2000

Effect of soaking and germination on trypsin inhibitor activity in desi chickpea

Maneesha K. Ranabhor
San Jose State University

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.

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.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

EFFECT OF SOAKING AND GERMINATION ON TRYPSIN INHIBITOR ACTIVITY IN DESI CHICKPEA

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

Master of Science

in Nutritional Science

by

Maneesha K. Ranabhor

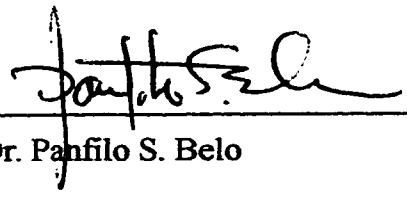
December 2000.

UMI Number: 1403002

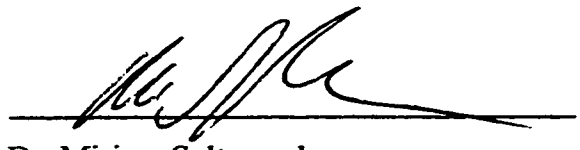
Bell & Howell Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

APPROVED FOR THE DEPARTMENT

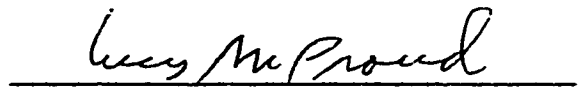
OF NUTRITION AND FOOD SCIENCE



Dr. Panfilo S. Belo

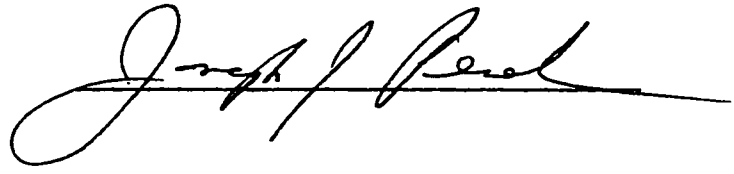


Dr. Miriam Saltmarch



Dr. Lucy McProud

APPROVED FOR THE UNIVERSITY



ABSTRACT

EFFECT OF SOAKING AND GERMINATION ON TRYPSIN INHIBITOR ACTIVITY IN DESI CHICKPEA

by Maneesha K. Ranabhor

The objective of the study was to determine the trypsin inhibitory activity (TIA) in raw, soaked and germinated desi chickpeas. Desi chickpea seeds obtained locally were soaked for 12 hrs and then germinated for 24 and 48 hours. Raw, soaked and germinated seeds were subsequently air dried, ground and defatted. The BAPA and TAME methods were used to assay TIA in these defatted powders.

Trypsin inhibitor activity in raw chickpea seeds, expressed as TI unit/ mg protein, using BAPA and TAME methods were 93.57 and 5.88, respectively. Soaking for 12 hrs in water significantly ($P \leq 0.05$) reduced TIA by 13.64 % and 11.56 %, as determined by BAPA and TAME method, respectively. Although increased TIA after 24 hrs of germination was observed compared to the soaked seeds, TIA was still lower than the raw chickpea seeds. Seeds germinated for 48 hrs showed the highest TIA among all of the samples.

ACKNOWLEDGMENT

I would like to acknowledge Dr. Panfilo S. Belo, my graduate adviser, for his support and expert advice throughout this research. His motivation, patience and direction helped deeply to the completion of this study. I would like to thank Dr. Miriam Saltmarch for her advice, expertise, and encouragement during my studies. I would also like to thank Dr. Lucy McProud for her helpful suggestions and input as a member of my graduate committee. I would also like to thank my parents for their support and encouragement throughout my studies.

Dedication

This thesis is dedicated to my husband, Kiran Ranabhor, whose interest, endless patience, encouragement, and support made it possible for me to complete my graduate studies at San Jose State University.

PREFACE

The following thesis is written in publication style. The second chapter is written in journal format 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 of the American Psychological Association, 4th edition, 1995.

Table of Contents

Page

List of Tables

Page

CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

Introduction

One of the serious problems facing most developing countries is the scarcity of food for growing human population (Carlini & Udedibie, 1997) and it is well accepted that supplying adequate amounts of high quality proteins to the increasing population in developing countries is not an easy endeavor (Bressani, 1975). The increasing cost of animal proteins has prompted food technologists, nutritionists, and biochemists to consider plant proteins as a major source of dietary proteins. The importance of using plant proteins, as a dietary source in areas in which protein calorie malnutrition (PCM) is prevalent, is now well recognized (Salunkhe, 1982).

Plant proteins are increasingly being used as an alternative to proteins from animal sources in human nutrition. Among plants, legume seeds represent a rich source of proteins and carbohydrates (Clemente, Vioque, Sanchez-Vioque, Pedroche, & Millan, 1999). They are important source staple foods, particularly in developing countries, due to their relatively low cost, long preservation time, and high nutritional value (Trugo, Donangelo, Trugo, & Bach Knudsen, 2000).

Chickpeas (*Cicer arietinum*) are among the legumes, which have potential as a protein-rich food source. They have long been one of the most important sources of protein in several developing countries, especially in rural populations (Nestares, Lopez-Frias, Barrionuevo, & Urbano, 1996). Chickpea seeds have been considered a suitable

source of dietary protein due to their good amino acid balance and high bioavailability (Newman, Roth, & Lockerman, 1987).

The two known biotypes of chickpea vary in their size, shape, and color of seeds. Kabuli chickpeas, large seeded with salmon white testa, are grown mainly in the Mediterranean area, and America. The desi chickpeas on the other hand are small seeded with a light brown testa and are cultivated mostly in India and East Africa (Kadam & Salunkhe, 1989).

Chickpeas are considered by many as a health food. They are an important component of vegetarian diets. Chickpeas are very palatable and can be consumed alone or mixed with other pulses and vegetables. They are popular in salads and may be canned in brine or with meats. Chickpea flour can be used to enrich cereal flours without impairing flavor, baking quality and the like in preparation of bread, cookies, and tortillas (Sotelo & Adsule, 1996). Ulloa, Valencia, and Garcia (1988) have suggested the use of chickpea based concentrate for infant feeding formulas.

Chickpeas contain several antinutritional factors such as phytic acid, amylase inhibitors, polyphenolic compounds, protease inhibitors, and a flatulence factor (Salunkhe & Kadam, 1989). Chickpea seeds are known to contain trypsin as well as chymotrypsin inhibitors. Desi type cultivars have been reported to contain higher concentrations of trypsin inhibitors compared to kabuli cultivars (Singh & Jambunathan, 1981). Antinutritional effects of trypsin inhibitors (TI) are due to their combination with trypsin to form inactive complexes, which interfere with the protein digestion. They have been shown to cause pancreatic hypertrophy in animals (Birk, 1994).

Chickpeas are subjected to various processing methods including soaking and germination, prior to their consumption. These processing methods have shown to exert an effect on the trypsin inhibitor activity (TIA) of legumes like chickpeas (Singh, 1985).

Although, effects of several processing treatments including soaking and germination on the TIA in legumes are reported in the literature, little information is available in the desi chickpeas.

Objective

The objective of the present study was to determine the effect of soaking and germination on the trypsin inhibitory activity in desi chickpeas. Trypsin inhibitor activity after 12 hrs of soaking as well as 24 and 48 hrs of germination was determined.

Significance of the Study

To alleviate the widely prevailing protein calorie malnutrition (PCM) in developing countries, food technologists, and nutritionists have been searching for alternative sources of food (Vijayakumari, Siddhuraju, Pugalenthi, & Janardhanan, 1998). Salunkhe (1982) has emphasized the need for development of low cost food using indigenous sources of vegetable proteins to alleviate PCM in underdeveloped countries. As legumes and their products are a versatile source of medium quality low cost proteins, their significance in human nutrition cannot be neglected (Salunkhe & Kadam, 1989). The nutritional value of legumes as a source of protein and carbohydrates in the diet is undeniable, not only for vegetarians, but more especially for those living in developing countries where large segments of the population suffer from PCM and where legumes are of utmost importance (Bravo, Siddhuraju, & Saura-Calixto, 1999).

Together with the other legumes, chickpeas have been one of the most important sources of protein in rural populations. They are also widespread in Asia and Central and South America, where they satisfy a considerable portion of the protein requirements of the population (Nestares et al., 1996). Sotelo, Flores, and Hernandez (1987) have mentioned that the protein quality of chickpeas is one of the best in the legume family.

Legumes including chickpeas are usually processed before consumption, depending upon cultural and taste preferences. The most commonly used domestic methods of legume processing include soaking for different periods of time as well as germination, and these methods are reported to be beneficial for enhancing the nutritive value of various legumes (Duhan, Khetarpaul, & Bishnoi, 2000).

Although legumes are consumed mainly after cooking, in some countries, raw sprouted legumes (soaked in water and germinated for few days) are also commonly consumed (Aman, 1979). Along with the other legumes, germinated chickpeas constitute a fair portion of the total amounts of legume consumed in several parts of India (Jaya, Krishnamurthy, & Venkataraman, 1975). Due to the increased usage of chickpeas in developing countries to alleviate PCM, it is important to study the TIA in chickpeas in order to maximize their potential for protein quality.

Effects of several processing treatments on the TI s in legumes including soaking and germination have been reported in the literature. However, the major emphasis so far has been on soybean and the *Phaseolus* species and there is comparatively less emphasis on chickpeas, especially desi chickpeas, despite its importance in the human nutrition. Therefore, reliable and consistent information on chickpeas is lacking. Although a few

researchers have studied the effect of soaking and germination on the TIA in desi chickpeas, the information in the literature is not consistent. Therefore, it would be of interest to study the effects of soaking and germination of desi chickpeas on their trypsin inhibitory activity.

The present study was conducted to determine how and to what extent soaking and germination affect the TIA in desi chickpeas.

Review of the Literature

Importance of Plant Proteins

As population growth continues to increase, and as the main sources of food (farms and oceans) may be approaching maximum *per capita* output, demand seems likely to outpace food production. There is a need to avoid or alleviate shortage of high-quality and high-energy foods and thus counter or mitigate one of the more serious threats to the quality of life for ourselves and our descendants. Our objective should be to improve the quality, quantity, and safety of available food and feed sources by all feasible methods (Friedman, 1996).

Protein energy malnutrition is widespread (Waterlow, 1994). Friedman (1996) reported that if the major problem in the occurrence of malnutrition is inadequate energy (food) intake, emphasis should be given to developing new food sources of high quality protein that will yield more total energy. The supplementation of cereals with protein rich legumes is considered one of the best solutions to PCM in the world (Chitra, Singh, & Venkateswararao, 1996).

Legumes are widely grown throughout the world and their dietary and economic importance is globally appreciated and recognized. They serve as one of the richest inexpensive sources of dietary protein, constituting an integral part of the human diet in the Indian subcontinent, Latin America, and African countries (Sharma & Sehgal, 1992). Legumes constitute an important source of dietary protein for a large segment of the World's population, particularly in those countries where the consumption of animal protein is limited by availability or is self-imposed because of religious or cultural habits (Jyothi & Sumathi, 1995). Gahlawat and Sehgal (1993) also reported that legumes along with the cereals constitute an important source of dietary calories and proteins for many segments of the world's population, especially in developing countries. Plant proteins are increasingly being used as an alternative to proteins from animal origin in human nutrition. Among plants, legume seeds represent a rich source of proteins and carbohydrates (Clement et al., 1999). Recently, along with soybeans, other legumes, such as common beans, lentils, lupins, and chickpeas have been recognized as valuable protein sources (Friedman, 1996).

Nevertheless, utilization of legumes in human nutrition is constrained due to their inherent antinutritional factors such as phytates, alkaloids, phytohemagglutinins, polyphenolic compounds, saponins, tannins, cyanogenic factors, and protease inhibitors, which have a negative influence on protein digestibility in monogastrics including humans (Liener, 1989). The occurrence of some antinutritional factors hampers the nutritional potential of legume protein by interfering with the intake, availability or metabolism of nutrients. Furthermore, it is well known that legumes are, in general,

deficient in sulfur containing amino acids such as methionine and cysteine/cystine. These considerations as well as the relative lower digestibility of the legume protein, compared to animal origin, affect its nutritional utilization (Fernandez-Quintela et al., 1997; Jyothi & Sumathi, 1995).

The Chickpeas

Chickpeas (*Cicer arietinum*) are one of the major food legumes grown in all continents of the world, especially in developing countries. Among the world's grain food legumes, the chickpea is second to dry beans (*Phaseolus vulgaris*) in area grown and third in production to dry peas (*Pisum sativum*) (Singh, 1985). It is grown mainly in the Indian subcontinent, the Mediterranean Basin, East Africa, Mexico, and Australia (Gil, Nadal, Luna, Moreno, & De Haro, 1996). Developing countries contribute more than 90 % of the world production of chickpeas. Among developing countries, India produces nearly 80% of the world's total production (Kadam & Salunkhe, 1989).

Chickpea crops improve the physical and biological properties of the soil. It also fixes atmospheric nitrogen and thus requires less chemical fertilization, a characteristic with obvious ecological advantages (Nestares, Barrionuevo, Urbano, & Lopez-Frias, 1999). The chickpea is a small, herbaceous, annual shrub; normally grow to a height of 45-60 cm. It is frequently bluish-green in color and covered with glandular hairs. The seedpods are oblong and one about 2.5 cm long and 1cm broad, generally containing one to two relatively larger seeds, which vary in color (Kay, 1979).

Chickpea seeds are generally small, rounded or angular, approximately 7-11mm long and 5-8 mm broad, with pointed anterior ends and smooth, or wrinkled, seed coats.

Grouped into two types: the desi type with small, angular, dark colored seeds and the kabuli type with large, smooth coated, beige seeds (Gil et al., 1996). Desi chickpeas have small seeds, which are brown or yellowish in color and usually wrinkled with a beak at the end whereas Kabuli chickpeas have white, larger smooth seeds (Kay, 1979).

Chickpeas have been assigned a higher order of nutritional merit among all pulses; from nutrition stand point (Kachroo, 1970). They are a valuable source of proteins, minerals, and vitamins. They are an important source of proteins in several developing countries (Mansour, 1996).

The crude protein content of chickpea seeds range from 12.4 to 30.6 %. In addition to genetic make up, certain environmental factors such as location, soil-type, irrigation, and fertilization influence the protein content in the chickpeas (Chavan, Kadam, & Salunkhe, 1989). Gupta and Kapoor (1980) reported that based on the amino acid composition, the proteins of chickpea seeds are found to have a higher nutritive value than some of other grain legumes such as lentils, green gram, cowpeas, and pigeonpeas. Sotelo, Flores, and Hernandez (1987) also mentioned that the protein quality of the chickpeas is one of the best in the legume family.

In addition to being an important source of protein, chickpeas are also reported to be a good source of minerals (Singh & Jambunathan, 1981). This legume supplies larger amounts of calcium and phosphorus than do other legumes (Chavan et al., 1989) and contains more calcium than whole cow's milk i.e. 120 mg/100g (Nestares et al., 1999). Chavan, Kadam, and Salunkhe (1989), mentioned that chickpeas are also a good source of dietary fiber. The crude fiber content in chickpeas ranges from 7.1 to 13.5 %,

depending upon the amount of seedcoat present. As desi chickpeas contain higher amount of seed coat, its fiber content is significantly higher ($P \leq 0.05$) than that of kabuli chickpeas. Cellulose and hemi-cellulose are found to be major components of chickpea fiber.

Foods based on chickpeas are prepared by a wide range of recipes and preparation methods (Koksel, Sivri, Scanlon, & Bushuk, 1998). Seeds are eaten as fresh or as dry pulse, parched, boiled, fried, or in various dishes. Sprouted seeds are eaten as a vegetable or salad. Young plants and green pods are eaten like spinach (Duke, 1982). Khaleque, Elias, Braham, and Bressani (1985) reported the use of chickpeas for developing an infant food with high nutritive quality. Recently, the use of chickpeas as a source of high quality protein preparations has been investigated. Ulloa et al. (1988) reported the use of chickpea protein concentrates as a potential ingredient for infant formula. Sanchez-Vioque, Clemente, Vioque, Bautista, and Millan (1998) reported the use of chickpea protein isolate and its potential use for the preparation of cheese, bakery and meat products because of its high water and fat absorption. In addition, George, Sivasankar, Jayaraman, and Vijayalakshmi (1997) suggested the production of methionine -rich fractions from chickpea protein hydrolysate as a potential peptide fortifier in food products. Clemente et al. (1999) opined that chickpea protein hydrolysate could be useful for the development of specialized hypoallergenic food products.

Like other grain legumes, chickpea seeds contain a variety of antinutritional factors (Nestares et al., 1996). These include phytates, alkaloids, phytohemagglutinins, polyphenolic compounds, saponins, cyanogenic factors, and protease inhibitors. These

antinutritional factors limit utilization of chickpea seeds by exerting deleterious effects on human nutrition (Mansour, 1996; Salunkhe & Kadam, 1989; Singh, 1985). Savage and Frøkiær (1998) stated that chickpeas contain appreciable amounts of trypsin inhibitors. They also reported that protein utilization of chickpeas is compromised to a varying extent, by the presence of a range of protease inhibitors (TI s).

Trypsin Inhibitors

Trypsin Inhibitors in plants are both protein and non-protein in nature (Hafez & Mohamed, 1983). Nevertheless, only protein TI has been studied extensively. Protein TI s found in plants inhibit proteolytic enzymes. They are typically polypeptides and proteins composed of L-amino acids linked through peptide bonds. Plant inhibitors are typically low or devoid of methionine, histidine, and tryptophan but are rich in aspartic and glutamic acids, serine, and lysine residues. Plant proteinase inhibitors vary in size from 4000 to 60,000 MW (Ryan, 1981).

Trypsin inhibitors are found in a variety of foods, especially in food legumes. They have attracted the attention of nutritionist' because of their possible effect on the nutritive value of plant proteins in general, and legume seeds in particular. The increasing interest in protein rich legume seeds for use in human and animal nutrition is bringing also into perspective the possible, long-and short-term nutritional effects of the endogenous proteinase inhibitors (Birk, 1994).

Two problems are causally related to TI s in food, impaired growth and pancreatic hypertrophy (Macrae, Robinson, & Sadler, 1993). The impact of adverse effects is compounded by low protein intakes, a situation likely to coincide with high TI intakes in

individuals who place dietary emphasis on legumes rather than meat as a protein source (Savage & Frøkiær, 1998).

The antinutritive effects of TI s are due to their combination with the trypsin enzyme to form inactive complexes, which interfere with protein digestion. It is now well established that the increased intake of raw beans, high in trypsin and chymotrypsin inhibitor activity, stimulates pancreatic juice secretion and can cause pancreatic hypertrophy in animals (Birk, 1994).

The mechanism whereby the TI s exert antinutritional effect is not completely understood (Birk, 1994). It is observed that pancreatic secretion is controlled by a mechanism of feedback inhibition, which depends on the level of trypsin present at any given time in the small intestine. When the level of this enzyme falls below a certain critical threshold value, the pancreas is induced to produce more of the enzyme. The suppression of this negative feedback mechanism can thus occur if the trypsin is complexed with the inhibitor. It is believed that the mediating agent between trypsin and the pancreas is the hormone cholecystokinin (CCK), which is released from the intestinal mucosa when the level of trypsin in the intestine falls below its threshold level. As TI s bind to trypsin in the small intestine to form insoluble complexes, they make trypsin unavailable for protein digestion. Thus, they interfere with the digestibility of dietary proteins. The inactivation of the TI is accompanied by a concomitant increase in the nutritive value of proteins. As TI content is associated with lower protein quality, TIA of chickpeas needs to be minimized in order to increase their protein utilization (Huisamn & Jansman, 1991).

As TI s react with trypsin, the trypsin level in the small intestine falls below threshold level. This in turn stimulates the pancreas to secret more trypsin, which results in the hyperactive pancreas. It can cause hypertrophy and/or hyperplasia of the pancreas (Birk, 1994).

Presence of TI s in chickpeas is well reported in the literature (Belew & Eaker, 1975; Harsulkar, Giri, & Kothekar, 1997; Smirnoff, Khalef, Birk, & Applebaum, 1976). Savage and Frøkiær (1998) stated that chickpeas contain appreciable amounts of trypsin inhibitors. The levels of TI s in chickpeas are reported to be higher than field peas, mung beans, pigeon peas, and cowpeas (Salunkhe & Kadam, 1989) but lower than soybean, lima bean and the winged bean. Desi type chickpeas have been reported to have a higher concentration of TI s compared to kabuli chickpeas (Singh & Jambunathan, 1981). Like other legumes, large variations exist in the TI s levels in different varieties of chickpeas. The TI content of commonly used legumes is shown in Table 1.

It has been reported that genotype and environmental interaction account for large variations in TIA in chickpeas. Rincon, Martinez, and Ibanez (1998) reported the TIA content of desi chickpeas ranging from 3.24- 15.06 TIU/ mg defatted sample whereas Singh and Jambunathan (1981) reported a higher TIA of 9.3 to 14.6 TIU/ mg defatted chickpea sample. Hira and Chopra (1995) documented TIA in the range of 37.4-60.4 TIU/mg proteins. Sumathi and Pattabiraman reported lower values for TIA, of 1.83 TIU/mg chickpeas on a dry weight basis.

Table 1. Trypsin Inhibitor Content of Some Common legumes

Legumes	Trypsin Inhibitor Activity (TIU/mg sample)
Lima bean	46.81
Soybean	41.55
Winged bean	41.40
Navy bean	18.23
Northern bean	18.08
Chickpea	18.80
Cowpea	12.20
Green gram	9.96
Field pea	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.

Although TI levels in chickpeas are relatively low compared to certain legumes such as soybean, they might have adverse effects through chronic exposure and /or unbalanced diets relying on legume products for protein. The groups most likely to be at risk from potential adverse effects are people who have recently converted to a predominantly vegetarian diet, which is perceived as traditional and wholesome. It is possible that these people may not possess sufficient knowledge about appropriate preparation methods and may prefer food that has not been extensively processed. Among the legumes, Chickpeas appear to be preferred by vegetarians (Savage & Frøkiær, 1998).

Chickpea protease inhibitors affect the pancreatic serine proteins, trypsin and chymotrypsin. The content of inhibitors was found to be 1.5g/kg. Inhibitors were further separated into six isoinhibitors. Out of these six inhibitors, two accounted for 50 % of the isolated inhibitors. These two main inhibitors have a molecular weight of approximately 16000 (Belew, 1977). Until today, studies on these inhibitors have shown the existence of isoinhibitors, with the number ranging from six (Belew, 1977) to eight (Hamza, Shehata, & Stegemann, 1986).

The amino acid composition of inhibitors showed that they are rich in half-cystine, aspartic acid and serine residues, which together account for more than 40% of the amino acid content. Inhibitors lack tryptophan and cysteine (Belew, Porath, & Sundberg, 1975). Trypsin inhibitor of chickpeas is a double-headed inhibitor with two independent binding sites specific for trypsin and chymotrypsin. The main TI in

chickpeas resembles the Bowman-Birk inhibitor (BBI). These chickpea seed inhibitors are reported to inhibit the inhibition of human trypsin and chymotrypsin.

Chickpea Processing Treatments

It is widely accepted that simple and inexpensive domestic processing techniques are effective methods of achieving desirable changes in the composition of seeds. This is accomplished by the removal of undesirable components, which is essential to improve the nutritional quality of legumes and effectively utilize their full potential as a human food (Urbano, Lopez-Jurado, Hernandez, Fernandez, Moreu, Frias, Diaz-Pollan, Prodanov, & Vidal-Varvade, 1995). These processing treatments include soaking, decortication, grinding, cooking, roasting, fermentation, frying, heating, and germination of the legumes (Deshpande & Damodaran, 1990).

There are many aspects of food legume processing and utilization that merit further research. The goal should be to concentrate on developing inexpensive, acceptable, nutritious, and easily prepared food products. This will contribute to increased food supplies and an improved nutritional status for the large number of malnourished people in less developed countries (Siegel & Fawcett, 1976).

Therefore, much research has been devoted to the inactivation of antinutritional factors by ordinary processing treatments such as soaking and germination. These are simple and inexpensive processing methods, which can be carried out to increase the nutritive value of legumes (Vijayakumari et al., 1998).

In order to utilize beans effectively as a human food, it is essential to inactivate/ remove antinutritional and unwanted components. Such inactivation or removal of these

compounds depends on the type of bean, the amount of these compounds present, and the final product to be prepared. There are several methods of processing beans which include dehusking, milling, soaking, cooking, germination, fermentation, autoclaving, roasting, frying, parching, and protein extract extrusion depending on the type of bean (Sathe, 1996).

Like other legumes, chickpeas are generally subjected to various domestic processing treatments before consumption (Singh, 1985). These traditional domestic processing treatments not only remove toxic substances and antinutritional factors, but also improve the palatability and digestibility of legumes.

Several methods exist for the preparation of legumes at the domestic level. A variety of procedures is used for the purposes of eliminating toxic substances and antinutrients, removing the seed coat, and softening the cotyledons. Raw beans can be processed into a palatable form without any cooking (Siegel & Fawcett, 1976). Trypsin inhibitor activity becomes more of an issue if the diet is inadequate or dominated by legumes. Longer boiling times, which reduce residual inhibitory activity, are undesirable for convenience and fuel economy, particularly in developing countries. In addition, some seed nutrients may be degraded when the seed is heated for a long time (Savage & Frøkiær, 1998).

Soaking

Soaking of legumes usually forms an integral part of various bean-processing methods including germination. It is a first step in most methods of preparing legumes for consumption. It decreases toxin content and surface contamination. The time needed

for soaking varies with the variety and species. Traditionally, dry beans are soaked overnight for around 8 - 48 hrs in cold water (Macrae et al., 1993). No loss of nutrients occurs when the legumes are soaked in their skins, and the soaking water is not warmed (Aykroyd & Doughty, 1964). The soaking media include water, salt (or combination of salts) solution, and alkali.

Soaking is an integral part of traditional methods of processing grains, which offers the dual advantage of saving energy cost by shortening cooking time as well as rendering the grains nutritionally superior by removing antinutritional factors (Mulimani & Vadiraj, 1994). It facilitates cooking as well as decortication along with germination (Deshpande, Sathe, & Salunkhe, 1989). Variation in effect of soaking on TI content is reported in the literature. Some researchers reported no change in TIA after soaking (Liu & Markakis, 1987) whereas in some studies substantial loss of TIA was noticed (Batra et al., 1986). Bansal et al. (1988) observed that overnight soaking of desi chickpeas resulted in substantial loss of activity of trypsin inhibitors. Soni, Singh, and Singh (1978) observed that in soaked seeds, TIA was decreased compared to raw seeds.

Germination

The preparation of germinated legumes is a method developed over the years, using traditional home practices. In the Far East and India, the process is carried out both in the home and on a cottage scale. Germination as a means for processing legumes and allows the whole bean to be eaten in a palatable form. In itself, germination is the process by which the first piece of stem (sprout) is encouraged to develop and grow in length. In germination, the legume seeds are soaked for 12 to 24 hours. Then they are

allowed to germinate in a moist muslin cloth in the dark for 24 to 48 hours. In India, sprouts are allowed to grow only up to one to two centimeters (Kadam & Salunkhe, 1989). Sprouted grains are eaten raw with salts, or further seasoned, fried and boiled (Aykroyd & Doughty, 1964).

Germination procedures are receiving increasing attention because of the probability that flavor and nutritional qualities may be improved, particularly through the breakdown of certain heat-stable antinutrients such as phytates and flatulence factors (Deshpande, Sathe, & Salunkhe, 1984). Germination procedures have been documented to be an effective treatment to remove antinutritional factors in legumes. Germination is a very commonly used method of processing legumes throughout the world. In recent years, legume sprouts have been used in the preparation of legume based low cost infant foods (Sattar et al., 1990). Germination is considered a suitable procedure to improve the nutritional value of legume seeds by reducing levels of antinutritional factors (Jood, Chauhan, & Kapoor, 1989).

The direct effect of sprouting on both the physical and chemical changes and the nutritive value of legumes is of great interest. As described in a report by Aykroyd and Doughty (1964), it is proposed that during soaking, the seed constituents become more assimilable for human nutrition. Increase in nutrients induced by germination takes place when sprouts become visible.

The germination process promote many chemical and biochemical changes in the seed which may increase the nutritional value of the final product and also decrease the amount of some undesirable compounds (Bau, Villaume, Nicolas, & Mejean, 1997).

Germination improves the nutritive value of legumes including chickpeas, (Chattopadhyay & Banerjee, 1953) by bringing changes in vitamins, and limiting amino acids, digestibility coefficient, flatulence factors, and antigrowth factors. Nnanna and Philips (1990) stated that germination could increase the nutritive value of legumes by inducing the activation of enzymes, which eliminate or reduce the antinutritional and indigestible factors. In addition, germination induces changes in protein (Mostafa, Rahma, & Rady, 1987), and starch digestibility (Nnanna & Philips 1990), which probably also result from the enzymatic action. Germination has been used as a way to improve the protein quality of chickpeas (Mansour, 1996).

During germination, some of the reserve materials of the seeds are degraded (Ghorpade & Kadam, 1989). Enzymes become active in order to degrade starch, storage protein, and proteinaceous antinutritional factors. The degradation of storage protein is necessary to make peptides and amino acids available in order to stimulate the seed growth and early plant growth. Proteinaceous antinutritional factors such as TI s are present in legume seeds and protect them from predators. However, they degrade to a lower level by the action of several enzymes (Savelkoul, Van der Poel, & Tamminga, 1994). Generally, the raffinose family (raffinose, stachyose, and verbascose) oligosaccharides decline rapidly during the first days of germination, while sucrose, glucose, and fructose increase (Aman, 1979).

Germination is also known to improve the protein digestibility of chickpeas (Jood et al., 1989). It has a marked effect on improving the nutritional quality of some legumes including chickpeas.

Various attempts have been made to study the effect of germination on the TIA in chickpeas in order to improve their nutritive value, but contradictory results are reported in the literature (Khaleque et al., 1985). Several researchers have reported changes in the TIA during soaking and germination of legume seeds (Sharma & Sehgal, 1992). However, there have been conflicting findings about the effects of these processing treatments on the TIA in desi chickpeas. A Few researchers have studied the effect of soaking and germination in desi chickpeas, but information is not consistent.

Some studies have observed a decrease in the level of TI s in chickpeas after germination whereas a few reported an increase in the TIA for 24 to 72 hours. Jaya and Venkataraman (1980) reported an increase in the TIA after 48 hrs of germination while Chattopadhyay and Banerjee (1953) did not observe any change in trypsin inhibitor activity. Bansal et al. (1988) noted decrease in the activity of TI in desi chickpeas after the first day (24 hrs) of germination, but then there was gradual increase in TIA on the second day (48 hrs), although the increased TIA was lower than the raw seeds.

Determination of Trypsin Inhibitory Activity

Methods currently used for the determination of TI s in legumes are spectrophotometric methods based upon the reduction of the hydrolysis rate of trypsin substrate resulting from the addition of TI extract. Both natural trypsin substrate, casein (Kunitz, 1947), and 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 methods of TI analysis, BAPA has been used more extensively and is reported not only to be 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 & Markakis, 1989; Smith et al., 1980) to increase simplicity, accuracy, and reproducibility. Although, BAPA has been used more widely, when compared to the TAME method, it is tedious and time consuming. Substrate TAME is more rapidly hydrolizable by trypsin than α-benzoyl-L-argenine ethyl ester (BAEE) and is not hydrolyzed by chymotrypsin (Hummel, 1959). There are several studies reported in the literature (DeLumen & Belo, 1981; Sathe & Salunkhe, 1981) in which the TAME has been used successfully in determining TI in legumes.

The TIA is measured using either bovine or porcine trypsin. Although bovine trypsin is used extensively in the determination of TI activity, porcine trypsin is more stable under alkaline conditions (Buck et al., 1962). Compared to bovine, the structure of porcine trypsin is intrinsically more resistant to self-digestion (Liu & Markakis, 1989). 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).

To our knowledge, data on the soaking and germination effect on TIA in desi chickpeas is available only by using casein substrate and there is no data available by using the BAPA and TAME methods. Although, porcine trypsin is said to be a more appropriate source for the experimental conditions in the determination of TI (Liu & Markakis, 1989), in all of the methods used previously for TI in desi chickpeas, bovine

was the only source of trypsin that was used. Therefore, there is no data available on TIA in desi chickpeas using the TAME and BAPA method with porcine as a trypsin source. Therefore, there is a need to obtain data on TIA in desi chickpeas using these methods.

CHAPTER 2

JOURNAL ARTICLE

Authors' Title Page

EFFECT OF SOAKING AND GERMINATION ON TRYPSIN INHIBITOR ACTIVITY IN DESI CHICKPEA

Maneesha K. Ranabhor, Panfilo S. Belo*, Miriam Saltmarch, and Lucy McProud

Department of Nutrition and Food Science

San Jose State University

1 Washington Square

San Jose, CA 95192-0058

U.S.A.

* Address questions and comments to: Dr. Panfilo S. Belo,

Department of Nutrition and Food Science, San Jose State University,

1 Washington Square, San Jose, CA 95192-0058, U.S.A., 408-924-3108.

ABSTRACT

The objective of the study was to determine the trypsin inhibitory activity (TIA) in raw, soaked and germinated desi chickpeas. Desi chickpea seeds obtained locally were soaked for 12 hrs and then germinated for 24 and 48 hours. Raw, soaked and germinated seeds were subsequently air dried, ground and defatted. The BAPA and TAME methods were used to assay TIA in these defatted powders.

Trypsin inhibitor activity in raw chickpea seeds, expressed as TI unit/ mg protein, using BAPA and TAME methods were 93.57 and 5.88, respectively. Soaking for 12 hrs in water significantly ($P \leq 0.05$) reduced TIA by 13.64 % and 11.56 %, as determined by BAPA and TAME method, respectively. Although increased TIA after 24 hrs of germination was observed compared to the soaked seeds, TIA was still lower than the raw chickpea seeds. Seeds germinated for 48 hrs showed the highest TIA among all of the samples.

Keywords: *Desi chickpeas; trypsin inhibitory activity; soaking; germination; BAPA; TAME*

INTRODUCTION

To alleviate the widely prevailing protein calorie malnutrition (PCM) in developing countries, food technologists and nutritionists have been searching for alternative sources of food (Vijayakumari et al., 1998). The increasing cost of animal proteins has prompted food technologists, nutritionists and biochemists to consider plant proteins as a major source of dietary proteins. Therefore, the importance of using the plant proteins, as a dietary source in countries where protein calorie malnutrition (PCM) exists, is well recognized (Salunkhe, 1982).

The nutritional value of legumes as sources of protein and carbohydrates in the diet is undeniable, not only for vegetarians but more especially in developing countries where large segments of the population suffer from PCM and where legumes are of utmost importance (Bravo, Siddhuraju, & Saura-Calixto, 1999). They constitute an important source of dietary protein for a large segment of the World's population, particularly in those countries where the consumption of animal protein is limited in availability or is self-imposed because of religious or cultural habits (Jyothi & Sumathi, 1995).

Chickpeas (*Cicer arietinum*) are among the legumes, which have potential as a protein-rich food source. They have long been one of the most important sources of protein in several developing countries, especially in rural populations (Nestares et al., 1996). Chickpeas, among the world's grain food legumes, are second to dry beans (*Phaseolus vulgaris*) in area grown and third in production to dry beans and dry peas (*Pisum sativum*) (Singh, 1985). They are generally grouped into two types: the desi type

with small, angular, dark colored seeds and the kabuli type with large, smooth coated, beige seeds (Gil, Nadal, Luna, Moreno, & De Haro, 1996).

Chickpeas are a valuable source of proteins, minerals, and vitamins. They are an important source of proteins in several developing countries (Mansour, 1996). Together with other legumes, chickpeas have been one of the most important sources of protein in rural populations. They are also widespread in Asia and Central and South America, where they satisfy a considerable portion of the protein requirements of the population (Nestares et al., 1996).

The crude protein content of chickpea seeds range from 12.4 to 30.6 % (Chavan, Kadam, & Salunkhe, 1989). Gupta and Kapoor (1980) reported that, based on the amino acid composition, the proteins of chickpea seeds are found to have a higher nutritive value than some other grain legumes such as lentils, cowpeas, green gram, and pigeonpeas. Sotelo et al. (1987) also mentioned that the protein quality of the chickpeas is one of the best in the legume family. Ashur, Clark, Moon, and Malz (1973) reported that chickpeas meet adult human requirements for all essential amino acids except for methionine and cysteine /cystine.

Like other grain legumes, chickpea seeds contain a variety of antinutritional factors including trypsin inhibitors (Nestares et al., 1996). These antinutritional factors limit utilization of chickpea seeds by exerting deleterious effects on human nutrition (Mansour, 1996; Salunkhe & Kadam, 1989; Singh, 1985). Savage and Frøkiær (1998) stated that chickpeas contain appreciable amounts of trypsin inhibitors. They also

reported that the protein utilization of chickpeas is compromised to a varying extent, by the presence of a range of protease inhibitors (TI s).

Effects of several processing treatments, including soaking and germination on the TI s in legumes have been reported in the literature. However, the major emphasis in research so far has been on soybean and the *Phaseolus* species (Bansal et al., 1988) and comparatively few studied have been conducted on chickpeas, especially desi chickpeas, despite its significance in the human nutrition. Therefore, such reliable and consistent information in chickpeas is lacking. Although a few researchers have studied the effect of soaking and germination on the TIA in desi chickpeas, the information in the literature is not consistent. Due to the increased usage of chickpeas in developing countries to alleviate PCM, it is important to study the TIA in chickpeas in order to maximize their potential for protein quality.

The present study was carried out to determine how and to what extent soaking and germination affect the TIA in desi chickpeas.

MATERIALS AND METHODS

Samples. Raw, dried desi chickpeas were obtained from a local grocery store in bulk at the beginning of the study. They were cleaned physically to remove any unwanted materials such as dirt and broken seeds. The seeds were subjected to three treatments: 12 hrs soaking, 24 hrs germination and 48 hrs germination.

Sample Preparation. The common domestically used procedure for soaking and germination was followed. Initially seeds were thoroughly washed with water and were

soaked in the excess water (with the ratio of 1:5 weight /volume) for 12 hrs, at room temperature ($\simeq 21^{0}$ C). After 12 hrs of soaking, soaking water was discarded. One portion of the soaked seeds was collected for TI analysis. The remaining portion of seeds was allowed to germinate in a cotton cloth in the dark for 24 hrs at room temperature ($\simeq 21^{0}$ C). Distilled water was added occasionally to keep the seeds hydrated. After 24 hrs, one portion of the germinated seeds was collected and another portion was allowed to continue to germinate until 48 hrs at room temperature. After 48 hrs of germination, germinated seeds were collected for TI analysis. After each germination, proportion of germinated seeds to non-germinated seed was recorded.

Soaked as well as germinated seeds were air dried overnight by using APS food dehydrator of Harvest Maid Company and then ground in a grinding mill. Ground samples were then passed through a 60-mesh screen. Samples were defatted in a soxhlet apparatus for six to seven hrs using petroleum ether. Defatted powdered samples were used in the 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 of desi chickpeas according to the procedures used by the Association of Official Analytical Chemists (AOAC, 1990). The total carbohydrate content of the seeds was determined by difference, subtracting the percentage of moisture, crude fat, crude protein, and ash from 100 %. The measurements were conducted in triplicate.

Extraction of Trypsin Inhibitors. The procedure used for the extraction of TI s was the one described by Liu and Markakis (1989). A 0.5g 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 to 3 min before filtering through a Whatman No. 2 paper. Freshly prepared aqueous extracts were used for TI activity determination.

Determination of Protein Content of the TI Extracts. The protein content in the aqueous TI extracts of the samples was determined by the method described by Lowry, Rosebrough, Farr, and Randall (1951) by using the bovine protein as a standard.

Determination of Trypsin Inhibitor Activity. Trypsin inhibitor activity of the samples was determined by using benzoyl –DL-arginine-p-nitroanilide hydrochloride (BAPA) (Sigma Chemical Co.) as well as p-toluene-sulfonyl –L-arginine methyl ester (TAME) (Sigma Chemical Co.) substrate.

BAPA Method. The method used for TI analysis was that described by Liu and Markakis (1989). Samples were prepared by diluting aqueous extracts of defatted beans with water until there was 30 to 70 % trypsin inhibition (Liu & Markakis, 1989). Porcine trypsin (type IX, Sigma Chemical Co.) was used as a source of enzyme for all of the samples. The TIA of raw seeds was also measured by using bovine trypsin (type II, Sigma Chemical Co.) to compare its values with previously reported studies.

The absorbance was measured by using Shimadzu UV-Visible recording spectrophotometer at 410 ηm. The TIA is expressed as trypsin inhibitory unit (TIU) per mg protein where one TIU of trypsin inhibitors is defined as the amount of inhibitor that inhibits 1.0 μg of pure trypsin (Tan, Wong, & DeLumen, 1984).

TAME Method. The Worthington Biochemical Corporation (1972) method was used for TIA determination. Porcine trypsin (type IX, Sigma Chemical Co.) was used as a source of enzyme. The absorbance was recorded at 30 sec intervals for 3 min at 247 nm. The TIA was expressed as trypsin inhibitor unit per mg protein where one inhibitory unit of trypsin inhibitors (TIU) is defined as an amount of inhibitor that inhibits 1.0 μg of pure trypsin (Tan et al., 1984). All measurements were performed in triplicate.

Results of TIA were calculated based on the dry as well as fresh weight of the sample. They were also expressed as TIU per milligram of defatted flour and per milligram of protein.

Statistical Analysis. Results were analyzed statistically ($P \leq 0.05$) by analysis of variance (ANOVA). Statistically significant differences ($P \leq 0.05$) between control and treatments were determined by using Least Significant Difference (LSD).

RESULTS AND DISCUSSION

Proximate Analysis. The results of proximate analysis of full fat flour prepared from raw seeds of desi chickpeas are presented in Table 2. The values obtained for proximate composition were within the range of values reported in the literature (Duke, 1982; Gil et al., 1996; Rincon et al., 1998). The sample contained 2.59 % moisture. The average crude protein content was 19.56 %. The crude fat content was observed to be 3.94 %. The oil had a characteristic yellow color. Along with the protein, desi chickpeas are good source of carbohydrates. The total carbohydrate content (determined by difference) was observed to be 70.04 %. The ash content was found to be 3.87 %.

Percent Germination of the Samples. When soaked seeds were allowed to undergo germination, the percentage of germinated seeds was recorded. The average germination was found to be 90.66 % when soaked seeds were germinated for 24 hrs, whereas it was found to be 95.00 % when germinated seeds were allowed to germinated for 48 hours.

Moisture and Crude Fat Content of the Samples. Moisture and crude fat content of raw, soaked and germinated samples are given in the Table 3. Seeds soaked for12 hrs showed slightly higher moisture content (49.96 %) than those that were germinated for 24 and 48 hrs after soaking. This difference in the moisture could be due to loss of water during the metabolism of seeds during germination.

On a dry weight basis, crude fat content of soaked and germinated seeds were lower in comparison with the raw seeds (control). Reduction in the crude fat content during germination was also noted by Jaya and Venkataraman (1982). This variation in the crude fat content could be correlated to metabolic changes that take place in the seed during soaking and germination.

Protein Content of the Aqueous TI Extracts. The data obtained on the protein content in the aqueous TI extracts is presented in the Table 4. Protein content in the aqueous TI extracts of all of the samples was determined by the Lowry method (Lowry et al., 1951). There was a significant increase ($P \leq 0.05$) in the protein content of soaked as well as germinated samples as compared to raw seeds. The protein content of raw seeds on a dry weight basis (6. 04 %) was increased to 6.34 % after 12 hrs soaking. Whereas for 24 and 48 hrs germinated samples protein contents were 6.58 % and 6.57 %

respectively. During soaking and germination, seed proteins undergo a lot of metabolic changes. These catabolic as well as anabolic changes such as hydrolysis and synthesis of seed proteins might have brought about changes in the protein content. An increase in the protein content during chickpea germination was also observed by Sattar et al. (1990).

Trypsin Inhibitor Activity. The trypsin inhibitor activity was determined by the BAPA as well as TAME method. The results for both the methods are summarized in the Table 5.

BAPA Method. Using the BAPA method, along with the porcine trypsin, the TIA of raw seeds was also determined by using bovine trypsin in order to compare the value with the previously reported studies. The TIA was found to be 6.91 TIU/ mg of fresh sample (or 7.00 TIU/ mg of defatted sample) using bovine trypsin. Rincon et al. (1998) reported TIA between 3.24 to 15.06 / mg of defatted flour. The values obtained are also close to the range of 9.3 to 14.6 TIU / mg of defatted sample of desi chickpea reported by Singh and Jambunathan (1981). Nevertheless, the value obtained was significantly lower ($P \leq 0.05$) than the one presented in Table 1, as reported by Elkowicz and Sosulski (1982).

The TI content of raw chickpea seeds by using porcine trypsin was 93.37 TIU/ mg of protein (5.50 TIU/ mg fresh sample). The level of TIA in desi chickpeas was found to be relatively low compared to some of the legumes such as soybean and winged beans.

When raw desi chickpea seeds were soaked for 12 hrs, a significant ($P \leq 0.05$) reduction of 13.64 % was observed in the TIA. Reduction in the TIA after soaking of desi chickpeas was also reported by Soni et al. (1978) and Bansal et al. (1989).

There was a gradual increase in the TI content during 24 hrs and 48 hrs of germination. After 24 hrs of germination of the soaked seeds, 9.98 % increase in the TI content was noticed. This increment in the TIA after 24 hrs of germination was significant ($P \leq 0.05$) than the soaked seeds, but was still lower than the raw seeds.

The increase in the TIA by 19.40 % was noticed in the 48 hrs germinated samples compared to the 24 hrs germinated samples. This increase in the TIA was significant ($P \leq 0.05$) compared to the soaked chickpeas, but was not significant ($P \leq 0.05$) compared to the raw and the 24 hrs germinated chickpeas.

TAME Method. Values for TIA obtained by the TAME method were low compared to the BAPA method. The same pattern was also observed by Samar (1992).

The raw chickpea seeds had a TIA of 5.88 TIU/mg of protein (0.35 TIU/ mg of fresh sample). Soaking of raw seeds in water for 12 hrs resulted in the significant ($P \leq 0.05$) decrease in TIA than raw seeds. The TIA was reduced by 11.56 % compared to raw seeds.

After germination of soaked seeds for 24 hrs, samples had significantly ($P \leq 0.05$) higher TIA than the soaked samples. The TIA was increased by 11.54 %. This was significantly higher ($P \leq 0.05$) than that of soaked seeds but was still lower than the raw chickpeas.

Germination for 48 hrs resulted in a higher TI content than the 24 hrs germinated sample. The TIA content was increased by 5.34 % compared to the 24 hrs germinated sample. However, this increase was not significant ($P \leq 0.05$). The increase was significant ($P \leq 0.05$) when compared to soaked and raw seeds.

The continuous increase in TIA after germination up to 48 hrs in desi chickpeas was also reported by Jaya and Venkataraman (1980). The increase in the TIA after germination has been reported elsewhere in the literature (Gupta, 1987).

The decrease in the TIA in the soaked seeds could have been attributed to the loss of TI in the soaking water. Sharma and Sehgal (1992) stated that soaking could lead to leaching of TI s into soaking water. Deshpande, Kadam, and Salunkhe (1989) also supported the finding that there is a loss of certain undesirable components including TI.

Savelkoul et al. (1994) studied the effect of germination in kidney beans and reported an increase in the TI content initially until the third day of germination (72 hrs) and then gradual decline in the TI activity after 7 days of germination. They theorized that the initial increase in TI s during germination would suggest that germination starts with the release of some additional TI s, which are already present, but cannot be detected because of their low molecular weights.

Wilson (1988) stated that during the course of germination, the individual isoinhibitors are observed to vary independently of each other, with some forms increasing in abundance while other declined. He mentioned that the available data are suggestive of inter-conversion of some inhibitor forms during germination (e.g. by proteolysis) but he also stated that there is no direct evidence presented for this system.

During germination, Hartl, Tan-Wilson, and Wilson (1980) observed new forms of Kunitz soybean TI in the cotyledon distinct from that of the resting seeds. They concluded that these forms arise from the Kunitz soybean TI present in the un-germinated seed by limited specific proteolysis.

CONCLUSION

Results of this study showed the presence of trypsin inhibitors in desi chickpeas as determined by BAPA and TAME method. The level of TIA in desi chickpeas is relatively low compared to some of the legumes such as soybean and winged beans. Levels of TIA varied in the raw, soaked as well as 24 and 48 hrs germinated samples. Trypsin inhibitor activity also varied with the method of TIA determination. Source of trypsin also affected the TI activity in the desi chickpeas.

Soaking of desi chickpeas in water for 12 hrs at room temperature was found to be effective in reducing the trypsin inhibitory activity. However, germination of soaked desi chickpea seeds for 24 and 48 hrs at room temperature showed increasing trend in the trypsin inhibitory activity. Germinated seeds, particularly those were allowed to germinate for 48 hrs, had the highest TI levels among all of the samples.

In conclusion, soaking of desi chickpeas in water for 12 hrs could be a beneficial procedure to decrease the TI content before consumption at domestic level whereas 24 and 48 hrs of germination at room temperature may not be desirable in reducing the TI content of desi chickpeas. Therefore, there is a further need to study and recommend desirable modifications in the domestic processing of desi chickpeas in order to improve its utilization from nutrition stand point.

Table 2. Proximate Analysis of Full Fat Flour of Raw Desi Chickpea Seeds [a]

Composition (%)	Raw Chickpeas
moisture	2.59 ± 0.07
crude protein (N × 6.25)	19.56 ± 0.18
crude fat	3.94 ± 0.07
ash	3.87 ± 0.01
total carbohydrates [b]	70.04

[a] Results are means of triplicate determination ± standard deviation, expressed on fresh weight basis

[b] Calculated by difference

Table 3. Moisture and Crude Fat Content of the Samples (%) [a]

Sample	Moisture [b]	Crude Fat [c]
Raw	2.59 ± 0.07	4.04 ± 0.07
Soaked	49.99 ± 0.20	3.93 ± 0.02
24 hrs Germination	49.83 ± 0.26	3.93 ± 0.01
48 hrs Germination	49.65 ± 0.32	3.92 ± .0.01

[a] mean ± standard deviation of three determinations

[b] Expressed on the wet (fresh) weight basis

[c] Expressed on the dry weight basis

Table 4. Protein Content of the Aqueous TI Extracts of the Samples (%) [a]

Sample	Protein [b]
Raw	6.05 ± 0.23
Soaked	6.34 ± 0.44
24 hrs Germination	6.58 ± 0.03
48 hrs Germination	6.57 ± 0.08

[a] mean ± standard deviation of three determinations

[b] Determined by Lowry method and expressed on the dry weight basis

Table 5. Trypsin Inhibitor Activity [a]

Trypsin Source	A	B	C	D
TAME Method				
(Porcine)				
Raw	0.36 ± 0.01	0.35 ± 0.05	0.36 ± 0.01	5.88 ± 0.11
Soaked	0.34 ± 0.02	0.17 ± 0.01	0.33 ± 0.02	5.20 ± 0.35
24 hrs Germination	0.42 ± 0.01	0.19 ± 0.01	0.41 ± 0.02	5.80 ± 0.29
48 hrs Germination	0.44 ± 0.01	0.20 ± 0.01	0.42 ± 0.01	6.11 ± 0.16
BAPA Method				
(Porcine)				
Raw	5.72 ± 0.27	5.50 ± 0.26	5.65 ± 0.26	93.57 ± 4.36
Soaked	5.31 ± 0.20	2.64 ± 0.13	5.11 ± 0.19	80.63 ± 3.07
24 hrs Germination	6.08 ± 0.17	3.00 ± 0.11	5.85 ± 0.16	88.68 ± 2.43
48 hrs Germination	6.57 ± 0.32	4.18 ± 0.21	6.33 ± 0.31	96.27 ± 4.53
(Bovine)				
Raw	7.00 ± 0.20	6.73 ± 0.20	6.91 ± 0.20	114.19 ± 3.26

[a] mean ± standard deviation of three determinations

A. TIU/mg Defatted Sample

B. TIU /mg Fresh Sample (Wet basis)

C. TIU /mg Dried Sample (Dry Basis)

D. TIU /mg Protein

LITERATURE CITED

Ashur, S. S.; Clark, H. E.; Moon, W. H.; Malzer, J. L. Nitrogen in adult human subjects who consumed wheat and rice supplemented with chickpea, sesame, milk or whey. *Am. J. Clin. Nutr.* **1973**, 26, 1195-1201.

Association of Analytical Chemists. *Official Methods of Analysis* (15th ed.). Arlington, VA: Authors, **1990**.

Bansal, K. K.; Dhindsa KS.; Batra, V. I. P. Trypsin inhibitor and hemagglutinin activities in chickpea (*Cicer arietinum* L.): effects of heat and germination. *J. Fd. Sci. Technol.* **1988**, 25, 1, 46-48.

Bravo, L.; Siddhuraju, P.; Saura-Calixto, F. Composition of underexploited Indian pulses. Comparison with common legumes. *Food Chem.* **1999**, 64, 185-192.

Chavan, J. K.; Kadam, S. S.; Salunkhe, D. K. Chickpea. In *Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization*; Salunkhe, D. K., Kadam, S. S., Eds.; CRC Press: Boca Raton, FL, **1989**; Vol. I, pp 247-288.

Deshpande, S. S.; Kadam, S. S.; Salunkhe, D. K. Soaking. In *Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization*; Salunkhe, D. K., Kadam, S. S., Eds.; CRC Press: Boca Raton, FL, **1989**; Vol. III, pp. 133-140.

Duke, J. A. Handbook of legumes of world economic importance. –legume species- *Cicer arietinum*. Plenum press: New York, **1982**; pp 52-56.

Elkowicz, K.; Sosulski, F. W. Antinutritive factors in eleven legumes and their air-classified protein and starch fractions. *J. Food Sci.* **1982**, 47, p. 1301-1304.

Gil, J.; Nadal, S.; Luna, D.; Moreno, M. T.; De Haro, A. Variability of some physico-chemical characters in desi and kabuli chickpea types. *J. Sci. Food Agric.* **1996**, 71, 179-184.

Gupta, Y. P. Antinutritional and toxic factors in food legumes: a review. *Plant Foods Hum. Nutr.* **1987**, 37, 201-228.

Gupta, Y. P.; Kapoor, A. C. Chemical evaluation of protein quality of various grain legumes. *Ind. J. Agric. Sci.,* **1980**, 50, 393-398.

Jaya, T. V.; Venkataraman, L. V. Effect of germination on the nitrogenous constituents, essential amino acids, carbohydrates, enzymes and antinutritional factors in chickpea and greengram. *Ind. Food Packer*. **1980**,1, 3-11.

Jyothi, V.; Sumathi, S. Effect of alkali treatments on the nutritive value of common bean (*Phaseolus vulgaris*). *Plant Foods Hum. Nutr.* **1995**, 48, 193-200.

Liu, K.; Markakis, P. An improved colorimetric method for determining antitryptic activity in soybean 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. Biol. Chem.* **1951**, 193, 265-275.

Mansour, E. H. Biological and chemical evaluation of chickpea seed proteins as affected by germination, extraction and α-amylase treatment. *Plant Foods Hum. Nutr.* **1996**, 49, 271-282.

Nestares, T.; Lopez-Frias, M.; Barrionuevo, M.; Urbano, G. Nutritional assessment of raw and processed chickpea (*Cicer arietinum* L.) protein in growing rats. *J. Agric. Food Chem.*, **1996**, 44, 2760-2765.

Rincon, F.; Martinez, B.; Ibenez, M. V. Proximate composition and antinutritive substances in chickpea *(Cicer arietinum* L.*)* as affected by the biotype factor. *J. Sci. Food Agric.* **1998**, 78, 382-388.

Salunkhe, D. K. Legumes in human nutrition: Current status and future research needs. *Curr. Sci.* **1982**, 51, 387-390.

Salunkhe, D. K.; Kadam, S. S. Legumes in human nutrition: Future prospects. In *Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization*; Salunkhe, D. K., Kadam, S. S., Eds.; CRC Press: Boca Raton, FL, **1989**; Vol. II, pp165-176.

Samar, P. L. Comparison of two synthetic substrates and two trypsin sources for measuring trypsin inhibitor activity in soybean, winged bean, and green gram. Unpublished master's thesis, San Jose State University, San Jose, CA: USA, **1992**, 33-35.

Savage, G. P.; Frøkiær, H. Antinutritive factors in cool season legume seeds for human nutrition- with particular reference to chickpeas. In *Recent Advances of Research in Antinutritional Factors in Legume Seeds and Rapeseed.* Jansman, A. M. J., Hill, G. D., Huisman, J., van der Poel, A. F. B., Eds., EAAP Publication No. 93, Wageningen Pers: Netherlands, **1998**; pp 97-106.

Savelkoul, F. H. M. G.; Tamminga, S.; Leenaars, P. P. A. M.; Schering, J. Ter Maat, D. W. The degradation of lectins, phaseolin and trypsin inhibitors during germination of white kidney beans (*Phaseolus vulgaris* L.). *Plant Foods Hum. Nutr.* **1994**, 45, 213-222.

Sharma, A.; Sehgal, S. Effect of domestic processing, cooking and germination on the trypsin inhibitor activity and tannin content of faba bean (*Vicia faba*). *Plant Foods Hum. Nutr.* **1992**, 42, 127-133.

Singh, U. Nutritional quality of chickpea (*Cicer arietinum* L.): Current status and future research needs. *Qual. Plant. Plant Foods Hum. Nutr.* **1985**, 35, 339-351.

Singh, U.; Jambunathan, R. Studies on desi and kabuli chickpea (*Cicer arietinum* L.) cultivars: levels of protease inhibitors, levels of polyphenolic compounds and in vitro protein digestibility. *J. Food Sci.* **1981**, 46, 1364-1367.

Soni, G. L.; Singh, T. P.; Singh, R. Comparative studies on the effects of certain treatments on the antitryptic activity of the common Indian pulses. *Indian. J. Nutr. Dietet.* **1978**, 15, 341-345.

Sotelo, A.; Flores, F.; Hernandez, M. Chemical composition and nutritional value of Mexican varieties of chickpea (*Cicer arietinum* L.). *Plant Foods Hum. Nutr.* **1987**, 37, 299-306.

Tan, N.; Wong, K.; DeLumen, B. O. 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.

Vijayakumari, K.; Siddhuraju, P.; Pugalenthi, M.; Janardhanan, K. Effect of soaking and heat processing on the levels of antinutrients and digestible proteins in seeds of *Vigna aconitifolia* and *Vigna sinensis*. *Food Chem.* **1998**, 63, 2, 259-264.

Wilson, K. A. The proteolysis of trypsin inhibitors in legume seeds. *CRC Crit. Rev. Biotech.* **1988**, 8, 197-216.

Worthington Biochemical Corporation. Trypsin (bovine pancreas). *Worthington enzymes manual*, Freehold: NJ. **1972**; pp 125-127.

CHAPTER 3

SUMMARY AND RECOMMENDATIONS

Summary

The objective of this study was to determine the effect of soaking and germination on the trypsin inhibitory activity in desi chickpeas. Desi chickpeas were subjected to the common, domestically used processing treatments of soaking and germination.

Raw seeds of desi chickpeas were subjected to soaking in water for 12 hrs at room temperature ($\simeq 21^0$ C). Soaked seeds were then allowed to germinate for 24 and 48 hrs at room temperature ($\simeq 21^0$ C). Aqueous TI extracts prepared from the air-dried, defatted samples were used to measure the trypsin inhibitory activity. One trypsin inhibitory unit was defined as the amount of inhibitor that inhibits 1.0 μg of pure trypsin. Trypsin inhibitory activity was determined by using the BAPA and TAME methods with porcine trypsin as a source of enzyme. Data collected were analyzed by using analysis of variance (ANOVA).

Results suggested that soaking for 12 hrs and germination of soaked seeds for 24 and 48 hrs affected the TIA in desi chickpea seeds. Soaking of the raw seeds led to significant reduction ($P \leq 0.05$) in the TIA. This statistically significant decrease ($P \leq 0.05$) in the TIA might be because of possible leaching of some TI into the soaking water. When these soaked seeds were allowed to undergo germination for 24 and 48 hrs, there was gradual increase in the TI activity. The sample that germinated for 48 hrs had highest TIA of all of the samples.

This increase in TI s during germination would suggest that germination starts with the release of some additional TI s, which are already present, but cannot be detected because of their low molecular weights. Another possibility is that the increase in the TI content could be because of the formation of new forms of TI from native ones by specific proteolysis of native TI of desi chickpeas.

Soaking of desi chickpeas for 12 hrs as described in this study could be a beneficial procedure to decrease the TI content before consumption or further processing. However, increased TIA during 24 and 48 hrs germination concluded that these two processing treatments are not desirable in reducing TI content of desi chickpeas.

Recommendations

The data available at present, on the effect of processing treatments on TI in desi chickpeas is by using casein as a substrate. Therefore, there is a need to compare the BAPA and TAME methods with the casein in desi chickpeas in order to compare and offer consistent and reliable results and recommend desirable modifications in these domestic processing treatments.

Desi chickpeas contain several antinutritional factors apart from trypsin inhibitors. Therefore, there is a need to study and develop desirable domestic processing treatments in order to minimize these antinutritional factors and in turn maximize the nutritional potential of this commonly consumed legume.

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

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

References

Aman, P. (1979). Carbohydrates in raw and germinated seeds from mung bean and chickpea. Journal of Science of Food and Agriculture, 30, 869-875.

Ashur, S. S., Clark, H. E., Moon, W. H., & Malzer, J. L. (1973) Nitrogen in adult human subjects who consumed wheat and rice supplemented with chickpea, sesame, milk or whey. American Journal of Clinical Nutrition , 26, 1195-1201.

Association of Analytical Chemists. (1990). Official Methods of Analysis (15th ed.). Arlington, VA: Authors.

Attia, R. S., Shehata, A. M., Aman, M. E., & Hamza, M. A. (1994). Effect of cooking and decortication on the physical properties, the chemical composition and the nutritive value of chickpea (*Cicer arietinum* L.). Food Chemistry, 50, 125-131.

Aykroyd, W. R., & Doughty, J. (1964). Legumes in human nutrition, Food and Agriculture Organization nutritional studies No. 19, FAO, Rome.

Bansal, K. K., Dhindsa K. S., & Batra, V. I. P. (1988). Trypsin inhibitor and hemagglutinin activities in chickpea (*Cicer arietinum* L.): Effects of heat and germination. Journal of Food Science and Technology, 25, 46-48.

Bau, H. M., Villaume, C., Nicolas, J. P., & Mejean, L. (1997). Effect of germination on chemical composition, biochemical constituents and antinutritional factors of soya bean (*Glycine max*) seeds. Journal of Science of Food and Agriculture, 42, 1-9.

Belew, M. (1977). The trypsin and chymotrypsin inhibitors in chickpeas (*Cicer arietinum* L.). The relationships among the six isoinhibitors. European Journal of Biochemistry, 73, 411-420.

Belew, M. & Eaker, D. (1975). The trypsin and chymotrypsin inhibitors in chickpeas (*Cicer arietinum* L.). European Journal of Biochemistry, 62, 499-508.

Belew, M., Porath, J., & Sundberg, L. (1975). The trypsin and chymotrypsin inhibitors in chickpeas (*Cicer arietinum* L.). Purification and properties of the inhibitors. European Journal of Biochemistry, 60, 247-258.

Birk, Y. (1994). Protein protease inhibitors in legume seeds-Overview. Archivos Lationoamericanos de Nutricion, 44, 26S-30S.

Bravo, L., Siddhuraju, P., & Saura-Calixto, F. (1999). Composition of underexploited Indian pulses. Comparison with common legumes. Food Chemistry, 64, 185-192.

Bressani, R. (1975). Legumes in human diets and how they might be improved. In Milner, M. (Ed.), Nutritional Improvement of food Legumes by Breeding; (pp. 15-42). New York: John Wiley and Sons.

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.

Carlini, C. R., & Udedibie, A. B. (1997). Comparative effects of processing methods on hemagglutinating and antitryptic activities of *Canavalia ensiformis* and *Canavalia braziliensis* seeds. Journal of Agricultural and Food Chemistry, 45, 4372-4377.

Chattopadhyay, H., & Banerjee, S. (1953). Effect of germination on the biological value of proteins and the trypsin-inhibitor activity of some common Indian pulses. Indian Journal of Medical Research, 41, 185-189.

Chavan, J. K., & Kadam, S. S. (1989). Protease inhibitors. In D. K. Salunkhe & S. S. Kadam (Eds.), Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization (Vol. 1, pp. 123-134). Boca Raton, FL: CRC Press.

Chavan, J. K., Kadam, S. S., & Salunkhe, D. K. (1989). Chickpea. In D. K. Salunkhe & S. S. Kadam (Eds.), Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization (Vol. 1, pp. 247-288). Boca Raton, FL: CRC Press.

Chitra, U., Singh, U., & Venkateswararao, P. (1996). Phytic acid, in vitro protein digestibility, dietary fiber, and minerals of pulses as influenced by processing methods. Plant Foods for Human Nutrition, 49, 307-316.

Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J., & Millan, F. (1999). Production of extensive chickpea (*Cicer arietinum* L.) protein hydrolysates with reduced antigenic activity. Journal of Agricultural and Food Chemistry, 47, 3776-3781.

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.

Deshpande, S. S., & Damodaran, S. (1990). Food legumes: chemistry and technology. In Pomeranz, Y. (Ed.), Advances in cereal science and technology (PP. 147-241). St. Paul, MN: American Association of Cereal Chemists.

Deshpande, S. S., Kadam, S. S., & Salunkhe, D. K. (1989). Soaking. In D. K. Salunkhe & S. S. Kadam (Eds.), Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization (Vol. III, pp. 133-140). Boca Raton, FL: CRC Press.

Deshpande, S. S., Sathe, S. K., & Salunkhe, D. K. (1984). Dry beans of Phaseolus: a review. CRC Critical Reviews in Food Science and Nutrition, 3, 21, 137-195.

Duhan, A., Khetarpaul, N., & Bishnoi, S. (2000). Optimum domestic processing and cooking methods for reducing the polyphenolic (antinutrients) content of pigeonpeas. Nutrition and Health, 13, 227-234.

Duke, J. A. (1982). Handbook of legumes of world economic importance -legume species- Cicer arietinum (pp. 52-56). New York: Plenum press.

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.

Estevez, A. M., Castillo, E., Figuerola, F., & Yanez, E. (1991). Effect of processing on some chemical and nutritional characteristics of pre-cooked and dehydrated legumes. Plant Foods for Human Nutrition, 41, 193-201.

Fernandez-Quintela, A., Macarulla, M. T., Del Barrio A. S., & Martinez, J. A. (1997). Composition and functional properties of protein isolates obtained from commercial legumes grown in northern spain. Plant Foods for Human Nutrition, 51, 331-342.

Friedman, M. (1996). Nutritional value of proteins from different food sources. a review. Journal of Agricultural and Food Chemistry, 44, 6-29.

Gahlawat, P., & Sehgal, S. (1993). Antinutritional content of developed weaning foods as affected by domestic processing. Food Chemistry, 47, 333-336.

George, S., Sivasankar, B., Jayaraman, K., & Vijayalakshmi, M. A. (1997). Production and separation of the methionine rich fraction from chickpea protein hydrolysate generated by proteases of *Bacillus amyloliquefaciens.* Process Biochemistry, 5, 401- 404.

Ghorpade, V. M. & Kadam, S. S. (1989). Germination. In D. K. Salunkhe & S. S. Kadam (Eds.), Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization (Vol. 2, pp. 165-176). Boca Raton, FL: CRC Press.

Gil, J., Nadal, S., Luna, D., Moreno, M. T., & De Haro, A. (1996). Variability of some physico-chemical characters in desi and kabuli chickpea types. Journal of Science of Food and Agriculture, 71, 179-184.

Gupta, Y. P. (1987). Antinutritional and toxic factors in food legumes: a review. Plant Foods for Human Nutrition, 37, 201-228.

Gupta, Y. P., & Kapoor, A. C. (1980). Chemical evaluation of protein quality of various grain legumes. Indian Journal of Agricultural Science, 50, 393-398.

Hafez, S. Y., & Mohammed, A. I. (1983). Biochemical study and non protein trypsin and chymotrypsin inhibitors in the soy bean. Journal of Food Science, 48, 1265-1268.

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.

Hamza, M A., Shehata, A. M. E., & Stegemann, H. (1986). Studies on α-amylase and trypsin inhibitors in legume seeds using agar diffusion and isoelectric focusing techniques. Qualitas Planatarum Plant Foods Human Nutrition, 36, 139-146.

Harsulkar, A. M., Giri, A. P., & Kothekar, V. S. (1997). Protease inhibitors of chickpea (*Cicer arietinum* L) during seed development. Journal of Science of Food and Agriculture, 74, 509-512.

Hartl, P. M., Tan-Wilson, A. L., & Wilson, K. A. (1980). Proteolysis of kunitz soybean trypsin inhibitor during germination. Phytology, 25, 23-26.

Hira, C. K., & Chopra, N. (1995). Effects of roasting on protein quality of chickpea *(Cicer arietinum)* and peanut (*Arachis hypogaea).* Journal of Food Science and Technology, 32, 501-503.

Huisamn, J., & Jansman, A. J. M. (1991). Dietary effects and some analytical aspects of antinutritional factors in peas (*Pisum sativum*), common beans (*Phaseolus vulgaris*) and soyabeans (*Glycine max* L.) in monogastrics farm animals. A literature review. Nutrition Abstracts and Reviews (Series B), 61, 901-921.

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., Krishnamurthy, K. S., & Venkataraman, L. V. (1975). Effect of germination and cooking on the protein efficiency ratio of some legumes. Nutrition Reports International, 12, 175-183.

Jaya, T. V., & Venkataraman, L. V. (1980). Effect of germination on the nitrogenous constituents, essential amino acids, carbohydrates, enzymes and antinutritional factors in chickpea and greengram. Indian Food Packer, 1, 3-11.

Jood, S., Chauhan, B. M., & Kapoor, A. C. (1989). Protein digestibility (in vitro) of chickpeas and black gram seeds as affected by domestic processing and cooking. Plant Foods for Human Nutrition, 39, 149-154.

Jyothi, V., & Sumathi, S. (1995). Effect of alkali treatments on the nutritive value of common bean (*Phaseolus vulgaris*). Plant Foods for Human Nutrition, 48, 193-200.

Kachroo, P. (1970). Gram. In Kachroo, P., (Ed)., Pulse Crops of India (PP. 54-135). New Delhi, India.: Indian Council of Agricultural Research.

Kadam, S. S. & Salunkhe, D. K. (1989). Production, distribution, and consumption. In D. K. Salunkhe & S. S. Kadam (Eds.), Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization (Vol. 1, pp. 5-21). Boca Raton, FL: CRC Press.

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 soybean samples. Cereal Chemistry, 46, 518-526.

Khaleque, A., Elias, L. G., Braham, J. E., & Bressani, R. (1985). Studies on the development of infant foods from plant protein sources. Part I. Effect of germination of chickpea (*Cicer arietinum*) on the nutritive value and digestibility of proteins. Archivos Latinoamericanos De Nutricion, 35, 315-325.

Koksel, H., Sivri, D., Scanlon, M. G., & Bushuk, W. (1998). Comparison of physical properties of raw and roasted chickpeas (leblebi). Food Research International, 31, 659-665.

Kunitz, M. (1947). Crystalline soybean trypsin inhibitor. II General properties. Journal of General Physiology, 30, 291-310.

Liener, I. E. (1989). Significance of humans of biologically active factors in soybean and other food legumes. Journal of American Oil Chemists' Society, 9, 203-242.

Liener, I. E., & Kakade, M. L. (1980). Protease inhibitors. In I. E. Liener (Ed.), Toxic constituents of plant food stuffs (pp. 7-68). New York: Academic Press.

Liu, K., & Markakis, P. (1987). Effect of maturity and processing on the trypsin inhibitor and oligosaccharides of soybeans. Journal of Food Science, 52, 222-225.

Liu, K., & Markakis, P. (1989). An improved colorimetric method for determining antitryptic activity in soybean 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.

Macrae, R., Robinson, R. K., & Sadler, M. J. (1993) Eds.; Trypsin Inhibitors. In Encyclopedia of Food Science, Food Technology and Nutrition, (Vol. 6, pp 3617-3622). New York: Academic Press.

Macrae, R., Robinson, R. K., & Sadler, M. J. (1993). Eds.; Legumes. In Encyclopedia of Food Science, Food Technology and Nutrition, (Vol. 4, pp 2718-2730). New York: Academic Press.

Mansour, E. H. (1996). Biological and chemical evaluation of chickpea seed proteins as affected by germination, extraction and α-amylase treatment. Plant Foods for Human Nutrition, 49, 271-28.

Mostafa, M. M., Rahma, E. H., & Rady, A. H. (1987). Chemical and nutritional changes in soybean during germination. Food Chemistry, 23, 257-263.

Mulimani V. H., & Vadiraj, S. (1994). Changes in trypsin and chymotrypsin inhibitory activity on soaking of sorghum (*Sorghum bicolor* L. Moench). Plant Foods for Human Nutrition, 46, 27-31.

Nestares, T., Barrionuevo, M., Urbano, G., & Lopez-Frias, M. (1999). Effects of processing methods on the calcium, phosphorus and phytic acid contents and nutritive utilization of chickpea (*Cicer arietinum* L.). Journal of Agricultural and Food Chemistry, 47, 2807-2812.

Nestares, T., Lopez-Frias, M., Barrionuevo, M., Urbano, G. (1996). Nutritional assessment of raw and processed chickpea (*Cicer arietinum* L.) protein in growing rats. Journal of Agricultural and Food Chemistry, 44, 2760-2765.

Newman, C. W., Roth, N. R., & Lockerman R. H. (1987). Protein quality of chickpea (*Cicer arietinum* L.). Nutrition Reports International, 36, 1-5.

Nnanna, I. A., & Philips, R. D. (1990). Protein and starch digestibility and flatulence potential of germinated cowpeas (*Vigna unguiculata).* Journal of Food Science, 55, 151-153.

Rincon, F., Martinez, B., & Ibenez, M. V. (1998). Proximate composition and antinutritive substances in chickpea (*Cicer arietinum* L.) as affected by the biotype factor. Journal of Science of Food and Agriculture, 78, 382-388.

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.

Salunkhe, D. K. & Kadam, S. S. (1989). Legumes in human nutrition: Future prospects. In D. K. Salunkhe & S. S. Kadam (Eds.), Handbook of world food legumes: Nutritional chemistry, processing technology, and utilization (Vol. 2, pp. 165-176). Boca Raton, FL: CRC Press.

Samar P. L. (1992). Comparison of two synthetic substrates and two trypsin sources for measuring trypsin inhibitor activity in soybean, winged bean, and green gram. Unpublished master's thesis, San Jose State University, San Jose, CA: USA, 33-35.

Sanchez-Vioque, R., Clemente, A., Vioque, J. Bautista, J., & Millan, F. (1998). Chickpea (*Cicer arietinum* L.) protein isolates: chemical composition, functional properties and protein characterization. Food Chemistry, 64, 1-7.

Sathe, S. K. (1996). The nutritional value of selected Asiatic pulses: chickpea, black gram, mung bean and pigeon pea. In Nwokolo, E., & Smartt, J., (Eds.), Food and feed from legumes and oilseeds, (pp. 12-33). London: Chapman & Hall.

Sattar, A., Atta, S., & Akhtar, M. A. (1990). Effect of irradiation and germination on trypsin inhibitor and protein content of chickpea (*Cicer arietinum* L.). International Journal of Vital Nutrition Research, 60, 402-406.

Savage, G. P., & Frøkiær, H. (1998). Antinutritive factors in cool season legume seeds for human nutrition- with particular reference to chickpeas. In Jansman, A. M. J., Hill, G. D., Huisman, J., & van der Poel, A. F. B., (Eds.), Recent advances of research in antinutritional factors in legume seeds and rapeseed (pp. 97-106). Wageningen Pers, Netherlands: EAAP Publication.

Savelkoul, F. H. M. G., Tamminga, S., Leenaars, P. P. A. M., Schering, J., & Ter Maat, D. W. (1994). The degradation of lectins, phaseolin and trypsin inhibitors during germination of white kidney beans (*Phaseolus vulgaris* L.). Plant Foods for Human Nutrition, 45, 213-222.

Sharma, A., & Sehgal, S. (1992). Effect of domestic processing, cooking and germination on the trypsin inhibitor activity and tannin content of faba bean (*Vicia faba*). Plant Foods for Human Nutrition, 42, 127-133.

Siegel, A., & Fawcett, B. (1976). Food legume processing and utilization. (With special emphasis on application in developing countries) (pp. 23-24). Ottawa, Canada: International Development Research Center.

Singh, U. (1985). Nutritional quality of chickpea (*Cicer arietinum* L.): Current status and future research needs. Qualitas Planatarum Plant Foods for Human Nutrition, 35, 339-351.

Singh, U. (1988). Antinutritional factors of chickpea and pigeonpea and their removal by processing. Plant Foods for Human Nutrition, 38, 251-261.

Singh, U., & Jambunathan, R. (1981). Studies on desi and kabuli chickpea (*Cicer arietinum* L.) cultivars: Levels of protease inhibitors, levels of polyphenolic compounds and in vitro protein digestibility. Journal of Food Science, 46, 1364–1367.

Smirnoff, P., Khalef, S., Birk, Y., & Applebaum, S. W. (1976). A trypsin and Chymotrypsin inhibitors from chickpeas (Cicer arietinum). Biochemistry Journal, 745-751.

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.

Soni, G. L., Singh, T. P., & Singh, R. (1978). Comparative studies on the effects of certain treatments on the antitryptic activity of the common Indian pulses. Indian Journal of Nutrition and Dietetics, 15, 341-345.

Sotelo A., & Adsule R. N. (1996). Chickpea. In Nwokolo & J. E. Smartt, (Eds.) Food and Feed from Legumes and Oilseeds (pp. 82-88). London: Chapman & Hall.

Sotelo, A., Flores, F., & Hernandez, M. (1987). Chemical composition and nutritional value of Mexican varieties of chickpea (*Cicer arietinum* L.). Plant Foods for Human Nutrition, 37, 299-306.

Stanton, W. R., Doughty, J., Orraca-Tetteh, R., & Steele, W. (1966). Grain legumes in Africa. Rome: Food and Agriculture Organization of the United Nations.

Sumathi, S., & Pattabiraman, T. N. (1976). Natural plant enzyme inhibitors: part two- Protease inhibitors of seeds. Indian Journal of Biochemistry and Biophysics, 13, 52-56.

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.

Trugo, L. C., Donangelo, C. M., Trugo, N. M. F., & Bach Knudsen, K. E. (2000). Effect of heat treatment on nutritional quality of germinated legume seeds. Journal of Agricultural and Food Chemistry, 48, 2082-208.

Ulloa, J. A., Valencia, M. E., & Garcia, Z. H. (1988). Protein concentrate from chickpea: Nutritive value of a protein concentrate from chickpea (Cicer *arietinum*) obtained by ultra filtration and its potential use in an infant formula. Journal of Food Science, 43, 1396-1401.

Urbano, G., Lopez-Jurado, M., Hernandez, J., Fernandez, M., Moreu, M., Frias, J., Diaz-Pollan, C., Prodanov, M., & Vidal-Varvade, C. (1995). Nutritional assessment of raw, heated, and germinated lentils. Journal of Agricultural and Food Chemistry, 43, 1871-1877.

Vidal-Valverde, C., Frias, J., Estrella, I., Gorospe, M. J., Ruiz, R., & Bacon, J. (1994). Effect of processing on some antinutritional factors of lentils. Journal of Agricultural and Food Chemistry, 42, 2291-2295.

Vijayakumari, K., Siddhuraju, P., Pugalenthi, M., Janardhanan, K. (1998). Effect of soaking and heat processing on the levels of antinutrients and digestible proteins in seeds of *Vigna aconitifolia* and *Vigna sinensis*. Food Chemistry, 63, 259-264.

Waterlow, J. C. (1994). Childhood malnutrition in developing nations: Looking back and looking forward. Annual Reviews of Nutrition, 14, 1-19.

Williams, P. C., & Singh, U. (1987). Nutritional quality and the evaluation of protein quality in breeding programs. In Saxena, M. C., & K. B. Singh, (Eds.), The Chickpeas (pp.329-356). Walling ford, Oxon, UK: CAB international.

Wilson, K. A. (1988). The proteolysis of trypsin inhibitors in legume seeds. CRC Critical Reviews in Biotechnology, 8, 197-216.

Worthington Biochemical Corporation (1972). Trypsin (bovine pancreas). Worthington enzymes manual, (pp. 125-127). Freehold, NJ: Author.