of Isolated Trypsin Inhibitors From Winged Bean (*Psophocarpus tetragonolobus*) on rats. *J. Agric. Food Chem.* 1982a, 30, 46-50.
- Chan, J.; DeLumen, B. O. Properties of Trypsin Inhibitors from Winged Bean (*Psophocarpus tetragonolobus*) Seed Isolated by Affinity Chromatography. *J. Agric. Food Chem.* 1982b, 30, 42-46.
- Chernick, S. S.; Lepkovsky, S.; Chaikoff, I. L. A Dietary Factor Regulating the Enzyme Content of the Pancreas: Changes Induced in Size and Proteolytic Activity of the Chick Pancreas by the Ingestion of Raw Soybean Meal. *Am. J. Physiol.* 1948, 155, 33-39.
- DeLumen, B. O.; Belo, P. S. Inhibitors of Trypsin and Chymotrypsin in Winged Bean (*Psophocarpus tetragonolobus*) Tubers. *J. Agric. Food Chem.* 1981, 29, 884-886.
- DeLumen, B. O.; Salamat, L. A. Trypsin Inhibitor Activity in Winged Bean (*Psophocarpus tetragonolobus*) and the Possible Role of Tannin. *J. Agric. Food Chem.* 1980, 28, 533-536.

- Elias, L.G.; De Fernandez, D. G.; Bressani, R. J. Possible Effects of Seed Coat Polyphenolics on the Nutritional Quality of Bean Protein. *J. Food Sci.* 1979, 44, 524-527.
- Elkowicz, K.; Sosulski, F. W. Antinutritive Factors in Eleven Legumes and Their Air-Classified Protein and Starch Fractions. *J. Food Sci.* 1982, 47, 1301-1304.
- Erlanger, B. F.; Kokawsky, N.; Cohen, W. The Preparation and Properties of Two New Chromogenic Substrate of Trypsin. *Arch. Biochem. Biophys.* 1961, 95, 271-278.
- Fernandez, D. G., Elias, L.G., Braham, J. E.; Bressani, R. J. Trypsin Inhibitors and Hemagglutinins in Beans and Their Relationship with the Content of Tannins and Associated Polyphenols. *J. Agric. Food Chem.* 1982, 30, 734-739.
- Fritz, H.; Trautschold, I.; Werle. "Protease Inhibitors". In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; Vol. 2, pp 1064-1080.
- Hafez, S. Y.; Mohammed, A. I. Biochemical Study and Non Protein Trypsin and Chymotrypsin Inhibitors in the Soy Bean. *J. Food Sci.* 1983a, 48, 1265-1268.
- Hafez, S. Y.; Mohammed, A. I. Presence of Non Protein Inhibitor in Soy and Winged Beans. *J. Food Sci.* 1983b, 48, 75-76.
- Hamerstand, G. E.; Black, L. T.; Glover, J. D. Trypsin Inhibitors in Soy Products: Modification of the Standard Analysis Procedure. *Cereal Chem.* 1981, 58, 42-45.
- Hummel, B. C. W. A Modified Spectrophotometric Determination of Chymotrypsin, Trypsin, and Thrombin. *Can. J. Biochem. Phys.* 1959, 37,

1393-1399.

- Jaya, T. V.; Venkataraman, L. V. Effects of the Germination on the Nitrogenous Constituents, Essential Amino Acids, Carbohydrates, Enzymes and Antinutritional Factors in Chick Pea and Green Gram. *Ind. Food Packer*. 1980, 8, 3-11.
- Kadam, S. S.; Salunkhe, D. K. Winged Bean in Human Nutrition. *CRC Crit. Rev. in Food Sci. Nutr*. 1984, 21, 1-40.
- Kakade, M. L.; Hoffa, D. E.; Liener, I. E. Contribution of Trypsin Inhibitors to the Deleterious Effects of Unheated Soybeans Fed to Rats. *J. Nutr.*, 1973, 103, 1772-1775.
- Kakade, M.L.; Rackis, J.E.; McGhee, J. E.; Puski, G. Determination of Trypsin Inhibitor Activity of Soy Products: A Collaborative Analysis of an Improved Procedure. *Cereal Chem.* 1974, 51, 376-382
- Kakade, M.L.; Simons, N.; Liener, I. E. An Evaluation of Natural vs. Synthetic Substrates for Measuring the Antitryptic Activity of Soy bean Samples. *Cereal Chem.* 1969, 46, 518-526.
- Kunitz, M. Crystalline Soy bean Trypsin Inhibitor II. General Properties. *J. Gen. Physiol.* 1947, 30, 291-310.
- Liener, I. E.; Kakade, M. L. "Protease Inhibitors". In *Toxic Constituents of Plant Foodstuffs*; Liener, I. E., Ed.; Academic Press: New York, 1980; pp 7-68.
- Liu, K.; Markakis, P. An Improved Colorimetric Method for Determining Antitryptic Activity in Soy bean Products. *Cereal Chem.* 1989, 66, 415-422.
- Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein Measurement with the Folin Reagent. *J. Bio. Chem.*, 1951, 193, 265-275.

- Rackis, J. J. Physiological Properties of Soybean Trypsin Inhibitors and their Relationship to Pancreatic Hypertrophy and Growth Inhibition of Rats. *Fed. Proc.*, 1965, 24, 1488-1493.
- Salunkhe, D. K. Legumes in Human Nutrition: Current Status and Future Research Needs. *Curr. Sci.* 1982, 51, 387-390.
- Sathe, S. K.; Salunkhe, D. K. Investigations on Winged Bean Proteins and Antinutritional Factors. *J. Food Sci.* 1981, 46, 1389-1393.
- Smith, A. K.; Circle, S. J. "Chemical Composition of the Seed". In *Soybeans: Chemistry and Technology*; Smith, A. K.; Circle, S. J., Eds.; The AVI Publishing Company: Connecticut, 1978; pp 61-92.
- Smith, C.; Megan, W. V.; Twaalfhoven, L.; Hitchcock, C. The Determination of Trypsin Inhibitor Levels in Foodstuffs. *J. Sci. Food Agric.* 1980, 31, 341-350.
- Tan, N.; Wong, K. Thermal Stability of Trypsin Inhibitor Activity in Winged Bean. *J. Agric. Food Chem.* 1982, 30, 1140-1143.
- Tan, N.; Wong, K.; DeLumen. Relationship of Tannin Levels and Trypsin Inhibitor Activity with the in-vitro Protein Digestibilities of Raw and Heat-Treated Winged Bean, *J. Agric. Food Chem.* 1984, 32, 819-822.
- Worthington Biochemical Corporation. "Trypsin (Bovine Pancreas)". In *Worthington Enzymes Manual*; Freehold, NJ, 1972; pp 125-127.

CHAPTER 3

SUMMARY AND RECOMMENDATIONS

Summary

The objective of this study was to compare two synthetic substrates, BAPA and TAME, and two trypsin sources, bovine and porcine, in the determination of TI activity in soy bean, winged bean, and green gram. An aqueous extract from defatted beans was used for the measurement of TI. On the basis of the TAME method, the original BAPA method was modified to increase simplicity and speed as follows: 1) the assay was conducted in a total volume of 3.0 ml instead of 4.0 ml while maintaining the same enzyme and substrate concentration, 2) the reaction was conducted at room temperature for 3 min rather than at 37 °C for 10 min, and 3) the change in absorbance was monitored using a continuous recording spectrophotometer. The trypsin inhibitor activity obtained by the original BAPA, modified BAPA, and TAME methods were compared using a 3 x 3 x 2 factorial analysis of variance.

The trypsin inhibitor levels in soy bean, winged bean, and green gram determined by original BAPA were significantly higher ($p \leq .05$) compared to TAME which suggests that TI levels are affected by the substrate. Regardless of the substrate used, soy bean exhibited the highest TI activity followed by winged bean and green gram. Although the original BAPA method is used extensively, the TAME method has some advantages. The TAME method is faster, simpler, and cheaper and does not involve the use of an organic solvent which may effect the assay of TI (Fritz et al., 1974). Benzoyl-DL-arginine-p-nitroanilide hydrochloride was also found to be very sensitive to unclean surfaces since the formation of

precipitate was often observed. This was not seen with TAME. However, TAME requires a continuous recording spectrophotometer.

The modified BAPA method developed in this study gave significantly higher TI values ($p \leq .05$) compared to original BAPA. The difference in the TI activity by two methods may be attributed to the temperature differences. It is assumed that, at higher temperatures, enzymatic reaction between trypsin and the substrate is faster and less reactive sites of trypsin are available for TI resulting in low TI activity. It can also be suggested that, compared to porcine, bovine trypsin is affected more by temperature.

The trypsin inhibitor levels obtained by bovine were significantly higher ($p \leq .05$) than porcine trypsin in all three beans when the original and modified BAPA methods were used. Similar results were obtained for soy bean and green gram when TAME was used. This suggests that TI levels are affected by the source of enzyme. However, no significant difference between porcine and bovine was found in the winged bean using TAME, suggesting that the trypsin source does not affect winged bean TI when TAME is used as a substrate for the determination of TI activity. Regardless of the type of trypsin, the TI level was consistently highest in soy bean, followed by winged bean and green gram.

Although the modified BAPA method developed in this study gave significantly higher TI values ($p \leq .05$) compared to the original BAPA, the modified BAPA method is simpler and faster and is applicable to analysis of a large number of samples. However, the modified BAPA method requires a continuous recording spectrophotometer.

Recommendations

This study showed that the measured TI content varies with the type of method used for analysis. Hence, it is recommended that, while reporting TI content, the method used should also be indicated.

Although results of this study showed applicability of modified BAPA for the determination of TI levels in defatted soy bean, winged bean, and green gram, there is a need for further study using processed products containing these legumes.

When beans were analyzed by TAME, a significant difference was found between the two enzymes in all beans except winged bean. This suggests that there is an interaction among the type of trypsin inhibitor, the source of trypsin, and the type of substrate, and therefore, further research needs to be done.

It has been suggested that inhibition of bovine or porcine trypsin by TI may not be an index of their activity against human trypsin (Mallory & Travis, 1973). Therefore, a comparison of BAPA and TAME in measuring TI using human trypsin is recommended.

Currently, DL-BAPA is used as a substrate in the BAPA method. Since the D-isomer in DL-BAPA is not readily hydrolyzed (Erlanger et al., 1961), it may affect measurement of TI levels. Therefore, there is a need to compare D-BAPA and L-BAPA in the determination of TI activity in legumes.

REFERENCES

- Adsule, R. N., Kadam, S. S., & Salunkhe, D. K. (1986). Chemistry and technology of green gram (*Vigna radiata* [L] wilczek). CRC Critical Reviews in Food Science and Nutrition, 25, 73-105.
- American Association of Cereal Chemists. (1983). Approved methods of the AACC, Method 71-10. St. Paul, MN: Author.
- Association of Analytical Chemists. (1984). Official Methods of Analysis (13th ed.). Washington, DC: Author.
- Birk, Y. (1961). Purification and some properties of highly active inhibitor of trypsin and alpha-chymotrypsin from soybeans. Biochemica Et Biophysics Acta, 54, 378-381.
- Bowman, D. E. (1944). Fractions derived from soybeans and navy beans which retard tryptic digestion of casein. Proceedings of the Society of Experimental Biological Medicines, 57, 139-145.
- Buck, F. F., Vithsyathil, A. J., Bier, M., & Nord, F. F. (1962). On the mechanism of enzyme action LXXIII. Studies on trypsins from beef, sheep, and pig pancreas. Archives of Biochemistry and Biophysics, 97, 417-424.
- Chan, J., & DeLumen, B. O. (1982a). Biological effects of isolated trypsin inhibitors from winged bean (*Psophocarpus tetragonolobus*) on rats. Journal of Agricultural and Food Chemistry, 30, 46-50.
- Chan, J., & DeLumen, B. O. (1982b). Properties of trypsin inhibitors from winged bean (*Psophocarpus tetragonolobus*) seed isolated by affinity chromatography. Journal of Agricultural and Food Chemistry, 30, 42-46.

- Charpentier, B. A., & Lemmel, D. E. (1984). A rapid automated procedure for the determination of trypsin inhibitor activity in soy products and common food stuffs. Journal of Agricultural and Food Chemistry, 32, 908-911.
- Chernick, S. S., Lepkovsky, S., & Chaikoff, I. L. (1948). A dietary factor regulating the enzyme content of the pancreas: changes induced in size and proteolytic activity of the chick pancreas by the ingestion of raw soybean meal. American Journal of Physiology, 155, 33-39.
- Chrispeels, M. J., & Baumgartner, B. (1978). Trypsin inhibitor in mung bean cotyledons. Plant Physiology, 61, 617-623.
- Chu, H. M., & Chi, C. W. (1965). The isolation and crystallization of two trypsin inhibitors of low molecular weight from mung bean. Scientia Sinica, 14, 1441-1453.
- Chu, H., Shan L., Meihsuan, J., Chingwu, C., & Chintien, T. (1965). The relationship between trypsin inhibitor A and B from mung bean and some chemical characteristics of the inhibitors. Scientia Sinica, 14, 1454-1463.
- DeLumen, B. O., & Belo, P. S. (1981). Inhibitors of trypsin and chymotrypsin in winged bean (*Psophocarpus tetragonolobus*) tubers. Journal of Agricultural and Food Chemistry, 29, 884-886.
- DeLumen, B. O., & Salamat, L. A. (1980). Trypsin inhibitor activity in winged bean (*Psophocarpus tetragonolobus*) and the possible role of tannin. Journal of Agricultural and Food Chemistry, 28, 533-536.
- Doell, B. H., Ebden, C. J., & Smith, C. A. (1981). Trypsin inhibitor activity of conventional foods which are part of the British diet and some soya products. Qualitas Planatarum Plant Foods for Human Nutrition, 31, 139-146.

- Egberg, D. C., Potter, R. H., & Honold, G. R. (1975). The semi-automated determination of trypsin inhibitors in textured soy protein. Journal of Agricultural and Food Chemistry, 23, 603-609.
- Elias, L.G., De Fernandez, D. G., & Bressani, R. J. (1979). Possible effects of seed coat polyphenolics on the nutritional quality of bean protein. Journal of Food Science, 44, 524-527.
- Elkowicz, K., & Sosulski, F. W. (1982). Antinutritive factors in eleven legumes and their air-classified protein and starch fractions. Journal of Food Science, 47, 1301-1304.
- Erlanger, B. F., Kokawsky, N., & Cohen, W. (1961). The preparation and properties of two new chromogenic substrate of trypsin. Archives of Biochemistry and Biophysics, 95, 271-278.
- Feeney, R. E., Means, G. E., & Bigler, J. C. (1969). Inhibition of human trypsin, plasmin, and thrombin by naturally occurring inhibitors of proteolytic enzymes. Journal of Biological Chemistry, 244, 1957-1960.
- Fernandez, D. G., Elias, L.G., Braham, J. E., & Bressani, R. J. (1982). Trypsin inhibitors and hemagglutinins in beans and their relationship with the content of tannins and associated polyphenols. Journal of Agricultural and Food Chemistry, 30, 734-739.
- Freed, R. C., & Ryan, D. S. (1980). Isolation and characterization of genetic variants of the Kunitz soy bean trypsin inhibitor. Biochemistry Biophysics Acta, 624, 562-572.
- Fritz, H., Trautschold, I., & Werle, E. (1974). Protease inhibitors. In H. U. Bergmeyer (Ed.), Methods of enzymatic analysis (pp. 1064-1080). New York: Academic Press.

- Green, G. M., & Lyman, R. L. (1972). Feedback regulation of pancreatic enzyme secretion as a mechanism for trypsin inhibitor induced hypersecretion in rats. Proceedings of the Society of Experimental Biological Medicines, 140, 6-12.
- Green, G. M., & Lyman, R. L. (1973). Protein as a regulator of pancreatic enzyme secretion in rats. Proceedings of the Society of Experimental Biological Medicines, 142, 162-167.
- Hafez, S. Y. & Mohammed, A. I. (1983a). Biochemical study and non protein trypsin and chymotrypsin inhibitors in the soy bean. Journal of Food Science, 48, 1265-1268.
- Hafez, S. Y., & Mohammed, A. I. (1983b). Presence of non protein inhibitor in soy and winged beans. Journal of Food Science, 48, 75-76.
- Hamerstand, G. E., Black, L. T., & Glover, J. D. (1981). Trypsin inhibitors in soy products: Modification of the standard analysis procedure. Cereal Chemistry, 58, 42-45.
- Hummel, B. C. W. (1959). A modified spectrophotometric determination of chymotrypsin, trypsin, and thrombin. Canadian Journal of Biochemistry and Physiology, 37, 1393-1399.
- Jaya, T. V., & Venkataraman, L. V. (1980). Effects of the germination on the nitrogenous constituents, essential amino acids, carbohydrates, enzymes, and antinutritional factors in chick pea and green gram. Indian Food Packer, 8, 3-11.
- Kadam, S. S., & Salunkhe, D. K. (1984). Winged bean in human nutrition. CRC Critical Reviews in Food Science and Nutrition, 21, 1-40.

- Kakade, M. L., Hoffa, D. E., & Liener, I. E. (1973). Contribution of trypsin inhibitors to the deleterious effects of unheated soybeans fed to rats. Journal of Nutrition, 103, 1772-1775.
- Kakade, M.L., Rackis, J.E., McGhee, J. E., & Puski, G. (1974). Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. Cereal Chemistry, 51, 376-382.
- Kakade, M.L., Simons, N., & Liener, I. E. (1969). An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soy bean samples. Cereal Chemistry, 46, 518-526.
- Kortt, A. (1979). Isolation and characterization of the trypsin inhibitors from winged bean seeds. Biochimica Et Biophysica Acta, 577, 371-382.
- Kunitz, M. (1946). Crystalline soy bean trypsin inhibitor. Journal of General Physiology, 29, 149-154.
- Kunitz, M. (1947). Crystalline soy bean trypsin inhibitor. II General properties. Journal of General Physiology, 30, 291-310.
- Kunitz, M. (1945). Crystallization of a trypsin inhibitor from soybeans. Science, 101, 668-670.
- Laskowski, M., & Laskowski, M., Jr. (1954). Naturally occurring trypsin inhibitors. Advances in Protein Chemistry, 9, 203-242.
- Liener, I. E. (1979). Significance for humans of biologically active factors in soy bean and other food legumes. Journal of American Oil Chemists' Society, 56, 121-129.
- Liener, I. E. (1986). Trypsin inhibitors: Concerns for human nutrition or not? Journal of Nutrition, 116, 920-922.

- Liener, I. E., & Kakade, M. L. (1980). Protease inhibitors. In I. E. Liener (Ed.), Toxic constituents of plant foodstuffs (pp. 7-68). New York: Academic Press.
- Liu, K., & Markakis, P. (1989). An improved colorimetric method for determining antitryptic activity in soy bean products. Cereal Chemistry, 66, 415-422.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin reagent. Journal of Biological Chemistry, 193, 265-275.
- Mallory, P. A., & Travis, J. (1973). Human pancreatic enzymes: Characterization of anionic human trypsin. Biochemistry, 12, 2847-2851.
- McGuinness, E. E., Hopwood, D., & Wormsley, K. G. (1982). Further studies of the effects of raw soy flour on rat pancreas. Journal of Gastroenterology, 17, 273-276.
- Odani, S., & Ikenaka, T. (1972). Studies on soy bean trypsin inhibitors. IV. Complete amino acid sequence and the anti-proteinase sites of Bowman-Birk soy bean proteinase inhibitor. Journal of Biochemistry, 71, 839-842.
- Osborne, T. B., & Mendel, L. B. (1917). The use of soy bean as food. Journal of Biological Chemistry, 32, 369-371.
- Rackis, J. J. (1965). Physiological properties of soybean trypsin inhibitors and their relationship to pancreatic hypertrophy and growth inhibition of rats. Federal Proceedings, 24, 1488-1493.
- Rackis, J. J., & Gumbmann, M. R. (1981). Protease inhibitors: Physiological properties and nutritional significance. In R. L. Ory (Ed.), Antinutrients and natural toxicants in foods (pp. 203-218), Westport, CN: Food and Nutrition Press.

- Rackis, J. J., Gumbmann, M. R., & Liener, I. E. (1985). The USDA trypsin inhibitor study. I. Background, objectives and procedural details. Qualitas Planatarum Plant Foods for Human Nutrition, 35, 213-242.
- Rackis, J. J., McGhee, J. H., Liener, I. E., Kakade, M. L., & Puski, G. (1974). Problems encountered in measuring trypsin inhibitor activity of soyflour. Report of a collaborative analysis. Cereal Science Today, 19, 513-516.
- Richardson, M. (1977). A review: The proteinase inhibitors of plant and microorganisms. Phytochemistry, 16, 159-169.
- Ryan, C. A. (1981). Proteinase inhibitors. The Biochemistry of Plants, 6, 351-370.
- Salunkhe, D. K. (1982). Legumes in human nutrition: Current status and future research needs. Current Science, 51, 387-390.
- Sathe, S. K., & Salunkhe, D. K. (1981). Investigations on winged bean proteins and antinutritional factors. Journal of Food Science, 46, 1389-1393.
- Schwert, G. W., Neurath, H., Kaufman, S., & Snake, J.E. (1948). The specific esterase activity of trypsin. Journal of Biological Chemistry, 172, 221-239.
- Smith, A. K., & Circle, S. J. (1978). Chemical composition of the seed. In A. K. Smith & S. J. Circle (Eds.), Soybeans: Chemistry and technology (pp. 61-92). CN: The AVI Publishing Company.
- Smith, C., Megen, W. V., Twaalfhoven, L., & Hitchcock, C. (1980). The determination of trypsin inhibitor levels in foodstuffs. Journal of Science of Food and Agriculture, 31, 341-350.
- Sohonie, K., & Bhandarkar, A. P. (1955). Trypsin inhibitors in Indian food stuffs. Part II. Inhibitors in pulses. Journal of Scientific and Industrial Research, 14, 100-104.

- Tan, N., & Wong, K. (1982). Thermal stability of trypsin inhibitor activity in winged bean. Journal of Agricultural and Food Chemistry, 30, 1140-1143.
- Tan, N., Wong, K., & DeLumen, B. O. (1984). Relationship of tannin levels and trypsin inhibitor activity with the in vitro protein digestibilities of raw and heat-treated winged bean. Journal of Agricultural and Food Chemistry, 32, 819-822.
- Travis, J., & Roberts, R. C. (1969). Human trypsin: Isolation and physical characterization. Biochemistry, 8, 2884-2889.
- Worthington Biochemical Corporation. (1972). Trypsin (bovine pancreas). Worthington enzymes manual (pp. 125-127). Freehold, NJ: Author.