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Purification, characterization and kinetic studies of antinutrients in bambara groundnut (Vigna subterranea (L.) Verdc.) seeds

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Purification, characterization and kinetic studies of antinutrients in bambara groundnut (*Vigna subterranea* (L.) Verdc.) seeds

by

Redempta Boy Kegode

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has met the dissertation requirements of Iowa State University

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For the Major Program

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For the Graduate College

To George and Fadhili

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GENERAL INTRODUCTION

Introduction

Farming in Africa follows a seasonal and cyclical nature and during periods when labor is scarce, money becomes a limiting factor. The United Nations Food and Agricultural Organization identifies poverty as the fundamental cause of malnutrition (Dichter, 1987), although numerous other environmental, social and cultural factors play a significant role. Kwashiokor (severe protein deficiency) is the prevalent form of malnutrition seen in Africa. With increasing population and decreasing carrying capacity of the land, it is important to expand the use of a number of minor crops to increase their production, enhance rural development and eradicate malnutrition.

The bambara groundnut is a minor crop of potential importance in Africa. It has a high protein content (16-25 %) and is widely used on the continent to complement the staple foods which are basically carbohydrates. Creative schemes are needed to increase the quantity and quality of bambara groundnut for production and processing into an inexpensive and nutritious source of protein. Processing usually improves the protein quality of foods, however, nutritional consequences on other food constituents should be minimal. This goal can be achieved only if there is sufficient knowledge about the physical and chemical properties of the components of bambara groundnut. The bambara groundnut is a hard, smooth, edible seed that is usually round and varies in size and color. The variations in seed color are one of several characteristics that are used to distinguish between the various forms of bambara groundnut.

While several studies have been conducted on bambara groundnuts in general, there is still a lot of specific information that needs to be gathered. Additional studies are needed to properly identify the nature of the differences among the various forms and to determine the significance of bambara groundnut antinutrients to human nutrition. Therefore, the objectives of this study were to characterize the antinutrients found in bambara groundnut seeds with a view to designing appropriate detoxification processes that can be incorporated

within the procedures of food preparation. Another objective was to produce vegetable milk from bambara groundnuts with possibilities of fortified milks being used as a substitute for bovine milk where it is not available. The choice of origin of bambara groundnut seeds was incidental. Most of the documented studies on bambara groundnuts have been done on seeds obtained from Western Africa. At conception of this project, it was desired to obtain seeds from Western Africa in order to have some continuity of information, however, the seeds that were available were from East and South East Africa and these are the ones that were studied.

Dissertation Organization

This dissertation is composed of three manuscripts, each manuscript being a complete and separate chapter. The title of each manuscript is also the main chapter heading within the dissertation. In addition to the manuscripts, the dissertation contains a general introduction with a general literature review, general conclusion and an appendix. The primary author of this dissertation is the candidate for the degree of Doctor of Philosophy and is the person principally involved in the data collection, analysis, interpretation and writing of the papers.

Literature Review

The Bambara Groundnut (*Vigna subterranea* (L.) Verdc.)

The bambara groundnut is an indigenous African crop that has been cultivated for centuries in tropical regions south of the Sahara (Linneman and Azam-Ali, 1992). Although it is mostly produced in West Africa it is widely grown in Eastern and Southern Africa, and Madagascar (Williams, 1992). The crop is predominantly grown for human consumption but can also be used to feed pigs and poultry (Oluyemi, et al., 1976).

Botany and Ecology

Bambara groundnut belongs to the *leguminoseae* family and *papilionoideae* subfamily (Linneman and Azam-Ali, 1992). Two botanical varieties have been identified (Hepper, 1970); (1) var. *spontanea* (Harms) Hepper - which includes the slender and diffuse wild forms, and (2) var. *subterraneae* - which includes the more robust cultivated forms.

Little is known about how widely adapted the species is, although it can be cultivated at an elevation of 1,600 m, and it is typically a short-day plant (Harris and Azam-Ali, 1993; Linneman, 1996). For successful cultivation, frost-free days of at least 3.5 months are necessary. Bambara groundnut grows best under average temperatures of 20 - 28° C and cultivation requires evenly distributed rain from sowing to flowering. The crop tolerates heavy rainfall except at maturity. There are some bambara groundnut forms that are adapted to the cooler, moist highlands, e.g. Zimbabwe forms, and others that are adapted to areas too arid for other crops (Linneman and Azam-Ali, 1992). Soils rich in nitrogen encourage vegetative growth at the expense of pod production (Johnson, 1968). However, Dadson and Brooks (1989) reported that application of nitrogen is necessary for high seed yield. In addition, calciferous soils are also unsuitable, whereas, soils rich in potassium and phosphorus are beneficial (Hepper, 1970).

Morphology and Anatomy

Bambara groundnut is an indeterminate annual herb that grows to approx. 30 cm tall, with creeping, much branched, leafy lateral stems just above ground level. Its roots can form an association with nitrogen-fixing bacterium (*Rhizobium* spp.) and develop nodules. Bambara groundnut forms are either bunched, intermediate, or spreading types, and the cultivated forms usually have a bunched or intermediate growth habit

Pods usually develop underground, and may reach about 2.5 cm in diameter. Pods are usually almost spherical and contain 1 - 4 round or oblong seeds. Seeds are variable in color: white, cream, yellow, red, purple, brown, or black; the coloration can be uniform, mottled, blotched, or striped, and some have an eye with a dark color around the hilum. Seed color, size, and hardness characterize local cultivars along with the plant form: spreading, bunched, or semi-bunched (Linneman and Azam-Ali, 1992).

Germination and Development

Following planting, cultivated forms of bambara groundnut usualy take 7 - 15 days to emerge from soil, whereas the wild forms take >26 days (Linneman and Azam-Ali, 1992). The optimum temperature range for successful germination is between 22 and 36 °C. Bambara groundnuts have hard seed coats that can prevent water imbibition and impede germination. Any means of reducing the influence of the seed coat on imbibition, germination and emergence is likely to positively effect the uniformity of crop establishment.

Flowering starts 30 - 55 days after sowing and may continue until the plant dies Flowering in bambara groundnut can be day neutral, or show a quantitative or qualitative short-day response (1992; Harris and Azam-Ali, 1993; Linneman, 1996). There has been some confusion as to whether some forms are self-pollinated and others cross-pollinated. Fertilized flowers develop into fruits on or beneath the soil surface. Apparently reproductive development is not inhibited by light, unlike groundnut (peanut) where pegs must penetrate the soil before further development takes place (Linneman and Azam-Ali, 1992). Pods reach mature size about 30 days after fertilization, and seeds mature 10 days later. Early maturing genotypes take approximately 90 days, whereas late maturing genotypes take approximately 150 days, for seeds to reach maturity.

Fruit development may be influenced by photoperiod; long photoperiods delay or prevent fruit set in accessions with day neutral or delayed flowering, indicating a quantitative and qualitative short day response, respectively (Linneman and Azam-Ali, 1992). Photoregulation of development is an important trait in bambara groundnut that provides the plant with a flexible mechanism to adapt to circumstances that create seasonal fluctuations in the length of the growing period (Linneman, 1996).

Bambara groundnut is adapted to arid and drought conditions usually unsuitable for other crops (Doku and Karikari, 1971). However, it is unclear how the crop is able to withstand greater water stress than other pulses and still produce stable yields. Most researchers suggest that the bambara groundnut appears to maintain both a greater supply of water to its roots and a smaller demand for water by its shoots (Nyamudeza, 1989; Linneman and Azam-Ali, 1992). This strategy has clear advantages under drought conditions but may

be a disadvantage when water is readily available because in both situations assimilates are allocated in proximity to the roots rather than to the photosynthetic surfaces of reproductive structures.

Agronomy, Yield and Production

Bambara groundnuts are grown in small plots in many African traditional farming systems as a pure stand, or as an intercrop in association with one or more other species (Stanton 1968; Doku and Karikari 1971). Bambara groundnut can also be grown in rotation with other crops such as maize, cowpea, cassava or yam (as done in Nigeria) (Okigbo 1973). Bambara groundnut is useful in crop rotations because it may improve the nitrogen status of the soil (Mukurumbira 1985).

For best yields, bambara groundnut should be planted in deep free-draining soils with a light, friable seedbed which is conducive to the plants burying their pods (Johnson 1968). Soils should be deeply ploughed (Valentine 1963), or ridged if the soil is susceptible to waterlogging or is shallow, otherwise a flat seedbed is preferred (Johnson 1968).

Although crop durations are extremely variable, harvesting usually takes place between 90 and 170 days after sowing, depending on genotype, temperature, and other environmental factors (Linneman and Azam-Ali, 1992). Plants are usually uprooted by hand or by using a hoe. Since many pods are detached from the helm during harvesting, gleaning is important if maximum yield is to be obtained. Field losses as high as 50 - 65% of total yield have been reported (Johnson1968).

Under traditional African farming systems, yields vary considerably between sites, seasons and genotypes. Though the highest yield on record is 4,000 kg ha^{-1}, in most circumstances yields are rather low and those in the range of 60 - 110 kg ha^{-1} have been reported in Zambia, whereas for most of the semi-arid tropics, typical yields are often between 650 and 850 kg ha^{-1} (Stanton et al, 1966). Nonetheless, bambara groundnut produces reasonable yields under drought conditions on poor, pest- and disease-ridden soils where the cultivation of similar crops such as groundnut (peanut), common bean and cowpea is too risky.

Antinutritional Constituents

Legume seeds contain several antinutritional factors like trypsin inhibitors, plant polyphenolics (tannins), cyanogenic glucosides, alkaloids, phytohemagglutinins. The presence of trypsin inhbitors and tannins in bambara groundnuts has been reported. Trypsin inhibitory activity in eight bambara groundnut genotypes was found to range from 6.75-15.44 TIU/mg per 100 g samples (Poulter, 1981). The inhibitory activity was directly correlated with the quantity of protein in each cultivar. Bambara groundnut trypsin inhibitor was isolated and partially characterized by Martino-Ferrer and Ferrer (1983). The inhibitor was found to exist in two forms; a monomer (MW 13,200 Da) in the denatured form and a dimer (MW 26,300 Da) in the native form. Studies of enzyme kinetics revealed that the enzyme-inhibitor complex formed from one inhibitor molecule and two enzyme molecules and the dissociation constant is 1.28×10^{-8} M.

Poulter (1981) also investigated bambara groundnut tannins and found that the quantities in eight accessions ranged from 0.36-0.94 g/100 g seeds, and that the tannin content was inversely related to the trypsin inhibitor content of the seeds. Bambara groundnuts have no cyanogenic glucosides, alkaloids or phytohemagglutinins (Linnemann and Azam-Ali, 1993). The is no documentation of the presence of tri- and tetra-saccharides in bambara groundnuts.

Plant Phenolic Compounds

Plant phenolic compounds can be divided into two broad groups, (1) phenolic acids and coumarins, and (2) flavonoids including proanthocyanidins, hydrolyzable tannins and anthocyanidins (Salunkhe et al.,1990; Deshpande, et al,. 1986).

Phenolic acids in plants are synthesized through the shikimic acid pathway which gives rise to tryptophan, tyrosine and phenylalanine. These amino acids serve as precursors for the formation of cinnamic acid, which is further converted to various phenolic acids. Coumarins are produced from cinnamic acid, which is synthesized from tyrosine and phenylalanine.

Flavonoids include the largest and most diverse range of plant phenolics (Salunkhe et al., 1990). All flavonoids share the basic C_{15} (C_6-C_3-C_6) skeleton of flavone. Flavonoids consist of scarlet, crimson, and purple anthocyanin pigments of flowers; ivory or pale yellow flavones, flavonols, flavanols; yellow chalcones; and aurones and colorless flavonones and isoflavones (Salunkhe et al., 1990).

As depicted in Fig. 1, depending on the presence or absence of an OH group in the side chain of the C_9 unit of the C_{15} skeleton, two branches of flavonoid families are generated (Salunkhe et al., 1990). In the first flavonoid family, if an OH group is absent as in the case of cinnamic, p-coumaric, or caffeic acid, the condensation of C_9 and C_6 units results in the formation of 3-desoxyflavonoids. In the second flavonoid family, the presence of OH in the side chain of the C_9 as in the case of the enol form of phenylpyruvate or after the reaction of water with the quinoid radical of cinnamic acid results in the formation of 3-hydroxyflavonoids.

In both flavonoid families, accessory hydroxyl and methyl groups as well as different sugar residues in O- and C-glycosidic linkages can be introduced at different positions of the molecule. The positions, number and combinations of these substituents contribute to the enormous complexity of the flavonoid spectrum (Salunkhe et al., 1990). Both families are independent from each other in their origin as wells in occurrence, while flavanones and flavones are often found together. They are connected with each other and chalcones by specific enzymes, yet there is a certain mutual exclusion between flavones and flavonols in many plant families (Salunkhe et al., 1990). Most flavonoids occur as glycosides in which the C_6-C_3-C_6 aglycone part of the molecule is linked with a number of different sugars. Depending on whether the linkage of sugar to flavonoid aglycone is through an OH group or through a carbon-carbon bond, flavonoids are called O-glycosylflavonoids or C-glycosylflavonoids, respectively. Some flavonoids among the flavones, isoflavones and flavonones are C-C linked glycosides. Flavanones and dihydroflavonols are the simple reduction products of flavones and flavonols, and serve as precursors of the more highly oxidized flavonoids in plants. Isoflavones are isomeric with flavones and are produced from the same C_{15} skeleton by aryl migration. Flavanols are unique in that they do not occur as glycosides, but show reactivity through polymerization into 'proanthocyanidins' (Salunkhe et

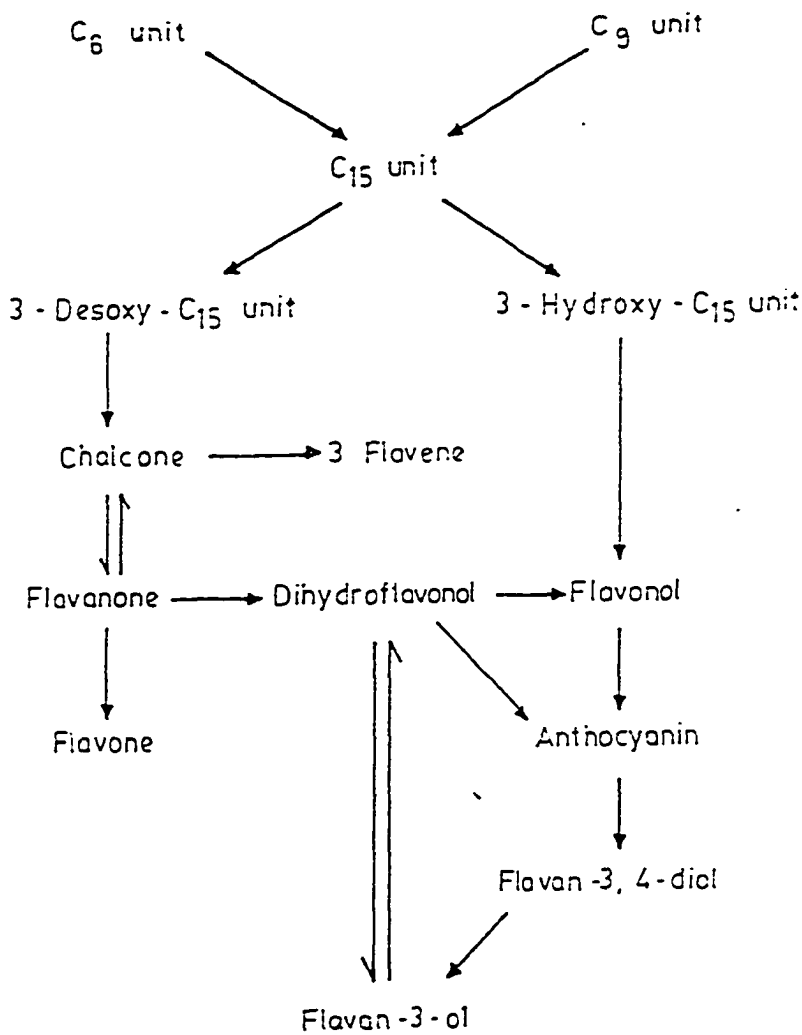


Fig.1 Origin and interrelationships of some flavonoids

al., 1990).

The flavonoid ring structure is of mixed biosynthetic origin (Fig . 2). Ring A is derived from three acetate units condensed head-to-tail, whereas ring B and the three carbon atoms of the central ring are derived from cinnamic acid (Salunkhe et al., 1990). As the acetate units are first converted to malonyl CoA, both the acetate-malonate and the shikimic acid pathways contribute to flavonoid biosynthesis.

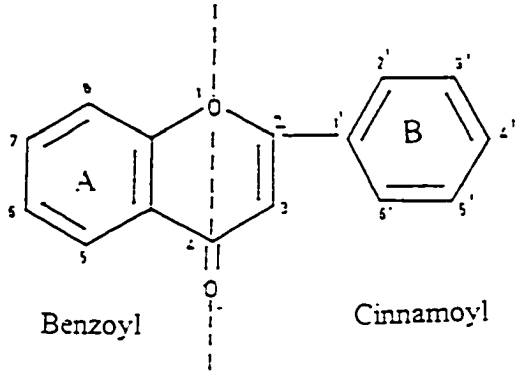


Fig 2. Flavonoid ring structure

Tannins

Tannins are are polymeric plant flavonoids having molecular weights ranging from 500-3000 Da, and are capable of binding and precipitating proteins. There are two classes of tannins that are distinguished: proanthocyanidins or condensed tannins which are flavonoid-based polymers, and hydrolyzable tannins which are polygalloyl esters (Loomis,1974; Hagerman, 1992).

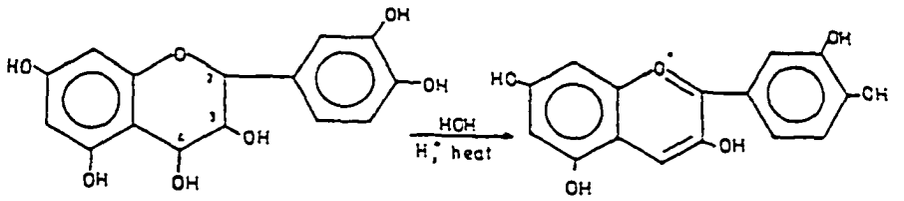
Proanthocyanidins or Condensed Tannins. The proanthocyanidins are a structurally diverse group having several variations in the pattern of hydroxylation on the A

and B rings of the flavanoid monomers (Hagerman, 1992). The most common proanthocyanidins are the procyanidins which are hydroxylated on carbons 5 and 7 of the A ring and on carbons 3' and 4' on the B ring. Thus, proanthocyanidins are mainly the polymerized products of flavan-3-ols and flavan-3,4-diols, or a mixture of the two (Fig. 3).

Flavan-3-ols are often referred to as catechins (Salunkhe et al., 1990). Since catechin molecules possess two asymmetric carbon atoms at C-2 and C-3, four isomers exist. They are (+) and (-) catechin in which the two phenyl and 3-hydroxyl groups are *trans*, and (+) and (-) epicatechin, in which these two groups are *cis*. Only (+) catechin and (-) epictechin are common in nature. Flavan-3,4-diols are called leucoanthocyanins because upon heating in acid solution, they not only polymerize to phlobaphene-like products, but also produce anthocyanidin (Fig. 3A). A flavan-3,4-diol molecule possesses three asymmetric carbon atoms, C-2, C-3 and C-4 therefore the occurence of eight isomers is possible. The heterocyclic ring of the flavanoid has three chiral centers giving rise to many possible stereoisomers (Hagerman, 1992).

The proanthocyanidins are not hydrolyzed in acid or base (Hagerman, 1992), but in hot alcohol, the flavanoid polymer is oxidatively cleaved to yield colored anthocyanidins. anthocyanidins. The dimeric forms are first produced, then trimers, tetramers and higher oligomers.The dimeric forms are first produced, then trimers, tetramers and higher oligomers.The degree of polymerization of procyanidins appears to be characteristic of a specific plant (Salunkhe et al., 1990). Any plant produces a range of procyanidins of differing molecular weights but only those flavan-3-ols with molecular weights of up to 3000 Da are soluble. Tannins are thought to be located in the vacuoles of intact plant cells. Flavan-3-ols with higher molecular weights
are either insoluble or attached in some way to structural tissues of plants (Salunkhe et al., 1990). The principal tannins present in sorghum, grain legumes, wine, several fruits, and forage plants are proanthocyanidins or condensed tannins.

Hydrolyzable Tannins. Hydrolyzable tannins (HT) are hydrolyzed in acid, base or by an esterase (tannase) to yield the parent polyol and the phenolic acids (Hagerman, 1992;



Leucocyanidin
(Flavan 3,4-diol)

Cyanidin

A

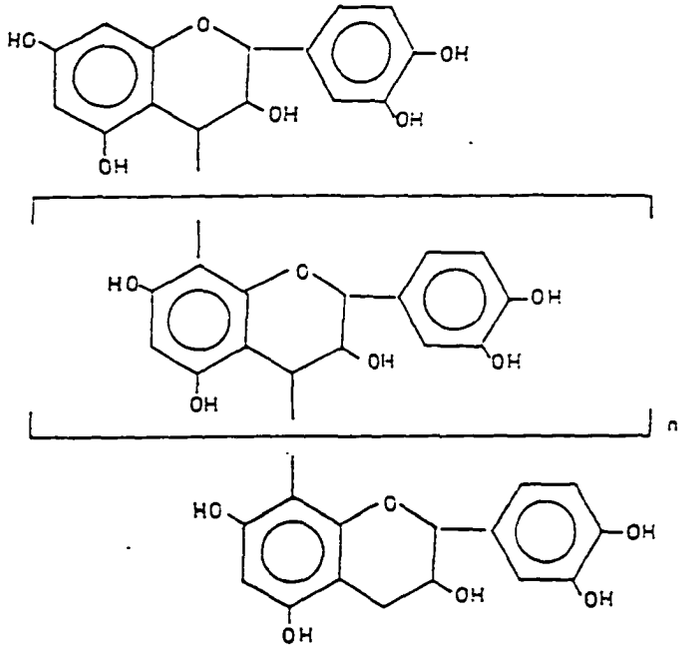


Fig. 3. Condensed tannin

Deshpande et al., 1986). Glucose is the most common alcohol. HT (Fig. 4) can further be subdivided into gallotannins and ellagitannins. If gallic acid is the only phenolic acid produced upon hydrolysis, the tannin is a gallotannin (Hagerman, 1992; Deshpande et al., 1986; Salunkhe, et al., 1990). The gallic acid can be esterified directly to a monosaccharide, or can be esterified to other gallic acid residues to form depside chains, which can be up to five gallic acid residues long (Hagerman,1992). Ellagitannins are hydrolyzed to hexahydroxydiphenic acid (HHDP) and the free acid spontaneously lactonizes to ellagic acid. HHDP is thought to form by oxidative coupling of adjacent galloyl residues on gallotannins (Salunkhe, et al., 1990).

Tannins are found in various plant tissues utilized as human food or animal feed (Makkar, 1989; Salunkhe et al., 1990). Tannins are widely distributed and their presence in plant tissue has several important consequences. The extracts of bark which have for a long time been used to convert animal skins into leather are rich in tannins. The interactions of tannins with salivary proteins and glycoproteins in the mouth makes plant material astringent to the taste and gives fruits such as blackberry, strawberry, cranberry and apple their improved acceptability (Salunkhe et al., 1990). Due to their protein binding property, tannins are of considerable importance in food processing e.g. tea processing, fruit ripening and wine manufacturing. Tannins have also been investigated for their role in herbivory, their association with lower nutritive value and lower bioavailability of macromolecules like proteins (Swain, 1979), control of bloat and improved protein utilization in ruminants (Broadhurst and Jones, 1978). Recently, tannins have generated interest as anti-cancer agents.

Tannins have been found in a variety of plants used for human food and animal feed. In food grains, tannins have been reported in sorghum, millets, barley, dry beans, faba beans, peas, cowpeas, pigeon peas, winged beans and bambara groundnuts. Tannins have been found in fruits such as apples, bananas, blackberries, cranberries, dates, grapes, peaches, pears, plums and strawberries. Processed foods and drinks such as wine and tea contain appreciable amounts of plant phenolic compounds. Higher animals cannot synthesize

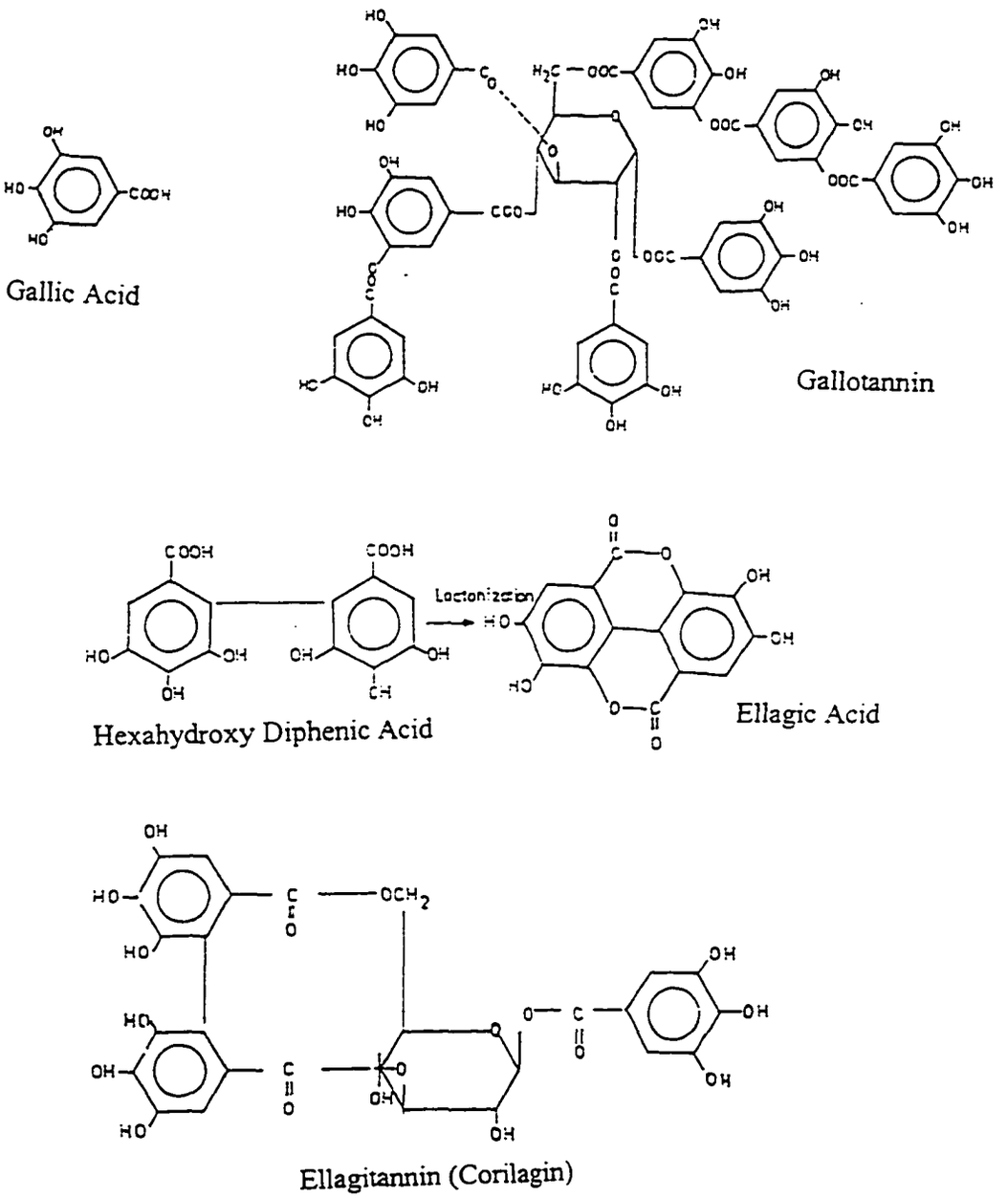


Fig.4. Hydrolyzable tannins

compounds with the benzenoid rings from aliphatic precursors, except estrone and related phenolic steroids (Salunkhe et al., 1990). Phenols that are essential for animals (catechol amines, tocopherols, thyroxine, tyrosine) are drawn directly or indirectly from plants (Salunkhe et al., 1990).

Tannins in plant food material amounts to about 5 - 50% of the material's dry weight (Singleton, 1981). The pigmented varieties of cereals and legumes contain 2 - 4% proanthocyanidins, although values of up to 7 -8% have been reported for some high tannin sorghum varieties. Foods that contain considerable amounts of proanthocyanidins include cider, cocoa, and red wines of which strong preparations may have as much as 1g tannin per liter (Salunkhe et al., 1990).

Relatively few studies of the nature of legume tannins have been reported. Legume tannins that have been fairly well studied are from *Phaseolus* beans, faba beans and peas (Salunkhe et al., 1990).

Tannin-Protein Interactions. Tannins are recognized by their ability to precipitate proteins from aqueous solution (Hagerman, 1992; Salunkhe et al., 1990). Lower molecular weight phenolic compounds, including phenolic acids and simple flavonoids, may bind proteins but cannot cross-link the complexes as is required for precipitation (Hagerman, 1992; Salunkhe et al., 1990), and those of higher molecular weight are ineffective because they are too large (Salunkhe et al., 1990). Hence, polymeric phenolic compounds that are capable of precipitating proteins constitute only a proportion of the total phenols in a plant tissue. Lignin, a highly methoxylated phenolic polymer does not precipitate proteins and is not a tannin.

Tannin-protein interactions are both tannin-specific as well as protein-specific (Asquith and Butler,1985) and they are also influenced by conditions of the reactions such as pH, temperature, solvent composition, time (Hagerman, 1992), reaction time and relative concentration of reactants (Hagerman and Butler, 1980). Protein and tannin can interact in four ways: hydrogen bonding, hydrophobic, ionic and covalent (Hagerman, 1992). Usually, only hydrogen bonding and hydrophobic interactions are involved in the formation of tannin-protein complexes (Hagerman, 1992). The phenolic hydroxyl group is a hydrogen bond donor and forms strong hydrogen bonds with the amide carbonyl of the peptide backbone

(Hagerman, 1992). At high pH values, the phenolic hydroxyl group is ionized and tannins do not interact with proteins (Hagerman and Butler, 1978). Neither ionic nor covalent bonds are important in stabilizing tannin-protein complexes (Hagerman, 1992).

Tannins are able to bind simultaneously to different points on the protein and also to cross-link separate protein molecules to form complexes which may be either soluble or insoluble (Hagerman,1992). Insoluble complexes are formed at pH values near the isoelectric point of the protein where electrostatic repulsions that prevent protein aggregation are minimized (Hagerman and Butler, 1978), and in the presence of excess tannin (Hagerman, 1992). In the presence of excess protein, the tannin-protein complexes remain soluble because each protein molecule is bound by only a few phenolic ligands.

The minimum and maximum molecular weight or length of a polymer and the nature of the three-dimensional structure of the tannins required to form stable complexes with proteins having different molecular weights, amino acid composition, tertiary or quaternary structures and solubility may vary (Salunkhe, et al., 1990). A minimum molecular weight of 350 is required for effective protein precipitation (Singleton, 1981) so with proanthocyanidins, the minimum would be the dimers and with hydrolyzable tannins, the minimum would be two gallic acids or one ellagic acid unit (Salunkhe, et al., 1990). The reactivity of tannins is influenced by the molecular size or molecular weight, molecular configuration and the number of reactive groups on the tannin. The removal of a tannin molecule from a tannin complex requires the simultaneous breaking of all effective bonds between the phenolic molecule and the substrate. As the number of linkages increases, the number of reactive groups on the tannin also increases (Salunkhe, et al., 1990). The increased reactivity also indicates a corresponding increase in spatial compatibility between pairs of active sites on the adjacent tannin and protein surfaces.

The interaction between tannin and protein is influenced by the number of phenolic groups available for interaction, the arrangement of the groups that are available, size of the tannin, structure and the heterogeniety of the preparation (Hagerman, 1992). The affinity of a tannin for protein increases as the degree of polymerization of the tannin increases. The stereochemistry and pattern of hydroxylation on either the A or the B rings of the condensed tannin influence the interaction with protein (Porter and Woodruffe, 1984). Thus, the

prodelphinidins, with three *o*-hydroxy groups on the B ring, have a higher affinity than procyanidins, which have only two *o*-hydroxy groups on the B ring (Hagerman, 1992).

Tannins are specific protein precipitating agents (Hagerman, 1992). The affinity of tannin for protein is a function of the amino acid composition (primary structure) and the flexibility of the protein. Protein and polypeptides rich in proline have a high affinity for tannin (Hagerman and Butler, 1981). Protein secondary and tertiary structure influence the interaction with tannin because proline obstructs the formation of secondary structures (α-helixes) such that the resulting random coil structures have peptide backbones which are accessible to tannin. Proteins with compact globular structures like ribonuclease, lysozyme and cytochrome C, have low affinity for tannin because the peptide backbone is inaccessible (Hagerman and Butler, 1981).

Molecular weight of the protein and post translational processing may also determines the affinity of tannin for protein (Hagerman,1992). Larger proteins and polymers have higher affinity for tannin than do smaller polymers, however, small proline-rich polymers are preferred over large proline-poor polymers (Hagerman and Butler, 1981).

Hydrogen bonds and hydrophobic interactions my be the principal linkages involved in the formation of tannin-protein complexes (Salunkhe, et al., 1990). Tannins bind with proteins primarily through the formation of multiple hydrogen bonds between the phenolic hydroxy groups of tannins and the carbonyl functions of the peptide linkages of proteins.

Hydrophobic interactions are predominant in the formation of tannin-protein complexes. The strength of hydrogen bonds decreases with an increase in ionic strength and temperature, and the reverse is true for hydrophobic interactions. Butler et al. (1984) carried out their experiments on sorghum tannins and concluded that the interactions between sorghum tannins and proteins involve both hydrogen bonding and nonpolar hydrophobic association and that the binding process is highly cooperative and involves multiple interactions.

Hagerman and Butler, (1981) studied the specificity of binding of proteins by purified sorghum tannins and found that the relative affinities of different proteins to tannins vary by as much as four orders of magnitude. They suggested the following characteristics that maximize the opportunity for forming multiple hydrogen bonds between tannin molecules

and the peptide backbone of the proteins for nonpolar interactions: generally, proteins that bind sorghum tannins strongly are relatively large, have a loose, open structure, and are rich in proline. Although proanthocyanidins are similar, each seems to be uniquely suited to bind tightly to a limited number of proteins (Salunkhe, et al., 1990) thus the interactions of tannins with proteins should be evaluated individually.

Salivary proline-rich proteins have a high affinity for tannins and the carbohydrate moiety of glycoproteins plays an important role in the affinity.

Nutritional Consequences of Dietary Tannins

The effect of plant phenolics on different species varies (Sakunkhe et al., 1990). Certain types of phenols are known to be quite toxic to some species while having little or no effect on others. Carnivores have the least exposure to plant phenols and these compounds seem to have a marked toxicity in strict carnivores and insectivores, less in omnivores, and least in strict herbivores. However, owing to efficient detoxification, common dietary plant phenols are not considered toxicants under normal amounts and conditions (Sakunkhe et al., 1990). Proanthocyanidins and hydrolyzable tannins are the major classes of tannins found in the plant kingdom. However, hydrolyzable tannins are rare in common plant foods, while proanthocyanidins are the predominant class of polyphenols in fruits, forages, cereals and legumes (Sakunkhe et al., 1990).

Reports on the nutritional consequences of dietary tannins are mostly based on either *in vitro* studies or experiments with nonruminants. Most of the data on tannin toxicity and the physiological effects of tannins have been obtained from mixtures that contain components varying widely in molecular weight and isomerism (Sakunkhe et al., 1990). The unavailability of purified and well characterized pure proanthocyanidins has led many researchers to use available commercial preparations of tannic acid, which is a mixture of hydrolyzable gallotannins (Salunkhe et al., 1990). The biological effects of hydrolyzable tannins and proanthocyanidins vary widely, and because hydrolyzable tannins are not common in food, reports on tannin toxicity based on the use of tannic acid (a hydrolyzable tannin) are of limited use. Reports on tannin toxicity that are based on the use of tannin

extracted from seed hulls in the diet are more important in assessing the nutritional significance of dietary tannins.

Tannins have been shown to have effects on food and feed intake, growth, feed efficiency, digestibility, inhibition of digestive enzymes, increased excretion of endogeneous protein, effects on the digestive tract and on vitamin and mineral uptake (Salunkhe, et al., 1990).

Direct absorption of tannins in healthy animals seems unlikely due to membrane barriers, however, the chronic ingestion of large amounts of tannins can damage the gastrointestinal surface such that tannins might be absorbed, producing possible harmful effects (Reddy et al., 1985). The acute toxicity of orally administered tannin is low, but it increases significantly when tannins are administered perenterally. The LD_{50} for rats, mice and rabbits after a single dose of tannic acid was given orally ranged from 2.25 to 6.00 g/kg of body weight (Singleton and Kratzer, 1969). The LD_{50} for subcutaneous or intraperitoneal administration of proanthocyanidins from different sources range from 70-300 mg/kg body weight (Reddy et al., 1985).

The monomers of condensed and hydrolyzable tannins do not precipitate tannins hence may not produce toxic effects similar to dimeric and polymeric tannins. Due to their lower molecular weights, breakdown products of tannins could be absorbed across the membrane, and may exert more toxic effects than the monomers (Salunkhe, et al., 1990). There are no known reports of tannin toxicity in humans due to consumption of large amounts of legume tannins.

Although tannins appear to protect proteins from microbial attack and prevent bloating in the rumen, their ultimate effects on ruminant nutrition may depend upon the availability of bypass protein to the animal (Salunkhe, et al., 1990). The same factors that cause tannins to have a deleterious effect on monogastric nutrition will presumably be important in the postrumen digestive tract (Price and Butler, 1980). Thus, if the tannin-protein complex does not dissociate in the abomasum or intestine, there will be no benefit to the animal from the protein having been protected in the rumen; if it dissociates, the liberated tannin may damage the intestinal tract or form new complexes at some point with endogenous proteins (Salunkhe, et al., 1990).

Proteinase Inhibitors

Proteinase inhibitors are proteins that inhibit the activities of proteinases and are quite specific in their interactions with proteolytic enzymes (Nielsen, 1985). Proteinase inhibitors are ubiquitous in nature, being found in microorganisms, plants and animals (Filho and Compos, 1989) with each individual tissue being capable of having several different kinds of inhibitors present (Birk, 1989). Proteinase inhibitors are found in almost all plant parts but are generally concentrated in storage organs like seeds and tubers (Ryan, 1973). In legumes seeds, proteinase inhibitors are found in both the cytosol and protein bodies (Wilson, 1981). Proteinases (enzymes) are widely distributed in the plant kingdom. They are found in seeds, fruits, tubers, roots, bulbs, leaves, or stems of dicotyledonous and monocotyledonous plants (Filho and Compos, 1989).

Interest in plant proteinase inhibitors arose when it was reported that raw soybean extract was found to inhibit growth of rats. Raw soybean extract was subsequently found to contain a trypsin inhibitor which was linked to the antinutritional effect of soybeans. Numerous studies with proteinase inhibitors have been carried out since then and a number of generalizations have been made about these inhibitors. Many proteinase inhibitors are resistant to heat, extreme pH and hydrolysis by many proteolytic enzymes (Laskowski and Kato, 1980). Their resistance is attributed to their compact three-dimensional structure which is stabilized by disulfide bonds. Generally, plant proteinase inhibitors have a high content of half-cystine residues and a low percentage of methionine, histidine and tryptophan. They are rich in aspartate and glutamate, the acidic amino acids and serine and lysine residues and can have either acidic or basic isoelectric points (Laskowski and Kato, 1980). Their molecular weights range from 3000 to 80,000 Da. The inhibitors associate with the enzymes they inhibit strongly with a $K_a=10^7 - 10^{14}$ M^{-1} at neutral pH (Norton, 1991).

Proteinases are divided into 4 mechanistic classes (Hartley, 1960) depending on the chemical nature of the groups involved directly in catalysis:

a) Sulphydryl (Thiol) Proteinase (EC 3.4.22) - papain, ficin, bromelain.

b) Acid (Carboxyl) Proteinase (EC 3.4.28) - pepsin, rennin.

c) Metallo Proteinase (EC 3.4.34) - carboxypeptidases, aminopeptidases.

d) Serine Proteinase (EC 3.4.21) - chymotrypsin, trypsin, elastase, thrombin.

Proteinase inhibitors of the above proteinases recognize the active sites and are also grouped into four corresponding classes (Norton, 1991). Individual reactive sites on the inhibitor can inhibit only proteinases from one of the four mechanistic classes. Inhibition of proteinases by proteinase inhibitors is strictly competitive forming a complex that has no enzymatic activity.

Mechanism of Action of Proteinase Inhibitors

The mechanism of action of most proteinase inhibitors with the proteinases (enzymes) they inhibit is known as the 'standard mechanism' (Laskowski and Kato, 1980; Norton, 1991). Evidence for the standard mechanism is based on studies of serine proteinases particularly soybean trypsin inhibitor and trypsin (Laskowski and Kato, 1980). The reactive site peptide bond on the inhibitor reacts specifically with the catalytic site of the enzyme leading to modification of the reactive site peptide bond. The difference between most inhibitors and most substrates is that the reactive sites of the inhibitor are held within disulfide bridges so that after hydrolysis at the reactive site, the modified inhibitor is still held together without a change in conformation (Birk, 1989). The stable complex formed between the enzyme and the unmodified inhibitor is similar to that formed between the enzyme and modified inhibitor indicating that both are strong inhibitors of the enzyme. Laskowski and Kato (1980) proposed the following overall mechanism of the enzyme-inhibitor interaction:

$$E + I \leftrightarrow L \leftrightarrow C \leftrightarrow X \leftrightarrow L^* \leftrightarrow E + I^*$$

where E is the enzyme, I and I* are unmodified and modified inhibitor respectively, L and L* are loose, non covalent, rapidly dissociable complexes of the enzyme with I and I* respectively. C is stable enzyme-inhibitor complex and X is a relatively long-lived intermediate in the E + I* reaction.

Serine Proteinase Inhibitors

The strength and specificity of the enzyme-inhibitor interaction is strongly affected by the specific nature of the proteinase inhibitor and the species specificity of the inhibited enzyme (Birk, 1989). Thus, strong inhibitors of bovine trypsin are not necessarily inhibitors of human trypsin. Serine proteinase inhibitors are the most widely studied of the four classes and they follow the standard mechanism of Laskowski and Kato, (1980). Each inhibitor molecule surface has at least one peptide bond, the reactive site. Multiheaded inhibitors have more than one reactive site. The inhibitor binds to proteinase in a similar way a normal substrate would, except that over normal times and at physiological conditions, products are not yielded.

Laskowski and Kato (1980) classified serine proteinase inhibitors into 8 families based on extensive homology among its members and topological relationships between the disulfide bridges and the location of the active site. Subsequent studies led to the addition of two more inhibitor families (Norton, 1991). Table 1 gives a list of the inhibitor families.

Of all the serine proteinase inhibitors studied, the most well characterized are those inhibitors from soybeans which fall into the Kunitz family and the Bowman-Birk family, which were named after the persons who pioneered research on these inhibitors.

Table 1. Families of serine proteinase inhibitors

Bovine pancreatic trypsin inhibitor (Kunitz) family
Pancreatic secretary trypsin inhibitor (Kazal) family
Streptomyces subtilisin inhibitor family
Soybean trypsin inhibitor family
Soybean proteinase inhibitor (Bowman-Birk) family
Potato I inhibitor family
Potato II inhibitor family
Ascaris trypsin inhibitor family
Barley trypsin inhibitor family
Squash inhibitor family
Other families

From Laskowski and Kato, 1980

Kunitz Family. Those proteinase inhibitors that have a molecular weight approximating 20,000 Da, with two disulfide bridges and have a specificity for trypsin belong to this family. The Kunitz inhibitor consists of 181 amino acid residues and the reactive site is located at residues Arg63 and Ile64. The molecule contains only four half-cystine residues which form the two disulfide bonds in the native protein. Only one of the disulfide bonds is essential for activity. When both disulfide bonds are reduced, the molecule loses its activity (Nielsen, 1985). One molecule of inhibitor inactivates one molecule of trypsin in a reaction that is stoichiometric. The inhibitor binds tightly to trypsin such that the inhibitor-trypsin complex does not readily dissociate into products and enzyme (Liener, 1994).

Bowman-Birk Family. This family of inhibitors consists of those that have a molecular weight ranging between 6000-10,000 Da with a high proportion of disulfide bonds and are able to inhibit both chymotrypsin and trypsin at independent binding sites (Liener, 1994). Bowman-Birk inhibitors consist of a single polypeptide chain made up of approximately 60-80 amino acid residues. The Bowman-Birk inhibitor has two independent binding sites; a trypsin-reactive site at Lys16 and Ser17 and a chymotrypsin-reactive site at Leu43 and Ser44. Bowman-Birk inhibitors are rich in disulfide bonds (7 disulfide bridges) which give the molecule a very tight, compact three-dimensional structure (Liener, 1994). A high degree of homology has been found between the Bowman-Birk inhibitor and other low molecular weight inhibitors from other legumes (Liener, 1994). Bowman-Birk inhibitors also exhibit internal homology (Wilson, 1981) with the first half of the inhibitor being homologous to the second part. Bowman-Birk inhibitors usually have no tryptophan and low levels of methionine, valine and the aromatic amino acids, phenylanlanine and tyrosine. Some characteristics of the Bowman-Birk inhibitor family include:

a) Multiple forms of the inhibitor: Within a given plant species, distinct isoinhibitors can be present (Table 2). The multiplicity of inhibitors arises from postsynthetic modification or from different gene products either by gene duplication or elongation (Wilson, 1981).
b) Double headedness: Bowman-Birk inhibitors have been shown to react simultaneously and independently with two proteinase molecules (Wilson, 1981). The formation of a complex at one site does not affect complex formation at the second site. Common

Table 2. Isoinhibitors present in some common legumes

Legume	Number of Isoinhibitors
Adzuki bean (*Phaseolus angularis*)	2+
Lima bean (*P. lunatus*)	6
Garden bean (*P. vulgaris*)	3+
Mung bean (*Vigna radiata*)	2
Soy bean (*Glycine max*)	4
Chick pea (*Cicer arietinum*)	6
Peanut (*Arachis hypogaea*)	1
Black-eyed pea (*Vigna sinensis*)	2

From Wilson, 1981

reactive site pairs studied include trypsin-chymotrypsin, trypsin-trypsin and trypsin-elastase (Wilson, 1981).

Physiological Role of Proteinase Inhibitors

The physiological role of plant proteinase inhibitors has yet to be determined conclusively, since only a few inhibitors are known to inhibit the endogeneous proteinases of seeds (Birk, 1989). Proteinase inhibitors in plants are involved in several functions: they regulate endogenous proteinases and protect plant tissues from unwanted proteolysis. In some cases the inhibitors act as protein stores since they are rich in amino acids which can be mobilized for the synthesis of new proteins. In some plants like soybeans and seeds, the presence of proteinase inhibitors offers the plant protection from insects and their larvae whose gut proteinases are inhibited when they feed on the seeds leading to starvation (Ryan, 1973; Birk, 1989). Ryan (1973) also showed that when potato or tomato leaves are wounded either mechanically or by insects, there is an accumulation of proteinase inhibitors in the leaves of the plant indicating the presence of a defensive mechanism.

Proteinase inhibitors are known to depress the growth of small animals by negative feedback mechanism where enzyme secretion is inversely related to the level of trypsin

present in the small intestine (Liener, 1994). Trypsin inhibitors in the diet inactivate trypsin in the gut thereby inducing the intestinal mucosa to release cholecystokinin (CCK). CCK is a hormone that stimulates the acinar cells of the pancrease to produce more trypsin with a concomitant increase in the secretion of chymotrypsin, elastase and amylase. Subsequently, the pancreas becomes enlarged due to hypertrophy (increase in the size of the acinar cells of the pancreas) and hyperplasia (increase in the number of acinar cells). Thus the depressed growth caused by the inhibitors is due to an endogenous loss of protein rich in sulfur containing amino acids by a hyperactive pancreas. Protease inhibitors have been implicated in the induction of pancreatic cancer in rats.

Conversely, Kennedy (1994) provided evidence that the soybean Bowman-Birk inhibitor can suppress carcinogenesis in animal models. The suppressive effect was evident with different types of carcinogens, species, tissues and organs, routes of administration, types of tumors and cell types.

Properly processed soybean products in which TI have been destroyed provide a nutritious source of proteins (Liener, 1994). There is a need to balance the amount of heat necessary to destroy all of the TI and that which results in damage to the nutritional or functional properties of the protein. Thus most commercially available edible-grade soybean products retain 5-20% of the TI activity originally present in the raw soybeans (Rakis and Gumbmann, 1981). Most of the information on the nutritional effects of TI are derived from short-term experiments using animal models. Short-term experiments in humans involving the use of high levels of TI have shown that the soybean TI can adversely affect the normal human pancreatic function (Liener, 1994). Most humans are exposed to low levels of TI over an extended period; therefore, studies on the chronic ingestion of low levels of TI are more relevant.

Methods of Detoxification

Processing. The destruction of TI by heat is a function of temperature, duration of heating, particle size and moisture conditions (Liener, 1994). Most commercially available soybean products intended for human consumption have a residual TI activity of 20% of the original raw beans. Methods of heat treatment used include toasting (live steam), boiling in

water, dry roasting, dielectric heating, microwave irradiation, extrusion cooking, gamma irradiation and infrared irradiation. Other processing techniques include direct steam infusion, immersion boiling prior to extraction, treatment with 10% $Ca(OH)_2$ at 80°C for 1 hour (preparation of Mexican tortillas).

Germination has been reported to improve the nutritive value of the proteins. However, this effect appears to be unrelated to the level of TI in the germinated bean (Liener, 1994). The germination of the soybean increased the *in vivo* digestibility of the protein implying either a decrease in TI activity and/or a greater susceptibility to enzymatic attack of the degraded protein formed during germination.

Chemical Treatment. Various chemicals in combination with heat treatment have been shown to inactivate TI at lower temperatures (Liener, 1994). Thiol-containing compounds (cysteine, N-acetyl-cysteine, glutathione) and sodium sulfite or sodium metasulfite cleave disulfide bonds and reduce the temperature necessary to inactivate the TI in soybean flour. The disulfide bonds of the TI interact through the formation of mixed disulfides, the reduction of methionine sulfoxide to methionine and the introduction of new half-cystine residues (Liener, 1994).

Other. Methods of preparation of soybean dishes (e.g. soymilk and tofu which involve soaking, cooking and precipitation) lead to dishes with low TI activity (Liener, 1994). Tempeh, miso and natto are fermented soybean dishes which are low in TI activity and have a higher nutritive value than the raw soy beans.

Thermal Inactivation of Bambara Groundnut Trypsin Inhibitor

During soymilk production, thermal inactivation of trypsin inhibitor can be carried out by several processes, blanching the whole beans before grinding with water, cooking the soy flour/water slurry or by heating the soymilk after filtration (Kwok and Niranjan, 1995). Inactivation of the trypsin inhibitor can be done either by heat alone or by a combination of heat and chemicals.

Complete destruction of trypsin inhibitor in soymilk can only be achieved at the expense of nutrient destruction. Consequently, optimum conditions must be determined that maximize retention of nutrients and other quality attributes like color, flavor and texture, while inactivating deleterious enzymes, microbes and antinutrients. Hackler *et al.* (1965) determined that 90% of trypsin inhibitor reduction in soymilk results in maximum nutrient retention. The residual trypsin inhibitor activity in soymilk is thought to be due to the Bowman-Birk inhibitor (BBI) which is more heat stable than the Kunitz inhibitor (KSTI). The adequacy of heat treatment is dependent on several factors (a) pH of the aqueous extract, (b) heating temperature and (c) holding time.

Previous studies (Kunitz, 1947; Obara and Watanabe, 1971) have shown that trypsin inhibitors are unstable under alkaline conditions. Wallace *et al.* (1971) found that as pH increased, there was an increased rate of trypsin inhibitor inactivation when soymilk was heated at 98°C. As pH increased from 6.8 to 9.9, under the same conditions, the time for 100% destruction of typsin inhibitor activity destruction decreased by 86%. Disulfide bonds of trypsin inhibitors are important for their stability (Birk, 1968; Dibella and Liener, 1969) thus their hydrolysis under alkaline conditions in the presence of heat will destabilize the trypsin inhibitor molecule and make it more heat labile (Kwok and Niranjan, 1995).

When soymilk is heated below 100°C, the time taken to inactivate 90% of the original activity is long leading to problems with amino acid degradation and protein insolubilization (Kwok and Niranjan, 1995). Higher temperatures (>100 °C) and therefore shorter heating times are desirable. Dipietro and Liener (1989) investigated the effect of heat treatment on thermal inactivation of soy bean proteinase inhibitors (SBPI) in aqueous extracts of raw soy flour, pure KSTI and pure BBI. They found that SBPI in the extract lost their inhibitory activity faster than the pure inhibitors possibly due to the interaction of the inhibitors with other components of the extract, e.g. free thiol groups available for disulfide bond interchange and protein-protein interactions, which lead to denaturation and precipitation and, therefore, loss of inhibitory activity.

Several studies to evaluate the effects of high temperature and short time cooking (HTST) and ultra high temperature (UHT) on trypsin inhibitor inactivation have been carried out. Johnson *et al.* (1980a) used HTST (121-154°C/ 0-240 s) to determine heat inactivation

of trypsin inhibitor in soymilk processed by direct steam infusion. HTST heat inactivation of trypsin inhibitor did not follow first-order reaction kinetics. When log trypsin inhibitor was plotted over time, the reaction kinetics were not first-order, but rather curvilinear with two distinct linear portion. The first linear portions had a large slope and was attributed to the KSTI which is more heat labile, while the latter part of the linear curve had a smaller slope and was attributed to the BBI which is more heat stable.

The effect of UHT at pH 2, 6.5 and 7.5 was studied by Kwok *et al.* (1993). At high holding temperatures (143 and 154°C), pH of the milk did not have an effect on rate of thermal inactivation of trypsin inhibitor. The UHT heat treatment inactivation of trypsin inhibitor in soymilk at pH 6.5 and 7.5 did not follow first-order reaction kinetics. However, at 93°C, the kinetics of trypsin inhibitor inactivation appeared to be first order.

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PURIFICATION AND CHARACTERIZATION OF THE PROANTHOCYANIDINS FROM BAMBARA GROUNDNUT (*Vigna subterranea* (L.) Verdc.) SEED HULLS

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ABSTRACT

A proanthocyanidin (PA) has been purified and characterized from the hulls of the bambara groundnut seed (Vigna subterranea (L.) Verdc.). Extraction of the hulls with aqueous acetone followed by chromatography on Sephadex LH-20 resulted in a pure proanthocyanidin with a degree of polymerization of six. Spectral analysis and ^{13}C NMR determined that the PA was probably a dihydroflavanol. The purified PA was used as the standard in subsequent tannin assays. Eight different bambara groundnut seed samples were assayed for tannin using four different tannin assays; total phenol, vanillin, butanol and protein precipitation. All four assays were able to distinguish between the high and low tannin contents.

INTRODUCTION

The bambara groundnut (*Vigna subterranea* (L.) Verdc.) is an indigenous African crop that has been cultivated for centuries in tropical regions south of the Sahara (Linneman and Azam-Ali, 1992). Though it is mostly produced in West Africa it is widely grown in Eastern and Southern Africa, and Madagascar (Williams 1992). The bambara groundnut is predominantly grown for its seeds for human consumption. Seeds can be consumed either immature or mature. When immature, the seeds can either be shelled or unshelled. Mature seeds are hard and have to be shelled and cooked before consumption. Bambara groundnut seeds are variable in color: white, cream, yellow, red, purple, brown, or black; the coloration can be uniform, mottled, blotched, or striped (Linneman and Azam-Ali, 1992).

The nutritional value of legume seeds is generally poor unless the seeds are processed by soaking, dehulling and cooking. The poor nutritional value is attributed to the presence of antinutrients such as phytates, trypsin inhibitors and tannins. Tannins are plant polyphenolic compounds that are capable of binding proteins and interfering with protein digestibility (Liener, 1983) In legume seeds, tannins are concentrated in the seed coats where they contribute to seed coat color. Tannins can either be condensed or hydrolyzable. Most of the tannins identified in legume seeds are condensed tannins (Salunkhe, et al., 1990).

Few studies have been done on the bambara groundnut tannins. Poulter (1981) investigated bambara groundnut tannins in eight accessions and found that the quantities ranged from 0.36 to 0.94 g/100 g seeds. The objectives of this study were (1); to extract, purify and partially characterize proanthocyanidins from bambara groundnuts for subsequent use as a standard; (2) to determine the biological activity of the proanthocyanidin and to assay for tannin in eight bambara groundnut samples. This study is the first research to characterize the tannins in bambara groundnuts.

MATERIALS AND METHODS

Materials. Eight bambara groundnut seed samples were used in this study. Of these, six samples were originally from Malawi, Uganda and Zimbabwe and were kindly provided by Dr. Gillespie, Regional Plant Introduction station, Griffin, GA. Two other bambara groundnut samples were obtained from Kenya.

Extraction and Purification of Proanthocyanidin (PA). Extraction of the proanthocyanidins was accomplished by using the procedure of Hagerman and Butler (1980) as modified by Hussein, Fattah and Salem (1990). Bambara groundnut seeds were manually dehulled and the hulls were ground in a Thomas Wiley Sci.entific electric mill through a size 40-mesh. Ground seed hull (4.0 g) were extracted for 2 hr with 15 mL ethanol containing 2.8 mM/L ascorbic acid. The ethanol extract containing negligible amount of tannin was discarded, and the residue was extracted 3 times for 2 hr each time using 70% aqueous acetone containing 2.8 mM/L ascorbic acid. The acetone extracts were combined and extracted with an equal volume of 1 mM acetate buffer at pH 4 and the acetone was removed

by vacuum evaporation. The remaining aqueous solution was extracted 2 times with equal volumes of ethyl acetate to remove small molecular weight contaminants. The ethyl acetate fraction which contained some amorphous solid material was discarded. The aqueous phase was evaporated to dryness under reduced pressure and redissolved in a minimum volume of 80:20 ethanol-water (v/v) and mixed with Sephadex LH-20/ethanol slurry (2g/10 mL ethanol) for 30 minutes. The gel was repeatedly washed with ethanol to remove UV-absorbing contaminants until the absorbance at 280 nm reached a constant minimum value. The partially purified PA was eluted with 70% aqueous acetone washes until the absorbance at 540 nm reached a constant minimum value. The 70% aqueous acetone fraction containing the PA was evaporated *in vacuo* to remove acetone and subsequently extracted 3 times with an equal volume of liquefied phenol to dissociate the protein-PA complex. Residual phenol was removed by washing the aqueous phase with a small amount of diethyl ether. The aqueous phase was evaporated to dryness and redissolved in a minimum volume of ethanol (120mg/mL). A 5.0 mL aliquot was applied to the top of a Sephadex LH-20 column (2.5 x 36 cm) packed and equilibrated with ethanol and the column exhaustively washed with ethanol. The absorbance at 280 nm was monitored to a stable baseline after which the mobile phase was changed to 70% aqueous acetone at a flow rate of 72 mL/hr. Eluates were monitored at 400 nm and 540 nm and 5.0 mL fractions collected pooled, lyophilized and stored at 4°C.

UV Spectral Analysis. The purified PA from bambara groundnut was characterized by UV spectral analysis following the procedure of Mabry et al., (1970). The spectra were scanned on a UV-Visible spectrophotometer (HP 8452A Diode-Array, Hewlett-Packard Co.) set in general scanning mode between 200-400 nm. A stock solution (0.01 mg/mL) of the PA in methanol was prepared and the concentration adjusted so that the major peak gave an absorbance between 0.6 and 0.8. UV spectra were subsequently generated as follows:

1. The methanol spectrum was scanned using 2-3 mL of the stock solution,
2. The NaOMe spectrum was measured immediately after the addition of three drops of NaOMe stock solution to the solution used in # 1. After 5 min, the spectrum was rerun to check for decomposition and the sample discarded thereafter.

3. The $AlCl_3$ spectrum was measured immediately after the addition of six drops of $AlCl_3$ stock solution to 2-3 mL of fresh stock solution.
4. The $AlCl_3$/HCl spectrum was scanned immediately after the addition of three drops of stock HCl solution to the cuvette containing $AlCl_3$ from the preceeding step and the contents discarded thereafter.
5. The NaOAc spectrum was scanned after the addition, with shaking, of excess coarsely powdered, anhydrous reagent grade NaOAc to a cuvette containing 2-3 mL of fresh stock solution so that a 2 mm layer of NaOAc remained on the bottom of the cuvette. The spectrum was scanned within 2 min after the addition of NaOAc to the solution. A second spectrum was recorded after 5-10 min to check for decomposition.
6. The NaOAc/H_3BO_3 spectrum was determined as follows. Two procedures for obtaining NaOAc/H_3BO_3 spectra were used depending on whether or not decomposition was observed during the scanning of the NaOMe spectrum.
   Procedure I. Sufficient powdered anhydrous reagent grade H_3BO_3 to give a saturated solution was added with shaking to a cuvette (from # 5) containing NaOAc. The solution was discarded after the spectrum was recorded.
   Procedure II. Five drops of the H_3BO_3 stock solution was added to 2-3 ml of fresh stock solution of the flavonoid. The solution was then quickly saturated with coarsely powdered reagent grade NaOAc and the NaOAc/H_3BO_3 spectrum scanned.
   If no decomposition was observed when the NaOMe spectrum was rerun after 5 min, procedure I was employed; procedure II was employed if decomposition occurred in the presence of NaOMe.

The spectra obtained were used to provide information on shifts enabling a preliminary classification of bambara groundnut PA.

^{13}C NMR Spectroscopy. Purified PA (50mg) was dissolved in 1 mL hexadeuteroacetone ($(CD_3)_2CO$) and 0.7 mL of this solution was pipetted into an NMR tube. The tube was loaded into a Bruker AC-200D instrument, and the spectra measured at 50 MHz.

Extraction of Crude PA from Eight different Bambara groundnuts Cultivars. Crude PA extract was prepared from seeds of the eight bambara groundnut samples by the procedure of Hagerman and Butler (1978). Seeds were manually dehulled and the hulls ground to 40-mesh size. Ground sample (200 mg) was weighed into a screw-top test tube and defatted with 5 mL ethanol for 15 minutes. The ethanol extract was discarded and 10 mL methanol added to the tube. The grain was extracted for 15 minutes, centrifuged and the supernatant containing crude PA assayed within 8 hrs.

Total Phenols. To quantify total phenols from the crude bambara groundnut extracts, AOAC method 952.03 (1990) was used. Purified bambara groundnut PA was used as the standard. Water (3.75 mL) was pipetted into a test tube and 0.05 mL crude extract added. After mixing, 0.25 mL Folin-Denis reagent and 0.5 mL Na_2CO_3 was added and the mixture diluted to 5 mL with water and incubated for 30 minutes at room temperature. The absorbance at 760 nm was then determined and the mg PA/5 mL was calculated from the previously generated standard curve.

Vanillin Assay. To the quantify PA from crude extracts of bambara groundnut, the vanillin assay (Price *et al.*, 1978) was used. The test was carried out in a water bath set at 30°C. Vanillin reagent (5.0 mL) was added at 1 minute intervals to 1.0 mL crude extract. To another set of samples (background samples), 5.0 mL of 4% HCl solution was added. All the samples were incubated for 20 minutes at 30°C after which the absorbance at 500 nm was read. A blank sample was prepared by mixing 5.0 mL of 4% HCl in methanol and 1.0 mL aliquot of crude extract and incubated for 20 minutes at 500 nm before absorbance readings. The blank absorbance was subtracted from absorbance of samples with vanillin and the absorbance of each reaction tube was corrected for by the background absorbance. The PA of crude extract was determined from a standard curve using purified bambara groundnut PA.

Butanol Assay. Both purified bambara groundnut PA and crude extracts of bambara groundnut seeds were subjected to the butanol assay. The procedure of Porter et al. (1986) was used in which 6.0 mL of *n*-BuOH-conc HCL (95:5, v/v) and 0.2 mL of 2% (w/v) solution of ferric reagent was added to 1.0 mL pure PA extract in methanol in a screw-cap test tube. The mixture was suspended in a water bath at 95°C and heated for 40 minutes,

cooled and the the visible spectrum scanned between 500 and 600 nm to determine the wavelength of maximum absorption.

Degree of Polymerization. The degree of polymerization of bambara groundnut purified PA and crude extract was determined using catechin as the monomer (Butler *et al.*, 1982). The procedure is similar to the vanillin procedure except that glacial acetic acid is used instead of methanol. Determinations were made on equal weights of material.

Protein Binding/Precipitation Assay. To determine the biological activity of bambara groundnut tannin, the procedure of Asquith and Butler (1985) was followed. Methanol (1.0 mL) containing 0.1-0.7 mg PA was added to 4.0 mL of blue labeled BSA in acetate, pH 4.8 (solution A). The solution was mixed and allowed to stand at room temperature for 15 minutes after which it was centrifuged and the supernatant discarded. The precipitate was dissolved in 3.5 mL of solution B (1% (w/v) SDS, 5% (v/v) Triethanolamine, 20% (v/v) isopropanol) and the absorbance measured at 590 nm. A standard curve prepared using purified PA was used to calculate the quantity of tannin in the samples.

Relative Affinity. To compare the tannin-binding capacities of several proteins, the relative affinity of the tannin was determined using a competition assay (Asquith and Butler, 1985). Varying amounts of competitor (reagent grade trypsin, α-chymotrypsin, pepsin, BSA) were mixed with 1.0 mg of blue labeled BSA and made up to 1.6 mL with solution A (acetate, pH 4.8). Methanol (0.4 mL) containing enough tannin to precipitate 70-80% of the blue BSA (as determined in the absence of competitor) was added to the mixture and centrifuged. The supernatant was discarded and the pellet dissolved in 3.5 mL of solution B (1% (w/v) SDS, 5% (v/v) triethanolamine, 20% (v/v) isopropanol) and the absorbance measured at 590 nm.

Tannin Specific Activity. The tannin specific activity (Hagerman and Butler, 1980b) is a dimensionless ratio determined by dividing the amount of protein precipitated by tannin by the total phenols. Tannin specific activity was determined for all the samples.

Chromatography. *Thin-Layer Chromatography.* Two-dimensional thin-layer chromatography (TLC) was used to check the purity of the Sephadex LH-20 purified PA. TLC plates (20x20 cm) and 0.25 mm thickness were prepared using Kieselghur as the adsorbent and using a movable spreader (Stahl-type). For five 20x20 cm plates, 30 g kieselghur were slurried with 60 mL water, spread immediately, dried at room temperature

and activated by heating for 30 min at 110°C. Purified PA (5μg) was spotted onto the plates using a micro syringe and the chromatogram developed ascendingly in two dimensions. The first dimension was in the solvent butanol-acetic acid-water (BAW, 4:1:5) and the second dimension was in butanol-ethyl methyl ketone-acetic acid-water (BEAW, 2:5:1:2) solvent.

Paper Chromatography. One dimension paper chromatography of anthocyanidins was carried out on 3 MM whatman paper in an ascending manner. Anthocyanins (5 μL) were spotted and an ascending chromatogram developed in formic acid-conc. HCl-water (5:2:3).

All chromatograms were developed in rectangular glass tanks lined with filter paper using the 'chamber saturation' technique. After development of the chromatograms, PA were visualized with UV (long and short wavelength) and ferric chloride/potassium ferricyanide solution. Anthocyanidins did not require any reagent to visualize them because they are colored pigments. The R_f values were calculated from the formula:

$$R_f = \frac{\text{Distance of center of spot from start point (cm)}}{\text{Distance of solvent front from start point (cm)}}$$

Color Determination. A Hunter Lab Scan (Hunter lab, Fairfax, VA) was used to measure the color of the milled bambara groundnut hulls.

Statistical Analysis

Statistical analysis using the Statistical Analysis System (SAS,1992) was performed to determine differences among using an Analysis of Variance (ANOVA). Means were compared using Least Significance Differences (LSD). Regression equations were generated using Microsoft Excel (Windows 1995).

RESULTS AND DISCUSSION

Extraction and purification. Studies on bambara groundnut seeds showed that tannins, as measured by the total phenol procedure, were concentrated in the seed hulls with undetectable levels in the cotyledons (Table 1). The seed hull percentage of bambara

groundnuts varied between 9.5 to 12.2 % of the total seed weight which is similar to values of 7-14% for cow peas (Laurena et al., 1984) and 12% for faba beans (Helsper et al., 1993). The tannin content of hulls (1098 mg/g) was 2.5 times more than the tannin content of the whole seeds (435 mg/g). Low content found in whole seed flour as opposed to seed hulls has been reported by Deshpande and Cheryan (1985) and Laurena et al., (1984) all of whom found that tannin content in seed hulls were 1.2 - 2.5 and 7 -10 times higher respectively, than extractions from whole bean flours. Tannins have the ability to bind proteins and since seed proteins are concentrated in the cotyledons, the quantity of tannin assayed in the presence of protein is usually found to be lower than expected, thereby tannin content of whole seeds is always lower than that of hulls. The average protein content of bambara groundnut cotyledons was 19.2%, which is more than twice the average protein content of the seed hulls (7.9%). During extraction of tannins, it is advisable to extract as little of the hull proteins as possible to decrease non-tannin impurities. The extraction of proteins with tannins is minimized when the sample is first extracted with absolute ethanol. Hagerman and Butler (1980) found that absolute ethanol extracts only 10-15% of the total phenols as well as alcohol soluble protein.

Preliminary extraction studies using both methanol and acetone as the solvent showed that 70% aqueous acetone was equivalent to methanol in extracting tannins. Aqueous acetone (70%) containing 2.8 mM/L ascorbic acid was used to extract tannins from bambara groundnut hulls instead of methanol because acetone inhibits the binding of protein by tannins lowering the content of tannin-bound protein. Hussein et al. (1990) also found that aqueous acetone extracted relatively more proanthocyanindins from faba beans than acidified methanol or cold distilled water. Acetone has the ability to inhibit the binding of protein therefore, it is not advisable to use acetone to extract tannin if the tannin is to be used for protein precipitation assays. Tannin for protein precipitation assays should be extracted with a different solvent other than acetone.

After the crude tannin extract is loaded onto the column, non-tannin phenolics are washed out with ethanol because they do not bind to Sephadex LH-20. Three colors corresponding to seed hull pigment constituents were observed: the first fraction off the column was a pink pigment followed by a yellow, and then finally a brown fraction (Figure

1). Pale et al. (1997) detected five anthocyanin pigments from bambara groundnut seeds, three of which they isolated and identified as 3-*O*-β-glucosides of delphinidin, petunidin and malvinidin. It is probable that the colored pigments from bambara groundnut hulls that came off the column were anthocyanidins. PA are oligomers of leucoanthocyanidins, which are intermediates in the synthesis of anthocyanins.

Tannins were eluted from a Sephadex LH-20 column as a symmetrical peak at an elution volume of 185 mL and only fractions collected between 185 and 205 mL (totalling 20 mL) representing tannin fractions were subjected to spectral analysis at 400 nm (Figure 2). On lyophilization, the fractions yielded a fluffy brown powder that was readily soluble in methanol and water. The yield of bambara groundnut tannins was 3%, which was lower than the yield for faba bean PA (12%) (Hussein, et al., 1990) and for common bean (7%) (Hagerman and Butler, 1980). The low yield can be attributed to a low efficiency of extraction due to the smaller sample size (4.0 g) used or differences in bean types.

As non-tannin materials are removed from the sample, the tannin specific activity increased (Table 2). The tannin specific activity is a dimensionless ratio used to monitor the purification process. An increase in the tannin specific activity indicates a decrease in non-tannin material. For bambara groundnut tannins, the tannin specific activity increased from less than 0.5 in the crude extract to 2.06 in the purified material, constituting a 4.6 times increase in tannin specific activity. As purification progressed, there was a 50% reduction in protein concentration of purified bambara groundnut tannin (0.09%) compared to the crude extract (0.18%).

Purified bambara groundnut tannin reacted with acidified butanol to yield a red pigment which exhibited one major absorption peak at 552 nm (Figure 3). Pure cyanidin is a red pigment with a maximum absorption at 550 nm. The hydrolysis of purified bambara groundnut proanthocyanidin led to a depolymerization reaction in which anthocyanidin was the product. The production of anthocyanidin was further evidence that the purified tannin from bambara groundnut seeds was a proanthocyanidin (condensed tannin).

Estimation of the relative degree of polymerization gave a value for bambara groundnut PA of 5.9 monomers per oligomeric molecule which is consistent with

proanthocyanidins from cereals and legumes; sorghum tannins have been shown to have a relative degree of polymerization ranging between 5-7 (Butler, 1981).

Competitive binding assays were used to determine the relative affinity of bambara groundnut proanthocyanindin for BSA, pepsin, trypsin and α-chymotrypsin. Competition assays do not measure the concentration of tannin, but rather the relative affinity of proteins for the tannin. The amount of blue labeled BSA precipitated in the presence of competitor is expressed as a percentage of the amount precipitated in the absence of competitor. Bambara groundnut PA is competitively inhibited from binding with blue labeled BSA in the presence of pepsin or BSA (Figure 4). The relative affinity of BSA for bambara groundnut PA is slightly lower (0.14) than for pepsin (0.16) but both relative affinities are within the range of 0.17 found by Asquith and Butler (1985) for sorghum tannin with blue labeled BSA. Trypsin and α-chymotrypsin do not appear to have an affinity for bambara groundnut PA.

During protein digestion, pepsin enzyme in the stomach digests proteins to small peptides and basic amino acids. Trypsin and α-chymotrypsin are released into the intestinal lumen to continue the breakdown of proteins into amino acids. The affinity of bambara groudnut PA for pepsin implies that in the stomach, the enzyme would be preferentially bound and potentially inhibited by bambara groundnut PA even in the presence of other proteins. Thus, bambara groundnut PA is likely to interfere with proteolytic activty in the stomach leading to whole proteins passing untouched into the intestinal lumen.

Spectral Analysis. The methanol spectrum of bambara groundnut proanthocyanidin has a major absorption peak at 208 nm, and a low shoulder at 280 nm (Figure 5). Flavanones and dihydroflavonols typically have UV absorption spectra exhibiting one major band II absorption peak and a shoulder at band I (Markham, 1982; Harborne, 1970).

Addition of NaOAc to the proanthocyanidin produced a 20 nm bathochromic shift in the major absorption peak resulting in a spectrum with a maximum at 228 nm and increased intensity (Figure 6). NaOAc causes ionization of the most acidic of the flavonoid hydroxyl groups and is therefore used to detect the presence of a free 7-hydroxyl group. A 35 nm bathochromic shift indicates a flavanone with a free 7-hydroxyl and 5-hydroxyl, and a 60 nm bathochromic shift indicates a dihydroflavanol without a free 5-hydroxyl group. The 20 nm bathochromic shift obtained with the proanthocyanidin from bambara groundnut may be

interpreted as indicating the presence of either a flavanone with a free 7-hydroxyl or a dihydroflavanol without a free 5-hydroxyl group.

Addition of boric acid to a PA solution containing NaOAc shifted the peak to 230 nm; a bathochromic shift of 22 nm that was accompanied by an increase in intensity (Figure 7). $NaOAc/H_3BO_3$ is used to detect A-ring *o*-dihydroxy groups because it bridges the two hydroxyls. A bathochromic shift of 10-15 nm is indicative of A-ring *o*-dihydroxyls either at 6,7 or 7,8 positions. The 22 nm shift in the $NaOAc/H_3BO_3$ spectrum of this bambara groundnut proathocyanidin indicates the possible presence of A-ring *o*-dihydroxyls.

^{13}C NMR Spectroscopy. The spectrum of the purified PA showed intense signals at δ 74.7 and 76.4 and weak signals at 70.4, 88.8 and 92.3 (Table 3). Due to the effect of oxygen on the carbons at C-2 and C-3, shifts usually appear between 70.9 and 85.6 ppm (Agrawal 1989). Shifts associated with C-2 appear at 78.3- 85.6, and those for C-3 signals appear at 71.0-74.1. The chemical shifts obtained with bambara groundnut PA matched those documented for dihydroxyflavanols, corroborating the results obtained by UV spectral analysis.

This bambara groundnut proanthocyanidin was subsequently used as the standard in studies involving the quantitation of tannin in eight bambara groundnut samples.

Analysis of Eight Bambara groundnut Samples

Comparison of Tannin Content. The PA contents of eight bambara groundnut samples were measured by four different tannin quantitation procedures using purified bambara groundnut tannin as the standard (Table 3). For the total phenol assay, the tannin content ranged from 172 to 330 mg/g of hulls. The lowest tannin content was found in Zimbabwe 862 while the highest tannin content was in Uganda 298. Total phenols were measured by the Folin assay, which is not specific for tannins, therefore, all phenolics including non-tannins were quantified.

Tannin content as determined by the vanillin assay ranged from a low value of 165 mg/g for Malawi 396 to 265 mg/g for Uganda 298. Values for the vanillin assay were generally lower than for the total phenol assay as expected because the vanillin assay is specific for flavan-3-ols, and therefore, measures only a fraction of the total phenols.

The butanol assay measured the lowest tannin content in seeds from Zimbabwe 862 at 185 mg/g and the highest in Uganda 298 at 280 mg/g. The quantity of tannin determined by the butanol assay was consistently higher than for the vanillin assay for all samples. The butanol assay is specific for proanthocyanidins, which are depolymerized in acid butanol to produce anthocyanidins to generate the color that is measured.

The protein precipitation assay quantitated the largest values for tannin content among the four assays used. Tannin contents ranged from 211 mg/g for Zimbabwe 862 to 258 mg/g for Uganda 298. The protein precipitation assay is based on the interaction between tannins and protein leading to the formation of hydrogen bonds between the phenolic hydroxyl groups of the tannin and the peptide carbonyl groups of the protein (Hagerman, 1986). Proanthocyanidins have accessible *o*-phenolic hydroxyl groups which are available to form hydrogen bonds with proteins leading to the formation of insoluble complexes. The protein precipitation assay is regarded as being a direct measure of tannins *i.e.* phenolics that are capable of precipitating proteins.

Results of all four tannin assays are qualitatively similar (Table 4). Uganda 298, Kenya 665 and Uganda 245 are all ranked in the same order for all four assays, with Uganda 298 being ranked as having the highest tannin content, followed by Kenya 665 and Uganda 245 respectively. Zimbabwe 862 is ranked as having the lowest tannin content for three of the assays (total phenols, vanillin and protein precipitation) and second lowest for the vanillin assay. Samples with tannin contents between the highest and lowest are ranked somewhat randomly with these assay. Thus, the tannin assays agree with each other in as far as determining relative high and low tannin contents in bambara groundnut hulls but not in designating the intermediate rankings.

Comparison of Tannin Procedures. A quantitative comparison of the four assays reveals major differences between the tests (Table 4, across a row). The protein precipitation assay consistently gives the highest values for tannin for all samples (212 to 258 mg/g), and it exhibits the least range among samples, followed by the total phenol assay, then the butanol assay and finally the vanillin assay gives the lowest values (172 - 330 mg/g) compared to the other assays. Such differences are to be expected since the different tests measure tannins on

the basis of different properties. The vanillin and butanol assay are expected to give lower tannin contents than the total phenol assay because they do not measure all phenolics.

Various tannin assays are used by investigators to quantify tannins making comparisons of tannin data difficult. In order to see if the different tannin assays can be substituted for each other, we investigated the relationship between the tannin assays by regression analysis (Table 5). Fairly strong coefficents of determinations (R^2) are found between the protein precipitation assay and the total phenol assay ($R^2 = 0.78$), the butanol assay and the total phenol assay ($R^2 = 0.74$), and the protein precipitation assay and the butanol assay ($R^2 = 0.74$). Martin and Martin (1982) found no correlation between protein precipitation assay and either the total phenol or butanol assay when studying mature foliage from oaks. However, Naczk et al. (1994) found that tannin contents obtained by the butanol assay and the protein precipitation assay did not differ as much as tannin contents obtained by the vanillin assay. Table 4, across rows, compares the means of tannin content for each procedure within a sample. Both the vanillin and the butanol assay are functional group assays and are therefore expected to be in agreement. All samples except two, Uganda 298 and Kenya 665, have statistically similar tannin contents as measured by the functional group assays.

Tannin Content and Color. Means for the color variables for these seed samples were also compared (Table 6). Looking at L-values, Uganda 298 (tan) and Malawi 396 (tan/brown) were similar whereas Zimbabwe 862 (tan) was significantly different. Zimbabwe 522 (brown) and Uganda 245 (red) were similar. Malawi 458 (black/purple) was similar to Kenya 665 (black/brown) but not to Kenya 498 (black/brown). Kenya 498 (black/brown) was similar to Kenya 665 but not to Malawi 458. There were fewer similarities in redness and yellowness among seed samples; only Uganda 298 and Malawi 396, and Malawi 396 and Kenya 498 had similar redness values and Uganda 298 and Zimbabwe 862, and Uganda 245 and Kenya 498 had similar yellowness.

The relationship between tannin content and seed weight showing the seed coat color of the samples was studied (Figure 8). Only the tan seeds seem to show some relationship between seed weight and tannin content with a larger seed weight indicating a lower tannin

content. The rest of the seeds do not show any clear relationship. Studies carried out by Helpser (1993) on tannin-free and tannin-containing faba beans (*Vicia faba*) found that lower seed weight was associated with the absence of tannin.

Uganda 298 appears to be an outlier. It consistantly gave higher tannin contents with all four procedures implying that it may have some unique, unknown properties. Studies on sorghum grains (Price and Butler, 1977; Price et al., 1978) found that different solvents apparently extracted different types of tannins, and they subsequently classified sorghum tannins into three groups; I, II and III depending on their extractability. Bullard, et al. (1981) found that the relative degree of polymerization of sorghum tannins, affected their extractability. Group II tannins were found to be more polymerized and therefore less extractable than group III tannins. Maturity of the sorghum seeds seemed to affect their degree of polymerization and, therefore, their extractability. Group II tannins had colored testas, yet were found to be nutritionally equivalent to low tannin varieties. It is possible that PA in sample 298 are of the easily extractable type as compared to the rest of the samples. Further studies need to be done to determine the nature of the differences.

CONCLUSIONS

Extraction, purification and partial characterization of a proanthocyanidin from the hulls of Kenyan grown bambara groundnuts was successfully carried out. Functional group assays confirmed that the PA was a polymeric polyphenolic compound with a relative degree of polymerization equal to 5.9. Assays to determine the biological activity of the PA found that the PA had the ability to bind and precipitate blue labeled BSA, and that the PA was competitively inhibited from binding with protein in the presence of competitors like pepsin and BSA. Trypsin and α-chymotrypsin did not have any effect on bambara groundnut PA.

Spectral analysis of the purified PA showed that the PA was probably a dihydroflavanol without a free 5-hydroxyl group with the possible presence of A-ring *o*-dihydroxyls. ^{13}C NMR spectroscopy gave further evidence that the PA isolated from bambara groundnuts was a polymer of dihydroflavanol.

Four tannin assays were used to determine the tannin content and biological activity of eight bambara groundnut samples. All four assays were able to distinguish between

relatively high and low tannin contents, with the distinction blurring for the intermediate tannin contents. Functional group assays (vanillin and butanol) gave statistically similar results for tannin contents of the eight samples.

No relationship was found between the tannin content and seed coat color and tannin content and seed weight. There was also no relationship between the tannin content and geographic origin of the seeds. One seed sample, Uganda 298, consistantly gave much higher tannin contents for all four tannin assays indicating the prossibility of differences in its tannin types.

Further research is needed to determine the nature and basis of these differences in order to fully understand the nutritional implications of the proanthocyanidins present in bambara groundnuts.

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FIGURE LEGENDS

Figure 1. Elution profile of non-tannin phenolics from a Sephadex LH-20 column.

Figure 2. Elution profile of bambara groundnut proanthocyanidin from a Sephadex LH-20 column using 70% aqueous acetone.

Figure 3. Spectra of anthocyanidin formed from bambara groundnut proanthocyanidin.

Figure 4. Effectiveness of protein binding by proanthocyanidins in the presence of competitors.

Figure 5. UV spectrum of pure proanthocyanidin in methanol.

Figure 6. Effect of NaOAc UV spectrum of pure proanthocyanidin.

Figure 7. Effect of $NaOAc/H_3BO_3$ on the UV spectrum of pure proanthocyanidin.

Figure 8. Relationship between tannin content, seed coat color and seed weight.

Table 1: Characteristics of bambara groundnut seeds used in this study including seed coat color, proportion of cotyledons and hulls, seed weight, and tannin content of hulls

Geographic Origin[a]	Seed color	Composition[b]		Seed Weight[c]	Seed Hulls Tannin Content[d]	
		Cotyledons (%)	Hulls (%)	(g)	(mg/g)	Std Dev
Kenya 665	Black/Brown	89	10	0.4	293	20
Kenya 498	Black/Brown	89	10	0.4	240	23
Zimbabwe 862	Tan	88	11	0.6	172	20
Zimbabwe 522	Brown	88	11	0.4	211	17
Malawi 396	Brown/Tan	87	11	0.5	185	22
Malawi 458	Black/Purple	89	10	0.5	272	63
Uganda 298	Tan	86	12	0.4	330	28
Uganda 245	Red	90	10	0.7	288	21

[a] Includes identification number of samples

[b] Whole seed = 100%

[c] Weight of one seed = Weight of 100 seeds/100

[d] Determined by the Total Phenol procedure

Table 2: Steps in the purification of PA from bambara groundnut hulls

Purification Steps	Yield(%)[a]	Tannin Specific Activity[b]	Protein (%)
Ground Hulls	100[c]	-	7.90
Crude Extract	ND	0.45	0.18
LH-20 Batch	11.8	0.69	ND
Purified PA	3.0	2.06	0.09

[a] By weight

[b] Tannin Specific Activity = Protein Precipitation / Total Phenols

[c] Means are for two samples

ND Not determined

Table 3. ^{13}C NMR Spectral Data from Bambara groundnut proanthocyanidin (50 MHz, $(CD_3)_2CO$)

Carbon No.	Shift
2	76.4
3	70.4 74.7
8	92.3

Table 4. Comparison of tannin content for each bambara groundnut sample using four standard procedures for tannin content determination

Sample	Means (mg/g)			
	Total Phenols	Vanillin	Butanol	Protein Precipitation
Uganda 298	$329.86^{a\ x}$	$265.48^{a\ y}$	$280.08^{a\ y}$	$257.77^{a\ y}$
Kenya 665	$293.32^{b\ x}$	$195.75^{b\ z}$	$256.18^{b\ y}$	$253.56^{a\ y}$
Uganda 245	$288.17^{b\ x}$	$192.72^{b\ z}$	$249.08^{b\ y}$	$237.6^{ab\ y}$
Malawi 458	$271.66^{bc\ x}$	$187.41^{bc\ y}$	$196.09^{c\ y}$	$235.63^{ab\ xy}$
Kenya 498	$239.55^{cd\ x}$	$188.60^{bc\ y}$	$189.72^{c\ y}$	$233.39^{ab\ x}$
Zimbabwe 522	$210.86^{de\ x}$	$182.32^{c\ x}$	$188.42^{c\ x}$	$217.74^{b\ x}$
Malawi 396	$185.32^{ef\ y}$	$165.07^{d\ y}$	$195.42^{c\ y}$	$232.13^{ab\ x}$
Zimbabwe 862	$172.12^{f\ y}$	$169.41^{d\ y}$	$184.87^{c\ y}$	$211.48^{b\ x}$

Purified bambara groundnut proanthocyanidin was used as the standard in all assays
The standard was purified from sample 665
Means are for three samples
a to e Used to compare means within a column; Means within each column followed by the same letter are not different at alpha = 0.05.
x to z Used to compare means within a row.; Means within each row followed by the same letter are not different at alpha = 0.05.

Table 5. Regression equations for the relationship between the tannin assays used to analyze tannin levels in bambara groundnut samples

Parameters (Tannin Assays)		Equation	R^2
X	Y		
Total Phenol	Protein Precipitation	y = 0.3475x + 173.06	0.78
Total Phenol	Butanol	y = 0.8178x + 71.942	0.74
Total Phenol	Vanillin	y = 0.6312x + 81.021	0.66
Vanillin	Butanol	y = 0.9719x + 29.5	0.64
Vanillin	Protein Precipitation	y = 0.3732x + 162.75	0.54
Butanol	Protein Precipitation	y = 0.3576x − 157.14	0.74

Table 6. Measurement of color, L (darkness), a (redness), and b (yellowness) of bambara groundnut seed samples

Sample[a]	Color[b]		
	L	a	b
Uganda 298	53.9967[a]	4.6000[d]	10.5533[a]
Malawi 396	53.1133[a]	4.2900[de]	10.0800[b]
Zimbabwe 862	50.9233[b]	5.4700[b]	10.8933[a]
Zimbabwe 522	44.9733[c]	5.0433[c]	7.7467[c]
Uganda 245	43.1367[c]	6.6800[a]	5.7367[d]
Malawi 458	36.3133[d]	3.0500[f]	4.2533[f]
Kenya 665	35.2167[de]	3.9800[e]	4.8700[e]
Kenya 498	34.1833[e]	4.4167[d]	5.3700[d]

[a] Bambara groundnut samples from different geographical regions

[b] Measured by the Hunter Lab color instrument

[c] Means are of three samples. Means within a column followed by the same letter are not different at alpha = 0.05

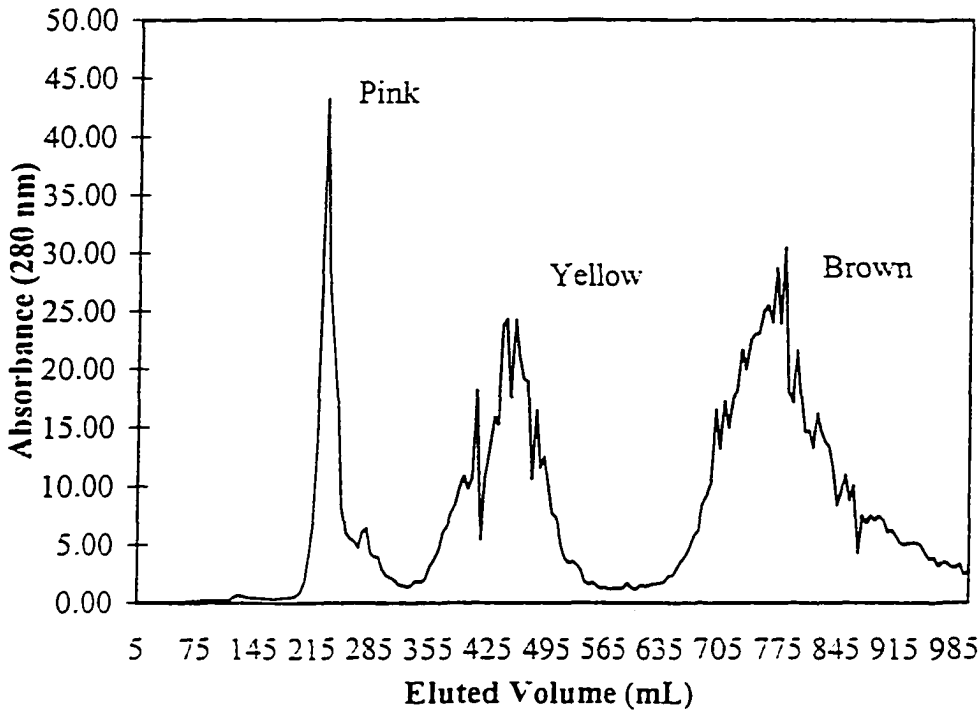
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Absorbance (280 nm)
Pink
Yellow
Brown
5 75 145 215 285 355 425 495 565 635 705 775 845 915 985
Eluted Volume (mL)

Figure 1

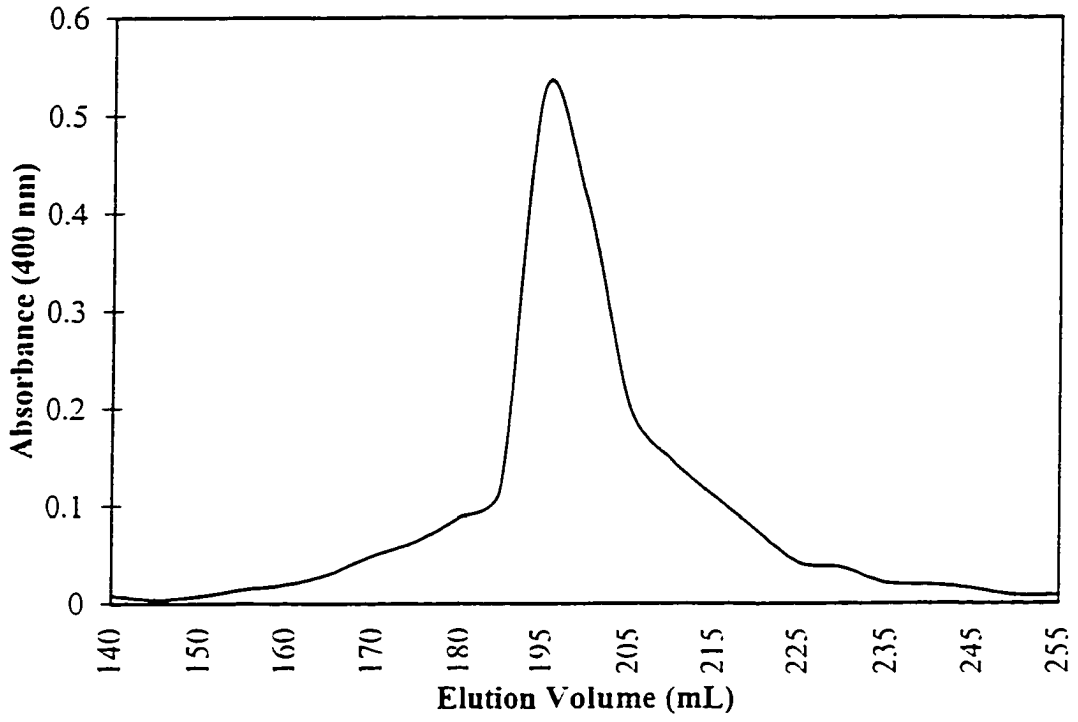
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (400 nm)
140
150
160
170
180
195
205
215
225
235
245
255
Elution Volume (mL)

Figure 2.

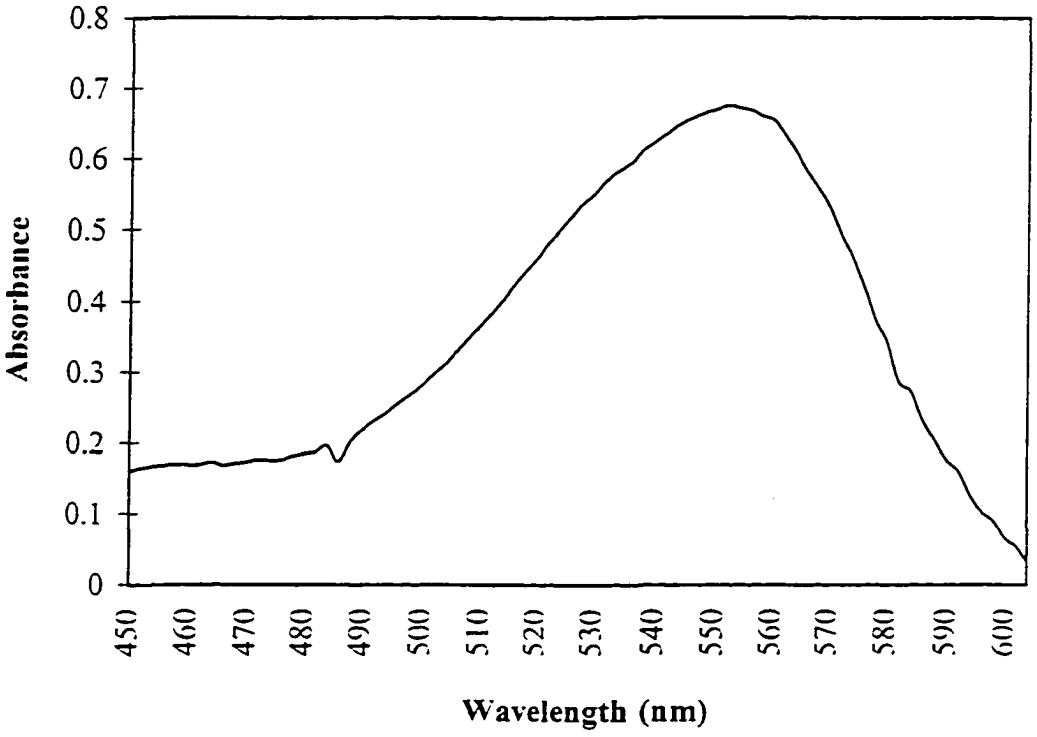
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
450
460
470
480
490
500
510
520
530
540
550
560
570
580
590
600
Wavelength (nm)

Figure 3.

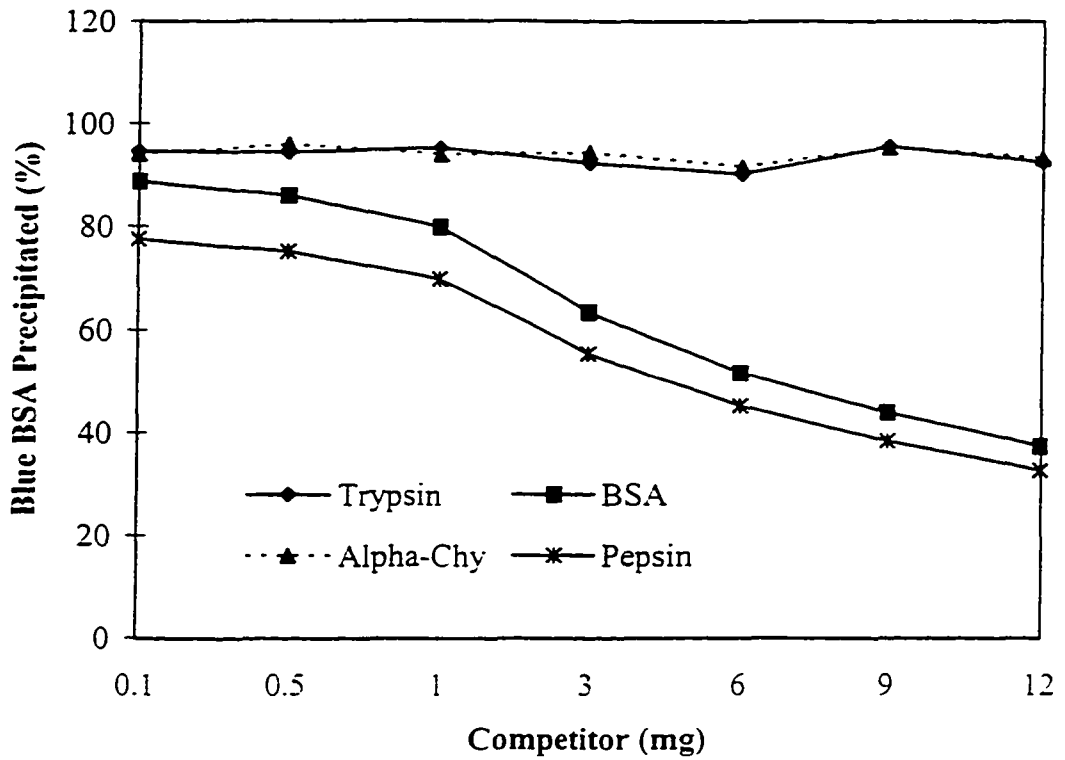
120
100
80
60
40
20
0
Blue BSA Precipitated (%)
Trypsin
BSA
Alpha-Chy
Pepsin
0.1
0.5
1
3
6
9
12
Competitor (mg)

Figure 4.

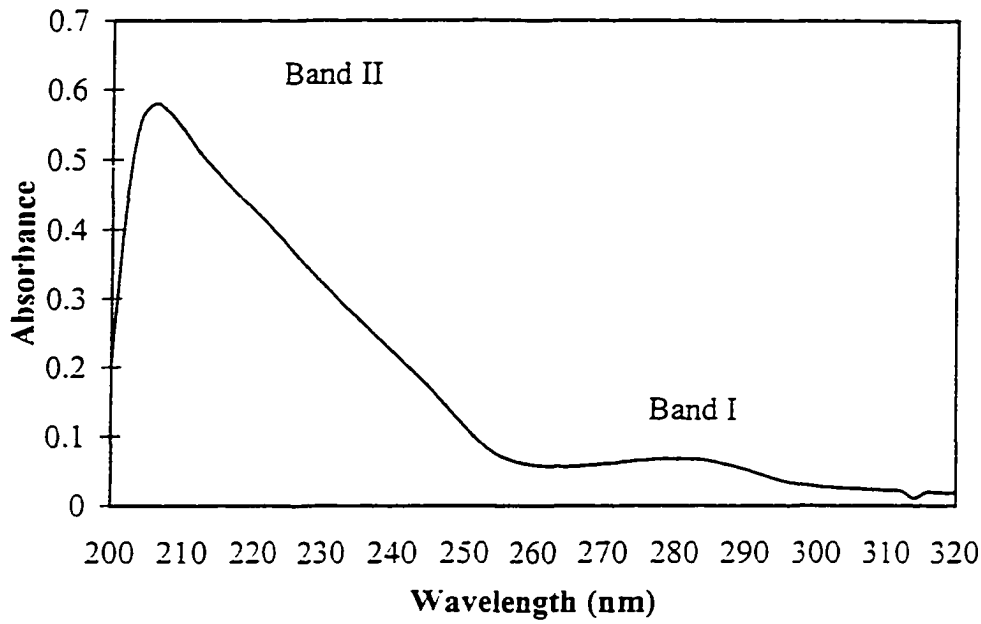
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
Band II
Band I
200 210 220 230 240 250 260 270 280 290 300 310 320
Wavelength (nm)

Figure 5.

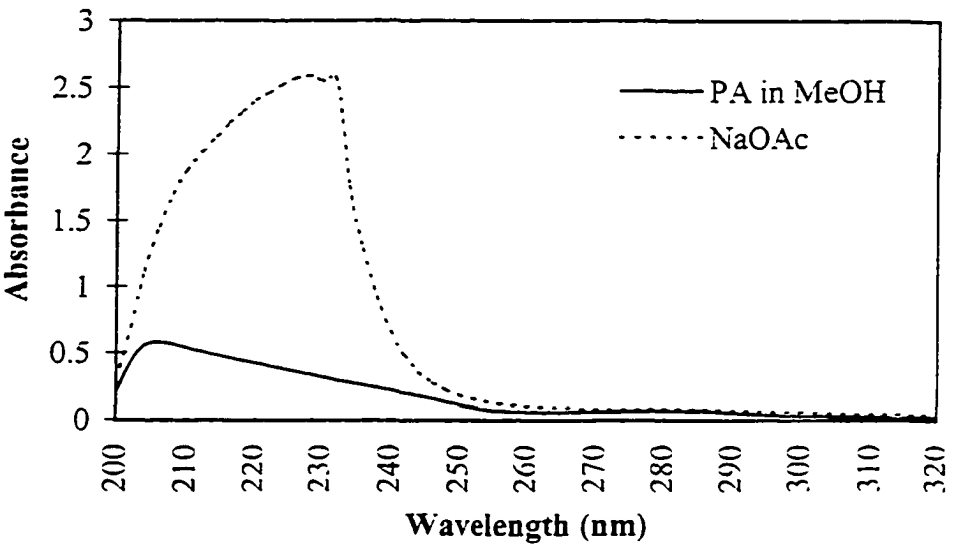
3
2.5
2
1.5
1
0.5
0
Absorbance
200
210
220
230
240
250
260
270
280
290
300
310
320
Wavelength (nm)
PA in MeOH
NaOAc

Figure 6.

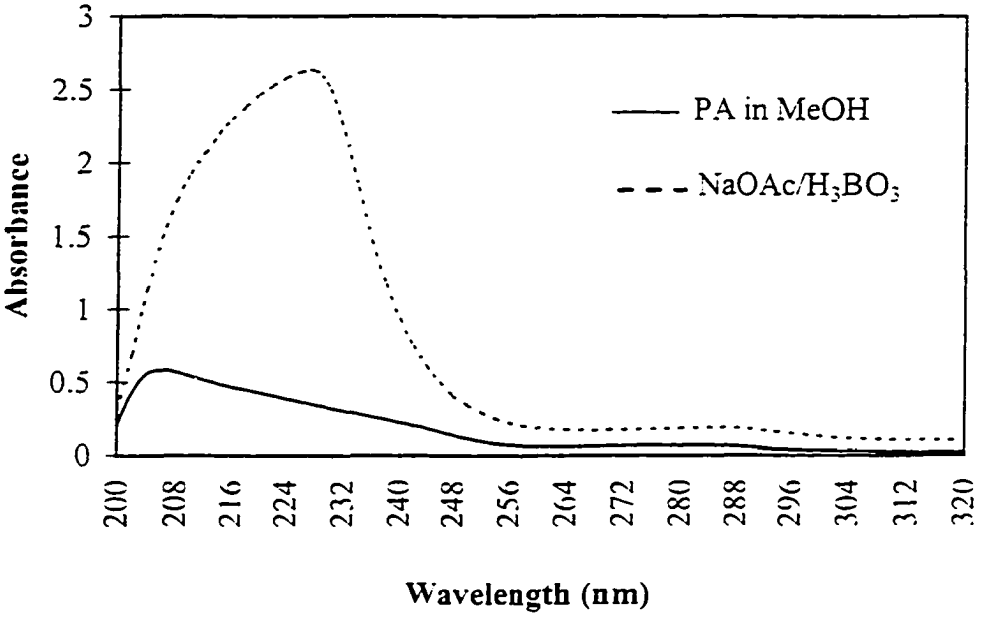
Absorbance
3
2.5
2
1.5
1
0.5
0
200
208
216
224
232
240
248
256
264
272
280
288
296
304
312
320
PA in MeOH
NaOAc/H3BO3
Wavelength (nm)

Figure 7.

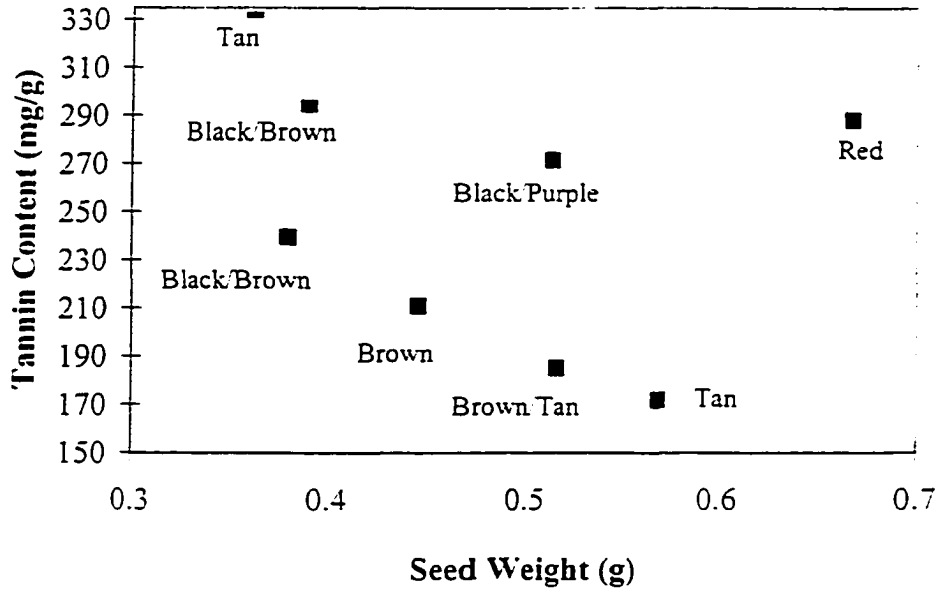
Tannin Content (mg/g)
330
310
290
270
250
230
210
190
170
150
0.3
0.4
0.5
0.6
0.7
Seed Weight (g)
Tan
Black/Brown
Black/Purple
Red
Black/Brown
Brown
Brown/Tan
Tan

Figure 8.

PURIFICATION, CHARACTERIZATION AND PARTIAL AMINO ACID SEQUENCE OF TRYPSIN ISOINHIBITORS FROM BAMBARA GROUNDNUT (*Vigna subterranea* (L.) Verdc.) SEEDS

A paper to be submitted to the Journal of Agriculture and Food Chemistry

Redempta B. Kegode and Mark H. Love

ABSTRACT

The presence of at least four major trypsin isoinhibitors has been detected in bambara groundnut seeds (*Vigna subterranea* (L.) Verdc.), which are leguminous seeds predominantly grown in Africa. The isoinhibitors denoted as BGTI I, BGTI II, BGTI III and BGTI IV were purified by ammonium sulfate precipitation, size-exclusion chromatography and ion exchange chromatography. Their p*I*s were determined by isoelectric focusing to be 4.5, 4.6, 6.0 and 6.4 respectively. Matrix assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOF) determined their molecular masses as 7895 Da BGTI I, 9195 Da BGTI II, 9013 Da BGTI III and 9017 Da BGTI IV which makes them low molecular weight trypsin inhibitors belonging to the Bowman-Birk family. The N-terminal amino acid sequences of the four isoinhibitors show some similarities to those of soybeans, cowpeas and lima beans.

INTRODUCTION

Proteinase inhibitors are ubiquitous in nature, however, the most widely studied ones are from legumes (Wilson, 1981; Filho and Compos, 1989) where they are found in almost all plant parts but are generally concentrated in storage organs like tubers and seeds (Ryan, 1973). Early interest in plant proteinase inhibitors was driven by findings that the inhibitors reduced the nutritive value of raw plant products. However, in recent years, plant proteinase inhibitors have been suggested as having a role in cancer suppression (Kennedy, 1994).

The bambara groundnut is a leguminous crop that is cultivated for its seeds which are used for human consumption. The presence of trypsin inhibitors in bambara groundnuts has been documented (Owusu-Domfeh, 1972; Poulter, 1981), and a trypsin inhibitor has been isolated and partially characterized (Martino-Ferrer and Ferrer, 1983). The trypsin inhibitor had a p*I* of 6.8 and exists as either a monomer (MW 13,200 Da) in the denatured form or a dimer (MW 26,300 Da) in its native form. Very little information is available about the physical and biochemical properties of bambara groundnut proteinase inhibitors.

This study was conducted to purify trypsin inhibitors from bambara groundnut seeds in order to determine their molecular mass and amino acid composition. This study is the first report of the presence of isoinhibitors in bambara groundnut seeds and documentation of their partial N-terminal peptide sequences.

MATERIALS AND METHODS

Materials. Bambara groundnut (*Vigna subterranea*) seeds were purchased locally in Kenya.
Extraction and Isolation. The isolation and purification of inhibitor was carried out as described by Ferrasson et al. (1997) with some modifications. Bambara groundnut seeds were first manually dehulled and the cotyledons ground to a flour (100-mesh) in a Wiley mill. Flour (150 g) was extracted with 50 mM sodium acetate (1500 mL), pH 4.9 at 4°C for 3 hr. The slurry was clarified by centrifugation at 9000*g* for 30 min and the pellet was discarded. Solid ammonium sulfate was added to the supernatant to 80% saturation and stirred at 4°C for 2 hr. The saturated solution was centrifuged at 9000*g* for 15 min then suspended in 50 mM Tris-HCl, pH 8.8 and desalted by dialysis (Spectra/Por ®, Spectrum Medical Industries, Inc., Laguna Hills, CA), (MWCO: 3,500) at 4°C for 24 hr. The desalted fraction was centrifuged at 15000*g* for 30 min to yield the crude inhibitor as the supernatant.
Purification. Gel chromatography purification of the crude inhibitor preparation was carried out on a Sephadex G-75 preparative column (5 x 85 cm) equilibrated with 50 mM Tris-HCl, pH 8.8. The column was loaded with the crude inhibitor preparation and eluted with 50 mM Tris-HCl, pH 8.8. Protein and trypsin inhibitor activity (TIA) were monitored and fractions containing trypsin inhibitory activity were collected and pooled (G75 fraction).

The G75 fraction was purified on a DEAE-Sepharose FF anion exchange column (2.5 x 20 cm) equilibrated with 50 mM Tris-HCl, pH 8.8. A linear gradient of 0-0.5 M NaCl in 50 mM sodium acetate, pH 4.9 was used to elute adsorbed trypsin inhibitor. Fractions showing trypsin inhibitory activity were collected, pooled and desalted by dialysis against deionized water for 24 hr at 4°C, and subsequently lyophilized to give the DEAE fraction.

The DEAE fraction was subjected to SP Sepharose cation exchange chromatography but due to an excessive loss of TIA an alternative procedure for further purification was used.

Carrier Ampholyte- Isoelectric Focusing (CA-IEF). Further purification of the DEAE fraction was done by CA-IEF which was also used to determine the p*I* of the isoinhibitors. The procedure followed the protocol as set out for Bio-Rad's Model 111 Mini IEF Cell. The carrier ampholytes (Bio-Lyte, Bio-Rad Lab, Hercules, CA) used as the standard spanned the pH range 5-8. The standard consisted of cytochrome C, lentil lectin, human hemoglobin C, human hemoglobin A, equine myoglobin, human carbonic anhydrase, bovine carbonic anhydrase, β-lactoglobulin and phycocyanin). The gels were cast with 25% polyacrylamide and run in a stepped up manner. On termination of IEF, the gel was immersed in 200 mL of fixing solution (70% distilled water, 30% MeOH, 11.5% w/v trichloroacetic acid, 3.5% w/v sulfosalicylic acid) to prevent diffusion of the proteins. After 20 min. the fixing solution was poured off and the gel was washed in 100 mL of destaining solution (30% MeOH, 5% Acetic acid) for two min. The gel was then cut into three pieces for subsequent analysis.

Gel #1 was stained with Coomasie brilliant blue (R-250) to visualize sample proteins and determine their p*I*s by comparing sample bands with standards. Gel #2 was used to detect protein bands that had trypsin inhibitory activity. The protein bands were cut out of the gel and incubated with Tris buffer (0.1 mL/band) in eppendorf tubes for 15 min. The tubes were them centrifuged in an Eppendorf Micro-centrifuge (Centrifuge 5414) for 2 min and the supernatant collected. The extraction was repeated a second time and the supernatants were pooled and TIA determined. Bands that showed TIA were termed the bambara groundnut isoinhibitors I-IV. Gel # 3 was electroblotted on PVDF in preparation for N-terminal peptide sequencing and amino acid analysis.

Peptide sequencing After electroblotting, of the isoinhibitors from gel onto polyvinylidinedifloride (PVDF), the active bands were cut out and then subjected to peptide

sequencing via Edman degradation on a 492 Procise Protein Sequencer/140C Analyzer from Applied Biosystems, Inc.

Amino Acid Analysis. Active protein bands that were cut out of PVDF were analyzed on a Beckman 6300 amino acid analyzer after perchloric acid oxidation and hydrolysis by HC. All amino acids except typtophan were determined.

Molecular Mass. Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) Mass Spectrometry was used to determine the molecular mass of the isoinhibitors. The sample was mixed with matrix (10 mg/mL α-cyano-4-hydroxycinnamic acid in 70% ACN/H_2O, 0.1% TFA) and the mixture allowed to dry and then was loaded onto a Fannigan MAT Lasermat 200 instrument that uses pulsed nitrogen laser at 337 nm. Positive polarity was used to generate ions.

Assays. Trypsin inhibitor activity was determined by the AACC Method 71.10 using BAPA as the synthetic substrate and bovine.trypsin as the enzyme. Trypsin inhibitory activity (TIA) was determined by measuring the absorbance of appropriately diluted samples at 410 nm in a DU Beckman UV-Visible Spectrophotometer against a standard containing bovine trypsin only. One trypsin unit is arbitrarily defined as an increase of 0.01 absorbance units at 410 nm per 10 mL of reaction mixture under procedure conditions. Trypsin inhibitor activity is expressed in terms of trypsin inhibitor units (TIU). Protein determination of fractions eluted off the column was estimated by near UV absorbance (280 nm).

RESULTS AND DISCUSSION

Purification of Inhibitor. Ammonium sulfate (80% saturation) precipitation of the aqueous extract resulted in 85% recovery of inhibitor activity and a 9-fold purification of the inhibitor (Table 1). The inhibitor-rich fraction was then loaded onto a Sephadex G75 column and purified inhibtor subsequently eluted (Figure 1). The inhibitor activity recovered was 52.4% which resulted in a 21-fold purification on the basis of the crude extract. Further purification of the inhibitor by anion exchange chromatography on DEAE Sepharose led to the recovery of 24.5% of the trypsin inhibitor activity which was eluted with a NaCl gradient. Elution of the trypsin inhibitor from the column resulted in a 25-fold purification (Figure 2).

Fractionation of the DEAE Sepharose fraction on a cation exchange column resulted in the identification of two isoinhibitors T1 and T2 (Figure 3), but since most of the TIA was lost, an alternative method aimed at maximizing activity of the inhibitors was used to separate and purifiy the isoinhibitors.

During purification by ion exchange, it was expected that the different bambara groundnut isoinhibitors would elute at different salt concentrations. Anion exchange chromatography was not effective in fractionating the isoinhibitors, however, cation exchange chromatography was partially effective in separating two isoinhibitors T1 and T2 (Figure 3), but with a loss in specific activity. Since IEF separated four major isoinhibitors, it is probable that isoinhibitor T2 was a coelution of BGTI I and II and isoinhibitor T1 was a coelution of BGTI III and IV. The coelution of the isoinhibitors (I , II and III, IV) at the same or very close salt concentrations can be explained by the very close p*I*s of BGTI I and II, 4.6 and 4.7 respectively. Similarly for BGTI III and IV with pIs of 6.0 and 6.4, respectively.

Isoelectric focusing was successful in separating and purifying the isohibitors as well as in determining their isoelectric points (p*I*). The isoelectric focusing pattern of the inhibitor preparation showed that the DEAE Sepharose fraction was heterogeneous and consisted of several protein bands (Figure 4). The protein bands were assayed for trypsin inhibitor activity and six of the bands were found to have trypsin inhibitory activity. The p*I*s of the trypsin isoinhibitors were acidic and ranged from 4.5 to 6.4. When the isoinhibitors were electroblotted from the gel, two of them, p*I*s 5.8 and 6.2 were not successfully transferred probably due to their lower concentration, hence only four of the isoinhibitors were further characterized, and they are referred to as BGTI I-IV having p*I*s of 4.5, 4.6, 6.0 and 6.4 respectively. The only other trypsin inhibitor purified from bambara groundnut had a pI of 6.8 (Martino-Ferrer and Ferrer, 1983). Purification by isoelectric focusing led to purification factors ranging from 57 to 743 (Table 1).

When the DEAE fraction was subjected to isoelectric focusing, six bands with inhibitor activity were identified. However, two of the bands could not be electroblotted for subsequent analysis, hence only four of the isoinhibitors were characterized further (Figure 4). These four new isoinhibitors from bambara groundnuts are closely related to the low

molecular weight inhibitors present in legumes. The specific activities of the isoinhibitors are different indicating that the extent to which they inhibit trypsin is different hence the relative proportions of the isoinhibitors in various bambara groundnut cultivars is likely to lead to variations in the nutritional value of different cultivars.

BGTI-I, III and IV had single peaks corresponding to molecular mass 7895, 9013 and 9017 Da respectively (Table 2) as determined by MALDI. BGTI-II gave a spectrum having four peaks which were close thus the peak with the largest molecular weight (9195 Da) was selected to represent the molecular weight of the isoinhibitor. The molecular weights were used to calculate the amino acid residues of the isoinhibitors.

Martino-Ferrer and Ferrer (1983) identified a single trypsin inhibitor from bambara groundnuts. They found that the native inhibitor had a molecular weight of 26,300 Da and a p*I* of 6.8 whereas the denatured inhibitor appeared to have a molecular weight of 13,200 Da. Our results indicate that bambara groundnuts have several isoinhibitors present in the seeds. The molecular weights of bambara groundnut isoinhibitors imply that they may belong to the Bowman-Birk family of inhibitors whose molecular weight ranges from 7000-9000 Da. It is possible that the isoinhibitors we characterized and the trypsin inhibitor identified by Martino-Ferrer and Ferrer (1983) belong to the same family of proteinase inhibitors. The larger molecular weight of the trypsin inhibitor of Martino-Ferrer and Ferrer (1983) may be a result of association since in aqueous solution Bowman-Birk inhibitors undergo self association which is concentration dependent (Norton, 1991).

All four isoinhibitors had similar amino acid compositions (Table 3). BGTI are rich in glycine, aspartic acid or asparagine and have fairly high contents of alanine, threonine, serine and lysine, while low in cysteine and methionine. BGTI-I had a total number of residues of 81, BGTI-II had 92, BGTI-III had 94 and BGTI-IV had 93 total residues (Table 3).

Although the molecular weights of the four bambara groundnut isoinhibitors (Table 2) indicate that they may belong to the Bowman-Birk family of inhibitors, their amino acid composition does not agree with that of the classical Bowman-Birk inhibitors which contain fourteen half-cystine residues forming seven disulfide bonds and is devoid of tryptophan and glycine. Tan-Wilson *et al.*, (1987) purified ten Bowman-Birk isoinhibitors from several

soybean cultivars and classified them into four subgroups, I-IV. Subgroup I included those isoinhibitors which were identified as the classical Bowman-Birk inhibitors. Subgroup II were similar to subgroup I except that they inhibited trypsin weakly. Subgroup III were the weakest inhibitors of trypsin and subgroup IV were different from the previous subgroups. Subgroup IV comprised of inhibitors which contained 200-210 amino acid residues, with molecular weights of 20,000 Da, high glycine content and low half cystine contents. Subgroup IV was assigned a separate class called the glycine-rich trypsin inhibitors. It is probable that bambara groundnut trypsin inhibitors belong to the glycine-rich family of inhibitors.

Martino-Ferrer and Ferrer (1983) determined that the trypsin inhibitor they purified from bambara groundnuts had four tyrosine residues and no tryptophan based on a molecular weight of 26,300 (dimer). Thus the monomer would probably have two tyrosines which would be in agreement with our results which found that BGTI I, III and IV had two tyrosines and BGTI II had 3 tryrosine residues.

N-terminal peptide sequences of BGTI isoinhibitors are presented in Figure 5. The sequences are aligned with respect to conserved amino acids. The first 19 amino acid residues of the isoinhibitors (BGTI I-IV) were identical to each other. The sequences of the isoinhibitors were compared to the sequences of Bowman-Birk inhibitors from other legumes. There is some homology between the sequences of bambara groundnut isoinhibitors and lima bean (LBI-VI S), cow pea (CPI-IV-B) and soybean (BBI) inhibitors.

Comparison of the primary structure of bambara groundnut trypsin isoinhibitors with the Bowman-Birk inhibitors from other legumes revealed some similarities (Figure 5). Both BGTI and soybean BBI have a homologous series consisting of the sequence D-E-S-S. BGTI and LBI both possess the sequence S-G-H-H at positions 1-4. BGTI and CPI have the H-H-Q sequence at positions 4-6.

The reactive site of BGTI was not determined, however, the classical BBIs have their trypsin reactive peptide bond located at Lys16-Ser17 and the amino acids around the reactive site are similar to each other and to other inhibitors within the family. The BGTIs do not possess any of the characteristics of the BBI reactive site, but they do possess the Lys17-

Pro18 bond that is similar to the trypsin reactive site found in the non-legume seed BBI from rice bran (RBTI). It is probable that for BGTI, the trypsin reactive bond is at Lys17-Pro18.

CONCLUSIONS

The study describes the purification, some biochemical and structural characterization of trypsin isoinhibitors from bambara groundnut seeds. This is the first time that the partial amino acid N-terminal sequence of trypsin inhibitor from bambara groundnut has been determined.

Bambara groundnuts contain at least four major trypsin isoinhibitors which have a molecular weight ranging between 7000 and 10,000 Da making them likely to belong to the Bowman-Birk family of inhibitors. However, Bowman-Birk inhibitors are known to have a high number of disufide bridges which were absent in the inhibitor from bambara groundnut. It may be that bambara groundnut inhibitor is yet another family of inhibitors previously unencountered. Our studies focused on the trypsin inhibitory activity of the inhibitors although Bowman-Birk inhibitors are known to be double-headed. The probable trypsin reactive site of bambara groundnut trypsin inhibitor was tentatively identified as Lys17-Pro18 but further studies are needed to definitely identify the site. Future studies should determine the chymotrypsin inhibitory activity of bambara groundnut proteinase inhibitors and also sequence the whole inhibitor in order to determine the chymotrypsin reactive site.

Plant proteinase inhibitors are known to be specific against various proteases. Since our study used only bovine trypsin as the protease, we do not know the specificity of bambara groundnut typsin inhibitors towards other proteases and future studies should be designed that can answer this question.

Various functions have been proposed for plant proteinase inhibitors; as storage proteins, as protective agents and as regulatory agents. Bambara groundnut plants are known to be more resistant to adverse environmental conditons and the reasons for this desirable trait is unknown. It is probable that proteinase inhibitors may have a role in the tenacity of bambara groundnut plants.

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FIGURE LEGENDS

Figure 1. Elution profile of bambara groundnut trypsin inhibitor from a Sephadex G75 Column. The sample was protein precipitated by ammonium sulfate. Protein (501 mg) was loaded onto the column. Fraction volume was 20 mL and protein was monitored by measuring absorbance at 280 nm. Standard trypsin reactions containing no bambara groundnut inhibitor gave an A_{410} of 0.320.

Figure 2. Anion exchange chromatography on DEAE sepharose column. Sample was pooled trypsin inhibitor fractions from G75 column and 137 mg protein were loaded onto the column. Figure 2A shows the elution of protein monitored at 280 nm Figure 2B shows the elution of trypsin inhibitor (solid line) with a linear NaCl gradient (broken line).

Figure 3. The elution pattern of two bambara groundnut trypsin isoinhibitors, T1 and T2 from a cation SP Sepharose column. The inhibitors were eluted with a NaCl gradient of 0-0.1M

Figure 4. CA-IEF pattern of purified bambara groundnut trypsin inhibitor on polyacrylamide gel. Lane (a) Standard consisting of cytochrome C, lentil lectin, human hemoglobin C, human hemoglobin A, equine myoglobin, human carbonic anhydrase, bovine carbonic anhydrase, β-lactoglobulin and phycocyanin), (b) Purified bambara groundnut isoinhibitors (BGTI I-IV).

Figure 5. N-terminal peptide sequences of bambara groundnut isoinhibitors (BGTI I-IV) compared with the Bowman-Birk inhibitors from lima beans (LBI-IV S), cowpea (CPI-IV-B), soybean (BBI) and rice bran trypsin inhibitor (RBTI). The enclosed sequences are homologous. The trypsin reactive site is shown by and arrow.

Table 1. Purification of Trypsin Inhibitor from Bambara Groundnuts[a]

Products of Purification Steps	Protein (mg)	TIU	Recovery (%)	Specific Activity (TUI/mg)	Purification Factor[a]
Aqueous Extract	5462	4680657	100.0	857	1
Crude Inhibitor	501	3982500	85.1	7954	9
G 75 Fraction	137	2452500	52.4	17888	21
DEAE Fraction	53	1147500	24.5	21692	25
IEF[b]					
BGTI I	0.0212	1520	0.03	71698	84
BGTI II	0.0138	1920	0.04	139130	162
BGTI III	0.0187	920	0.02	49198	57
BGTI IV	0.0152	9680	0.21	636842	743

[a] Dehulled seeds, 150 g

[b] Sample loaded was 0.0966 mg of the DEAE fraction

Table 2. Isoelectric point (p*I* s)[a] and Molecular Weights[b] of Bambara Groundnut Trypsin Inhibitors I-IV

Isoinhibitor	p*I*	Molecular Mass (Da)
BGTI I	4.80	7895
BGTI II	4.85	9195
BGTI III	5.55	9013
BGTI IV	5.69	9017

[a] p*I* determined from IEF

[b] Molecular mass as determined by MALDI

Table 3: Amino Acid Composition of Bambara Groundnut Trypsin Isoinhibitors I - IV

	Number of Residues[a]			
Amino Acid	**BGTI - I**	**BGTI - II**	**BGTI - III**	**BGTI - IV**
Cysta[b]	1	2	6	6
Asx	9	10	11	12
Thr	6	6	7	7
Ser	7	9	12	15
Glx	6	6	5	5
Pro	4	4	4	4
Gly	12	11	12	9
Ala	9	12	9	8
Val	6	6	3	3
Met	0	0	0	0
Ile	4	4	3	3
Leu	5	5	3	2
Tyr	2	3	2	2
Phe	3	3	2	2
His	2	2	4	4
Lys	5	6	6	7
Trp	ND	ND	ND	ND
Arg	2	3	4	3
No. of Residues	81	92	94	93
M.W.	7895	9195	9013	9015

[a] Residue values are integers calculated on the basis of molecular mass.

[b] Oxidation form of cysteine

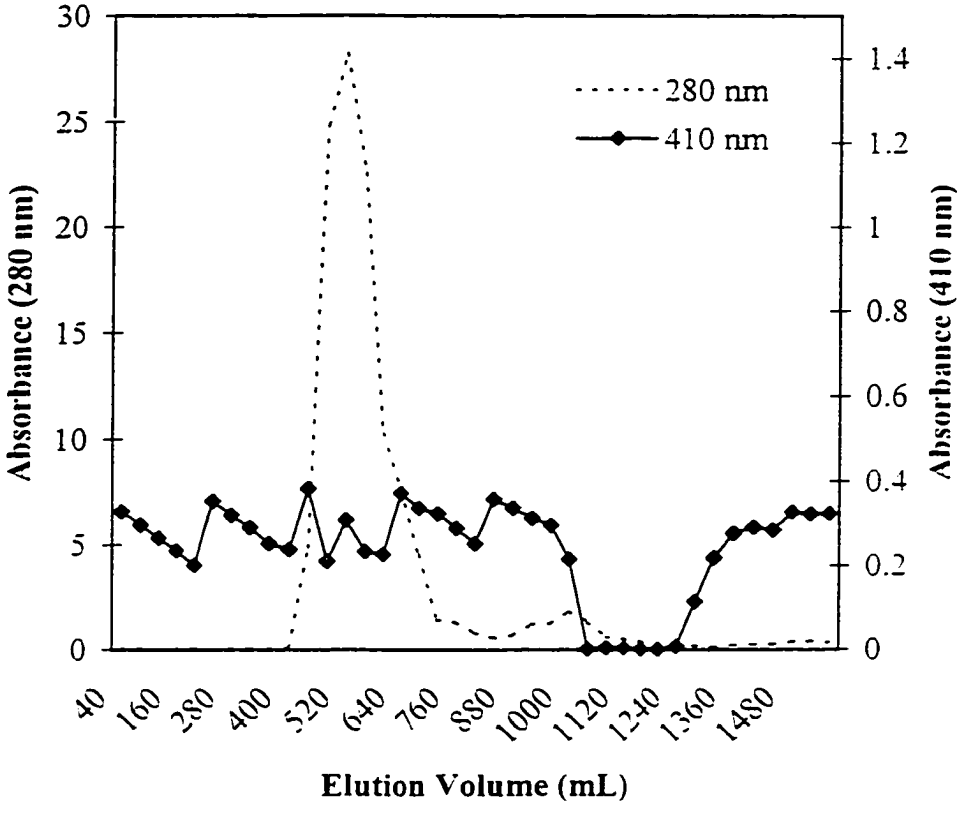
30
25
20
15
10
5
0
Absorbance (280 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance (410 nm)
280 nm
410 nm
40
160
280
400
520
640
760
880
1000
1120
1240
1360
1480
Elution Volume (mL)

Figure 1.

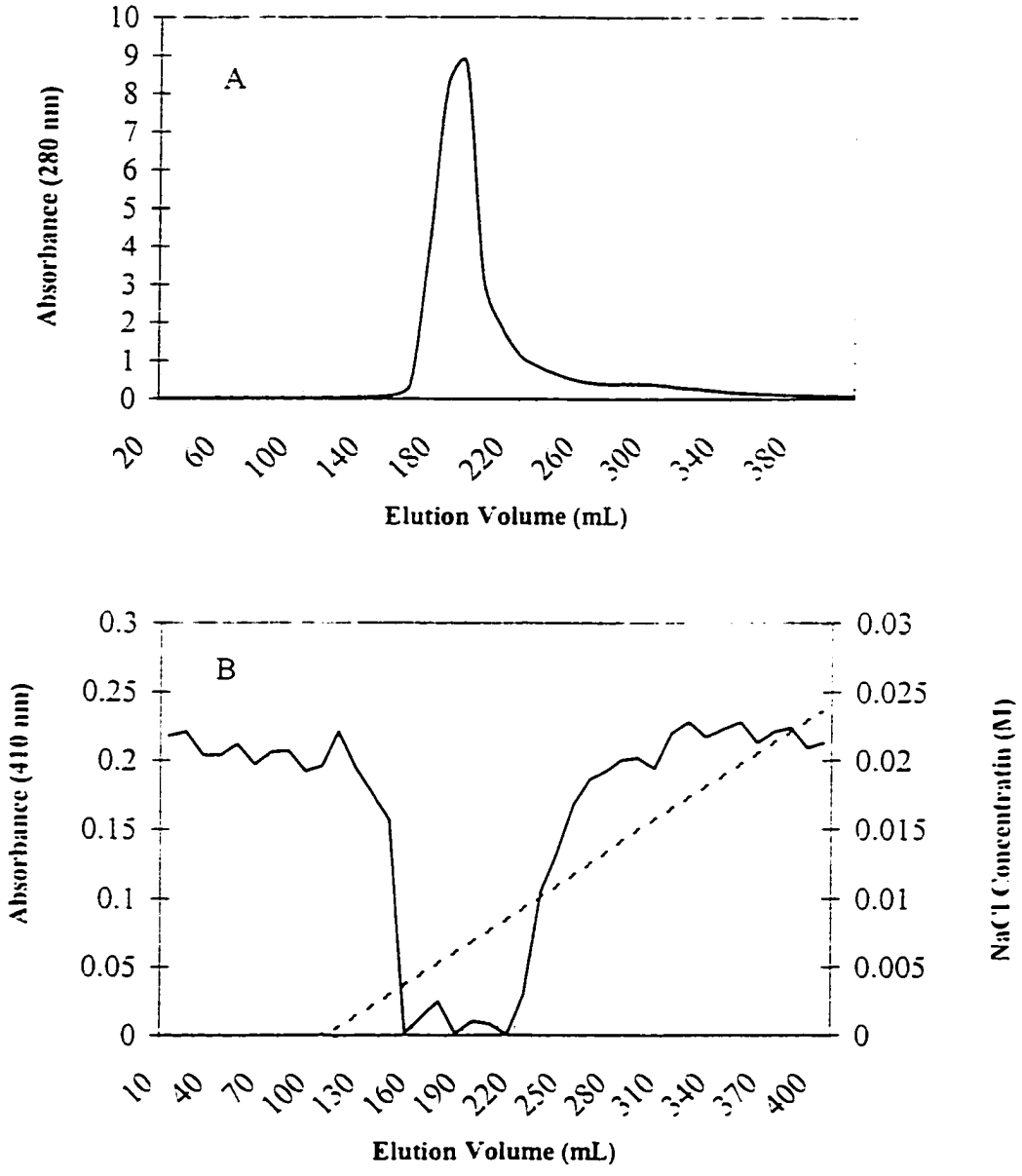
A
Absorbance (280 nm)
Elution Volume (mL)
B
Absorbance (410 nm)
NaCl Concentration (M)
Elution Volume (mL)

Figure 2.

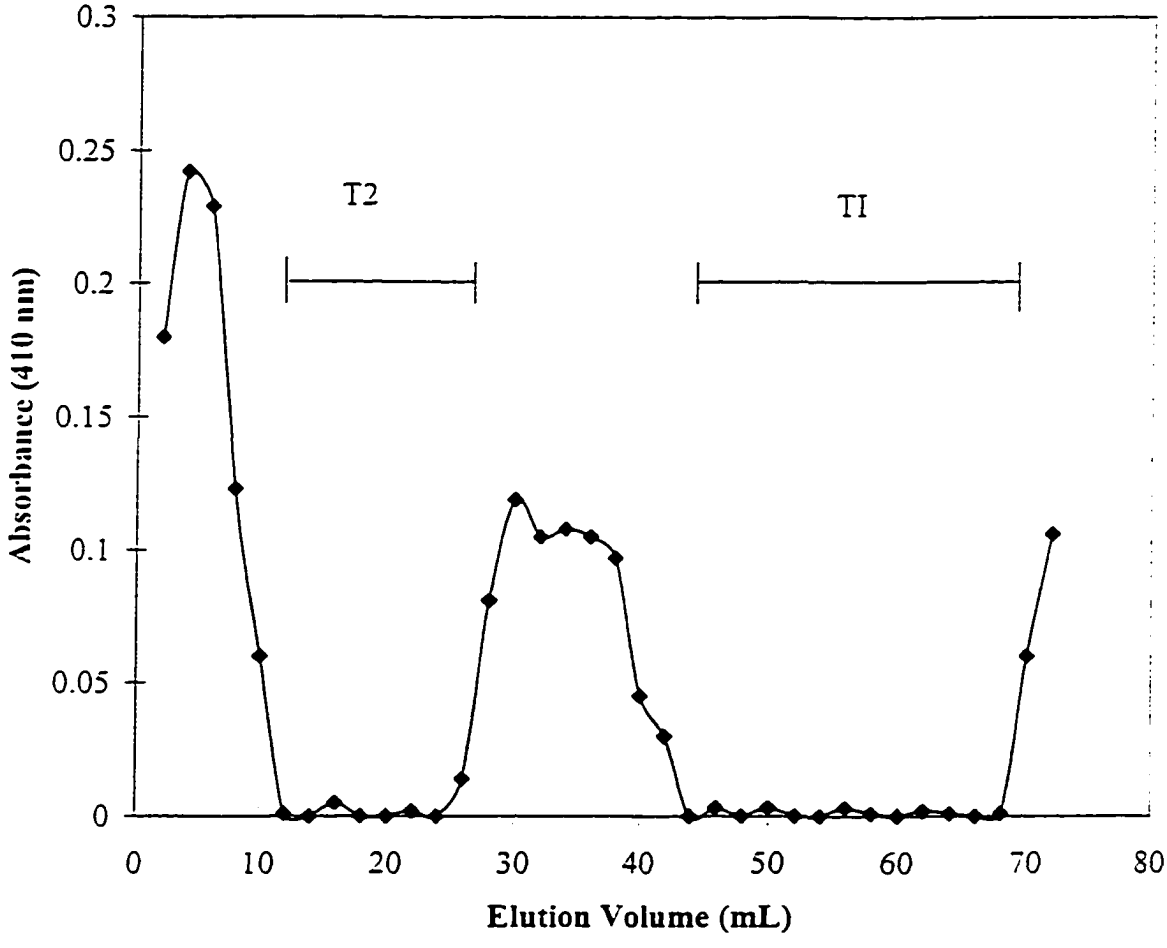
0.3
0.25
0.2
0.15
0.1
0.05
0
Absorbance (410 nm)
T2
TI
0
10
20
30
40
50
60
70
80
Elution Volume (mL)

Figure 3

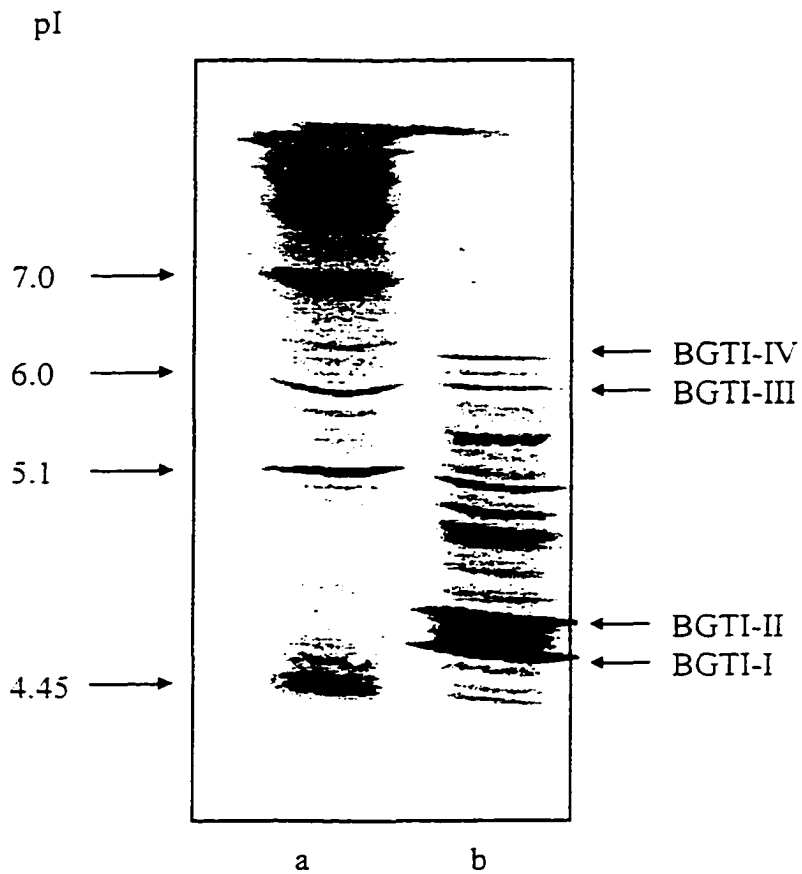

Figure 4.

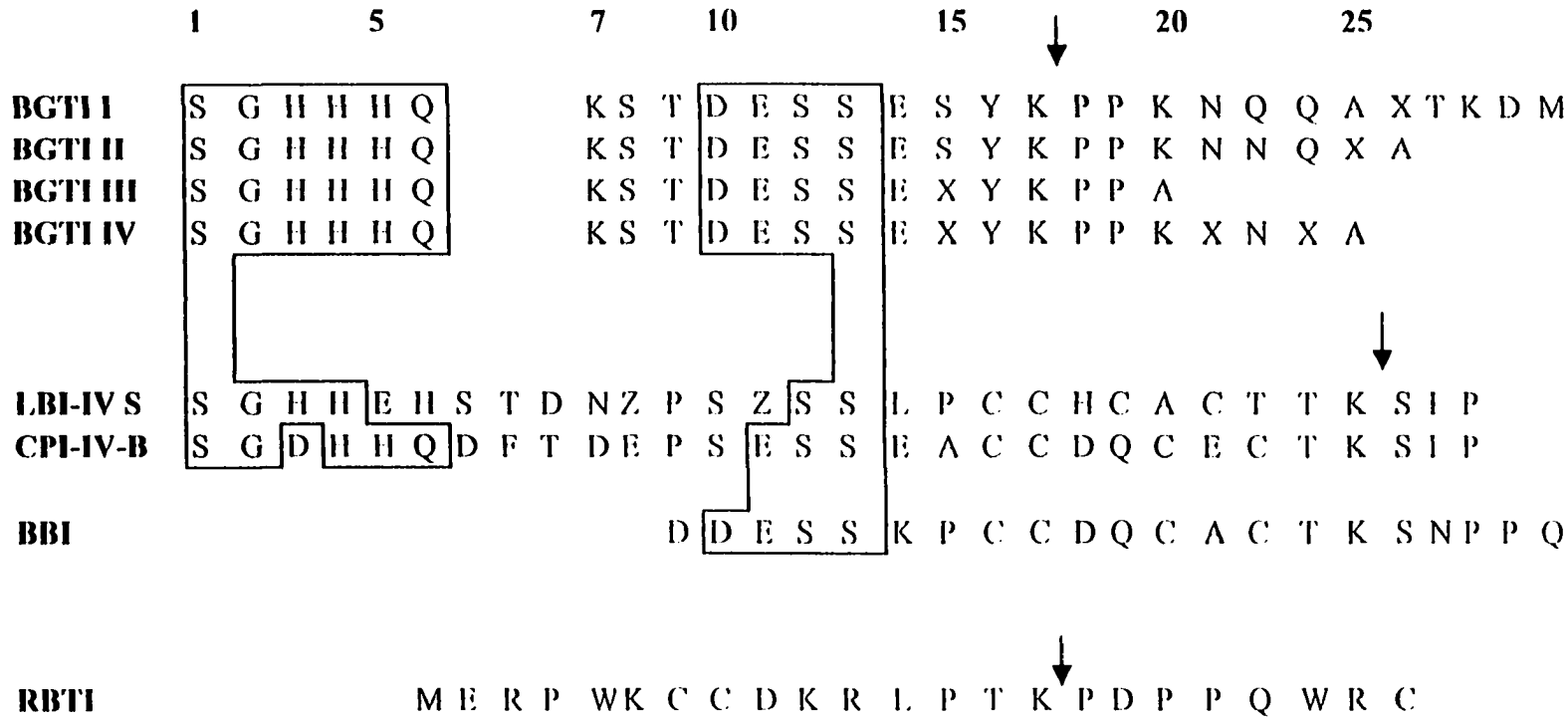
1 5 7 10 15 20 25
BGTI I S G H H H Q K S T D E S S E S Y K P P K N Q Q A X T K D M
BGTI II S G H H H Q K S T D E S S E S Y K P P K N N Q X A
BGTI III S G H H H Q K S T D E S S E X Y K P P A
BGTI IV S G H H H Q K S T D E S S E X Y K P P K X N X A
LBI-IV S S G H H E H S T D N Z P S Z S S L P C C H C A C T T K S I P
CPI-IV-B S G D H H Q D F T D E P S E S S E A C C D Q C E C T K S I P
BBI D D E S S K P C C D Q C A C T K S N P P Q
RBTI M E R P WK C C D K R L P T K P D P P Q W R C

Figure 5.

INACTIVATION KINETICS OF TRYPSIN INHIBITOR IN BAMBARA GROUNDNUT (*Vigna subterranea* (L.) Verdc.) MILK

A paper to be submitted to the Journal of Agriculture and Food Chemistry

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ABSTRACT

An aqueous extract (bambara groundnut milk) was produced from dehulled bambara groundnut seed flour by slurrying the flour and straining to remove undissolved solids. The milk had a pH of 6.7, and protein content of 27% (dwb), lipid 9% (dwb) and carbohydrate 64% (dwb). The viscosity of the milk increased on both heating and cooling. Inactivation kinetics of trypsin inhibitor extracted in the milk were determined by holding the milk at 70, 75 and 80°C. The inactivation of trypsin inhibitor in the milk followed first-order reaction kinetics. The time required to reduce TIA by 90% was D_{70}-677 min, D_{75}-490 min and D_{80}-235 min. The energy of activation for the inactivation of trysin inhibitor was calculated as 106 kJ mol^{-1}.

INTRODUCTION

The bambara groundnut is an indigenous African legume crop that is cultivated for human consumption of its seeds. The crop is popular because it can grow and flourish in arid and semi-arid areas where the soils are too poor to sustain the growth of other legumes. The mature seeds are very hard and require extended periods of cooking before they can be consumed. The high protein content (16-25%) of bambara groundnut seeds makes it an ideal crop for cultivation in areas where protein deficiency is high.

Proteinase inhibitors are found in almost all plant parts but are generally concentrated in storage organs like seeds and tubers (Ryan, 1973). In the bambara groundnut seeds, just like in most legume seeds, the proteinase inhibitors are found chiefly in the cotyledons.

The potential for bambara groundnut milk has been investigated by Brough et al., (1993) who used a sensory panel to determine that bambara groundnut milk was just as acceptable as soymilk, cowpea milk and pigeon pea milk. The presence of trypsin inhibitors in bambara groundnut would lower the nutritional quality of bambara groundnut milk, therefore the inactivation of trypsin inhibitor as a detoxification process in necessary. Detoxification methods include processing, germination, chemical treatment and methods of preparation of soybean dishes. The destruction of TI by heat is a function of temperature, duration of heating, particle size and moisture conditions (Liener, 1994). Most commercially available soybean products intended for human consumption have a residual TI activity of 20% of the original raw beans.

Inactivation of trypsin inhibitor in the milk can be achieved by heat, chemicals or a combination of treatments, however, complete destruction of trypsin inhibitor in bambara groundnut is likely to be detrimental to nutrients as well. Hackler et al. (1965) determined that 90% destruction of trypsin inhibitor results in maximum nutrient retention in soymilk.

In the conventional preparation of soymik, the heating step is designed to achieve maximum reduction of trypsin inhibitor activity. This study involved the production of bambara groundnut milk with the objective of investigating the kinetics of trypsin inhibitor inactivation for the purpose of developing adequate processing techniques that would result in minimum residual trypsin inhibitory activity.

MATERIALS AND METHODS

Materials. Bambara groundnut seeds (*Vigna subterranea*) were purchased locally from Kenya.

Preparation of bambara groundnut milk. Bambara groundnuts (40 g) were manually dehulled and soaked in distilled water at 4°C overnight. The hydrated beans were drained,

rinsed with distilled water and ground with 250 mL water in a Waring Commercial Blender (Dynamics Corporation of America, Hew Hartford, CT) for 1 min. The slurry was filtered using a nylon 100-mesh filter sack (Kawanishi Shoko Co. Ltd., CA) and the extract obtained was bambara groundnut milk (5.2°Brix).

Proximate Analysis. Moisture content was determined by AOAC Method 925.10 (1990). Crude lipid was determined by the AACC Method 30-25 (1983), and nitrogen was determined by micro Kjeldahl, AOAC Method 955.04 (c) and 954.01 (1990) . Carbohydrate was calculated by difference.

Viscosity Measurements. Viscosity of the bambara groundnut milk was measured by a Brookfield DV-II+ Calculating Digital Viscometer, model HB (Brookfield Engineering Labs, Inc., Stroughton, MA) using spindle #2. The milk was heated in a 250 mL beaker with stirring on a hot plate and after every 10 sec., stirring was stopped and viscosity was automatically measured and recorded. Heating was carried out to a temperature of 93°C after which the milk was cooled down to a temperature of 79°C. At least four readings were taken at each temperature.

Heat inactivation. Screw-cap test tubes (12.0 x 1.0 cm) with teflon lined caps were filled with 5.0 mL aliquots of bambara groundnut milk (pH 6.6). Thirteen test-tubes per heat treatment were immersed in water baths maintained at 70, 75 and 80°C. Come-up time was 3 min and samples were removed from the water bath at 15 min intervals up to 180 min and cooled rapidly in an ice water bath. The procedure was carreid out in duplicate.

Trypsin Inhibitor Assay. Trypsin inhibitor activity was measured according to the AACC Method 71-10 (1990) with some modification. The substrate used was *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) (Sigma Chemical Co. St. Louis, MO). The trypsin used was type III from Bovine pancreas (Sigma Chemical Co. St. Louis, MO). Trypsin inhibitory activity (TIA) was determined by measuring the absorbance of appropriately diluted samples at 410 nm in a DU Beckman UV-Visible Spectrophotometer against a standard containing trypsin only. One trypsin unit is arbitrarily defined as an increase of 0.01 absorbance units at 410 nm per 10 mL of reaction mixture under procedure conditions. Trypsin inhibitor activity is expressed in terms of trypsin inhibitor units (TIU). Residual TIA (%) was calculated by dividing the TIA of the heated sample by the TIA of the raw milk and multiplying by 100.

RESULTS AND DISCUSSION

The pH of the bambara groundnut milk produced was 6.7. Proximate analysis of bambara groundnut (Table 1) shows that 60% of the solids are carbohydrate. Protein makes up 27% of the total solids, while 9% is crude lipid. The high proportion of carbohydrate in bambara groundnut milk presents difficulties during heating because of the tendency of the carbohydrate to gel at pasteurization temperatures (>90°C). When bambara groundnut milk is subjected to heating, the viscosity begins to increase dramatically after 75°C (Figure 1). The viscosity continues to increases both during heating and cooling of the milk. The increase in viscosity of bambara groundnut milk may be attributed to the high percentage of starch extracted into the milk. At high temperatures, starch gels and causes an increase in viscosity of the milk.

Heating and holding bambara groundnut milk at 80°C was more effective at inactivating trypsin inhibitor than heating at either 75°C or 70°C (Figure 2). When the temperature was increased from 70°C to 75°C, residual trypsin inhibitor activity was reduced from 57% to 49% in 15 min. An increase of temperature from 75°C to 80°C resulted in a very rapid inactivation of trypsin to 13% in 15 min. At 75°C and 70°C, heating for 3 hours resulted in 24% and 32% residual TIA respectively. An increase in the holding temperature of bambara groundnut milk resulted in the TIA being more quickly inactivated, an effect which was similar to that reported for soymilk (Obara and Watanabe,1971; Liener and Tomlinson 1981). During the first 15 min of heating there is a rapid loss of TIA in the milk, and subsequently, the TIA is lost more gradually. Further studies designed to investigate the loss of TIA in the first 15 min of holding are required.

Martino-Ferrer and Ferrer (1983) studied the thermal inactivation of purified bambara groundnut trypsin inhibitor in aqueous solution at 90°C. They found that the inhibitor was very resistant to thermal denaturation since exposure for 10 min at 90°C resulted in 50% residual trypsin inhibitory activity, and after 20 min, 20% residual activity is retained. DiPietro and Liener (1989) investigated the effect of heat inactivation on purified Kunitz soybean trypsin inhibitor (KSTI), purified Bowman-Birk trypsin inhibitor (BBI) both in solution and an extract of raw soyflour. They found that trypsin inhibitor in both solutions of

purified inhibitor were less susceptible to heat inactivation than the inhibitor in raw soy flour extract. The behavior of trypsin inhibitor in a pure solution is not similar to the behavior in a more complex system like an extract. It is possible that the inhibitors in a complex system interact with other materials in the extract thus promoting the inactivation of the inhibitors.

The kinetics of thermal inactivation of bambara groundnut milk were modeled on first-order reaction kinetics. During heating of bambara groundnut milk at 70, 75, and 80°C, the TIA declined rapidly during the first 15 min although the rates of inactivation (k-value) were different at the different temperatures (Figure 2, Table 1). The reaction rate constants increased as holding temperature increased which is expected because as holding temperature is increased, more thermal energy is being transferred. The greatest reaction rate of inactivation was at 80°C. As the temperature increased from 70°C to 75°C to 80°C , the k-values increased by 38.2% and 109%, respectively. An increase in temperature increases the kinetic energy of the molecules and hence increases the rates of inactivation. The Q_{10} value determined for the inactivation reaction (70°C-80°C) of bambara groundnut trypsin inhibitor was 2.9.

The heating time required to inactivate 90% of the TIA (D-value) at a specific temperature was calculated for bambara groundnut milk. D_{70} is three times D_{80}, and D_{75}, is two times D_{80},. As holding temperature was increased from 70°C to 75°C to 80°C, the D-value at each temperature decreased, emphasizing the benefits of higher temperatures. Although high temperatures enhanced the heat inactivation of trypsin inhibitor in bambara groundnut, the temperatures used (70, 75, 80°C) were too mild to give adequate destruction within a reasonable time period. Kwok et al. (1993) found that D_{93} at pH 6.5 for soymilk was 60 min.

The activation energy (E_a) of trypsin inhibitor in soymilk was determined to be 77.4 kJ mol^{-1} (Hackler, et al., 1965). Bambara groundnut milk had an E_a of 106 kJ mol^{-1} which is higher than that found by Hackler (1965) but is within the range of 42-135 $kJmol^{-1}$ for the activation energies for other quality changes in foods (Kwok and Niranjan, 1995). The E_a for bambara groundnut milk trypsin inhibitor is relatively small indicating that the inactivation of bambara groundnut milk trypsin inhibitor is not very temperature dependent. Other constituents in the extract contribute to the inactivation of trypsin inhibitor. Friedman et al.

(1982) showed that free thiol groups available for disulfide bond interchange aid in the inactivation of the inhibitors. DiPietro and Liener (1989) proposed that soy extract has a higher overall protein concentration compared to pure inhibitor solutions increasing the possibility of noncovalent protein-protein interactions leading to loss of inhibitory activity through denaturation and precipitation.

CONCLUSIONS

Although heating of bambara groundnut milk at 70, 75 and 80°C resulted in inactivation of trypsin inhibitor, the time periods required to reduce the activity to 10% is very long. The long heating times could lead to amino acid degradation and insolubilization of proteins. Bambara groundnut milk had a high proportion of starch which gelatinizes at normal pasteurization temperatures. The gelatinization of starch makes it difficult to use high temperatures that would inactivate trypsin inhibitor in a shorter time period. Therefore, if higher temperatures cannot be used, there is the risk of producing a milk with poor nutritional quality. Future research should look at the possibility of starch removal and the subsequent use of higher temperatures to inactivate trypsin inhibitors should be investigated.

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Hackler, L. R., Van Buren, J. P., Streinkraus, K. H., El Rawi, I. and Hand, D. B. Effect of heat treatment on nutritive value of soymilk protein fed to weanling rats. *J. Food Sci.*. **1965**, 30, 723-728.

Kwok, K. C., Qin, W. H. and Tsang, J. C. Heat inactivation of trypsin inhibitors in soymilk at ultra high temperatures. *J. Food Sci.*. **1993**, 58, 859-862.

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Martino-Ferrer, di M. and Ferrer, A. Trypsin inhibitor in bambara pea. *Phytochemistry*. **1993**, 22, 813-816.

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FIGURE LEGENDS

Figure 1. Change in viscosity of bambara groundnut milk with change in temperature using continuous monitoring. Solid line indicates the heating process of the milk up to 93°C after which the milk is allowed to cool (broken line) to 79°C. Data points are means of four readings.

Figure 2. Loss of inhibitory activity of bambara groundnut trypsin inhibitor during heat treatment of bambara groundnut milk at different temperatures.

Figure 3. Determination of inactivation rate constants for trypsin inhibitor in bambara groundnut milk.

Figure 4. Arrhenius plot for the inactivation of trypsin inhibitor in bambara groundnut milk.

Table 1. Proximate Composition of Bambara Groundnut Milk

Component	%	Std Dev
Total Solids[a]	7.5	1.0
Crude Lipid[b]	9.3	-
Protein (N x 6.25)[c]	26.8	10.8
Carbohydrate[d]	63.9	-

[a] Mean of three samples
[b] One sample
[c] Mean of three samples
[d] By difference

Table 2. Reaction kinetics of trypsin inhibitor inactivation in bambara groundnut milk

Holding Temp (°C)	k x 10^{-3} (min^{-1})	D-value[a] (min)	E_a[b] (kJ mol^{-1})
70	3.4	677	106
75	4.7	490	
80	9.8	235	

[a] D = 2.303/k

[b] ln k = ln k_o - E_a/RT; E_a/RT = slope of Arrhenius plot; R = 8.31 J/K mol

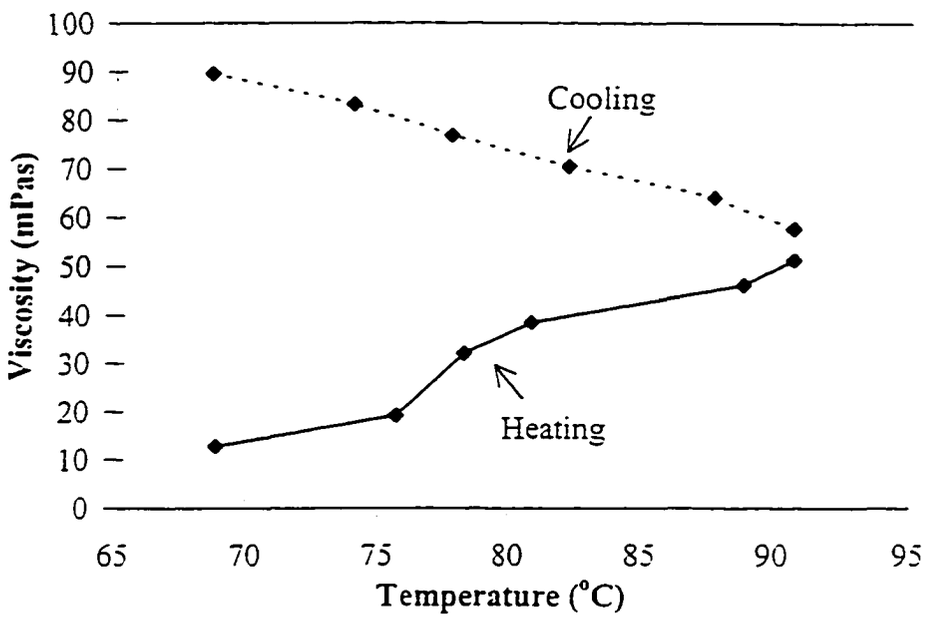
100
90
80
70
60
50
40
30
20
10
0
Viscosity (mPas)
Cooling
Heating
65
70
75
80
85
90
95
Temperature (°C)

Figure 1

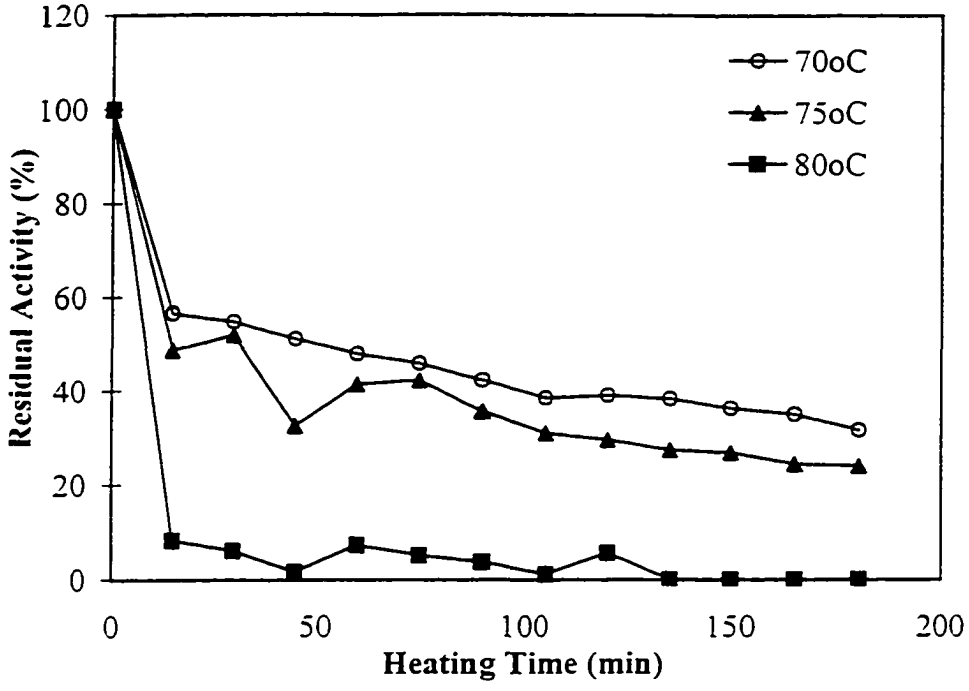
120
100
80
60
40
20
0
Residual Activity (%)
0
50
100
150
200
Heating Time (min)
70oC
75oC
80oC

Figure 2.

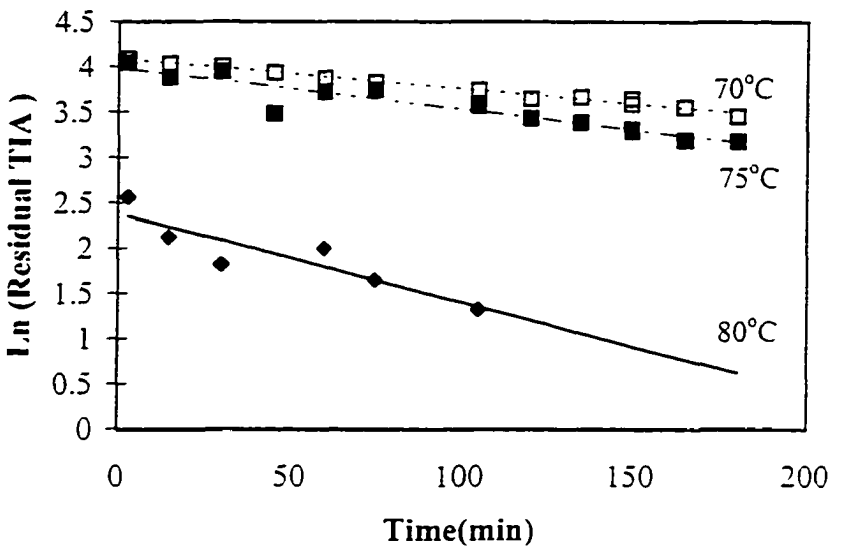
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Ln (Residual TIA)
0
50
100
150
200
Time(min)
70°C
75°C
80°C

Figure 3.

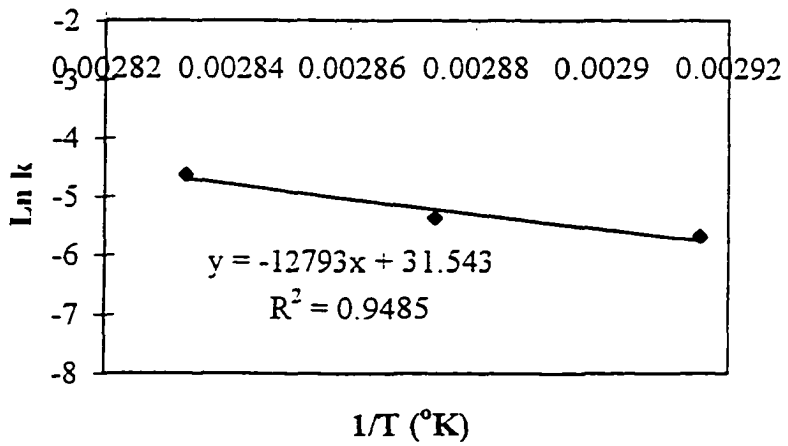
-2
-3
-4
-5
-6
-7
-8
Ln k
0.00282
0.00284
0.00286
0.00288
0.0029
0.00292
y = -12793x + 31.543
R2 = 0.9485
1/T (°K)

Figure 4.

GENERAL CONCLUSIONS

Bambara groundnuts contain antinutritional factors similar to other legume seeds. The antinutrients found in bambara seed coats are tannins and the predominant ones proanthocyanidins. The monomeric units of bambara groundnut proanthocyanidins are dihydroflavanols, of which six units polymerize to form the polymer. Assays to determine the biological activity of the PA found that it was competitively inhibited from binding with protein in the presence of competitors like pepsin and BSA.

Four tannin assays were used to determine the tannin content and biological activity of eight bambara groundnut samples. All four assays were able to distinguish between relatively high and low tannin contents, with the distinction blurring for the intermediate tannin contents. Functional group assays (vanillin and butanol) gave statistically similar results for tannin contents of the eight samples.

No relationship was found between the tannin content and seed coat color and tannin content and seed weight. There was also no relationship between the tannin content and geographic origin of the seeds. One seed sample, Uganda 298, consistantly gave much higher tannin contents for all four tannin assays indicating the prossibility of differences in its tannin types.

Bambara groundnuts contain at least four major trypsin isoinhibitors which have a molecular weight ranging between 7000 and 10,000 Da making them likely to belong to the Bowman-Birk family of inhibitors. However, Bowman-Birk inhibitors are known to have a high number of disufide bridges which were absent in the inhibitor from bambara groundnut. It is possible that bambara groundnut inhibitors represents yet another family of inhibitors previously unencountered.

Our studies focused on the trypsin inhibitory activity of the inhibitors although Bowman-Birk inhibitors are known to be double-headed. The probable trypsin reactive site of bambara groundnut trypsin inhibitor was tentatively identified as Lys17-Pro18 but further studies are needed to definitely identify the site. Future studies should determine the

chymotrypsin inhibitory activity of bambara groundnut proteinase inhibitors and also sequence the whole inhibitor in order to determine the chymotrypsin reactive site.

Plant proteinase inhibitors are known to be specific against various proteases. Since our study used only bovine trypsin as the protease, we do not know the specificity of bambara groundnut typsin inhibitors towards other proteases and future studies should be designed that can answer this question.

Although heating of bambara groundnut milk at 70, 75 and 80°C resulted in inactivation of trypsin inhibitor, the time periods required to reduce the activity to 10% are very long. The long heating times could lead to amino acid degradation and insolubilization of proteins, and with bambara groundnut milk, if starch is not removed first, long heating times lead to starch gelatinization. The possibilities of starch removal and the subsequent use of higher temperatures should be investigated. With proper heating, to inactivate proteinase inhibitors, bambara groundnut milk would have a better nutrient composition.

APPENDIX

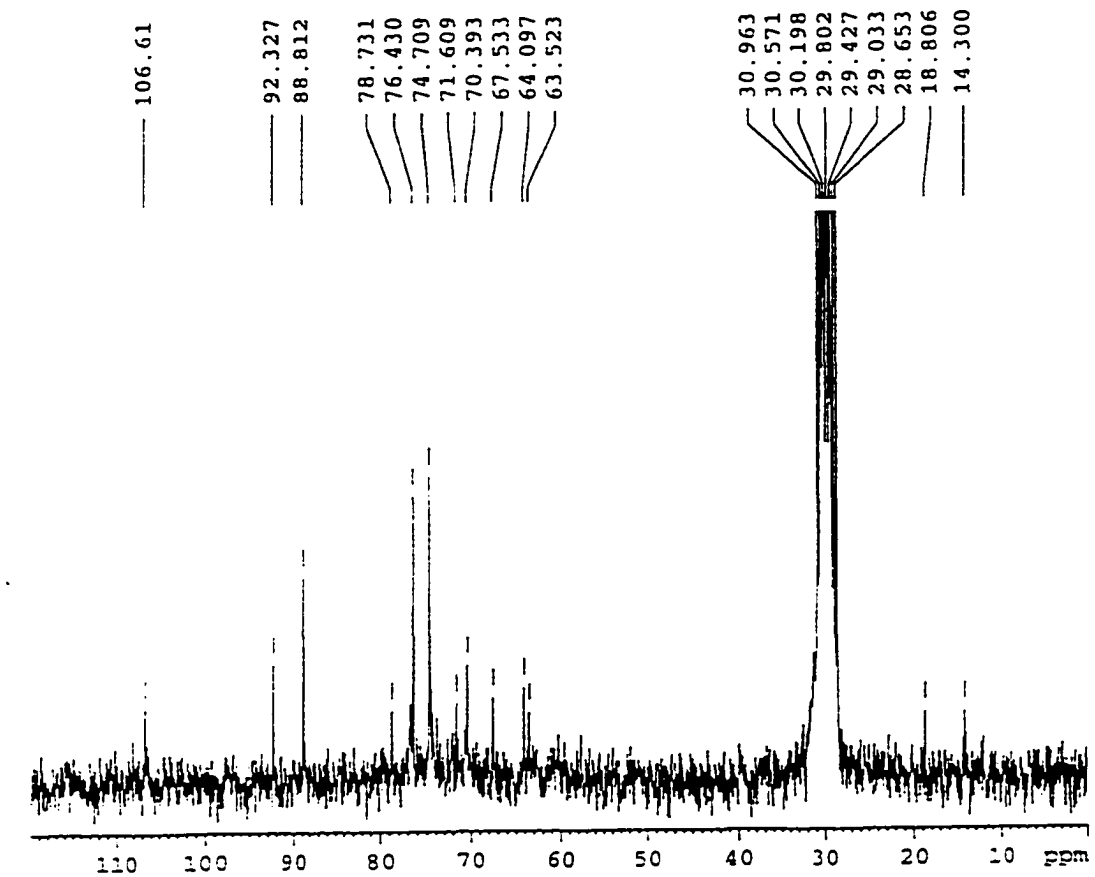

Figure A-1. ^{13}C NMR spectrum of purified proanthocyanidin from Bambara Groundnuts

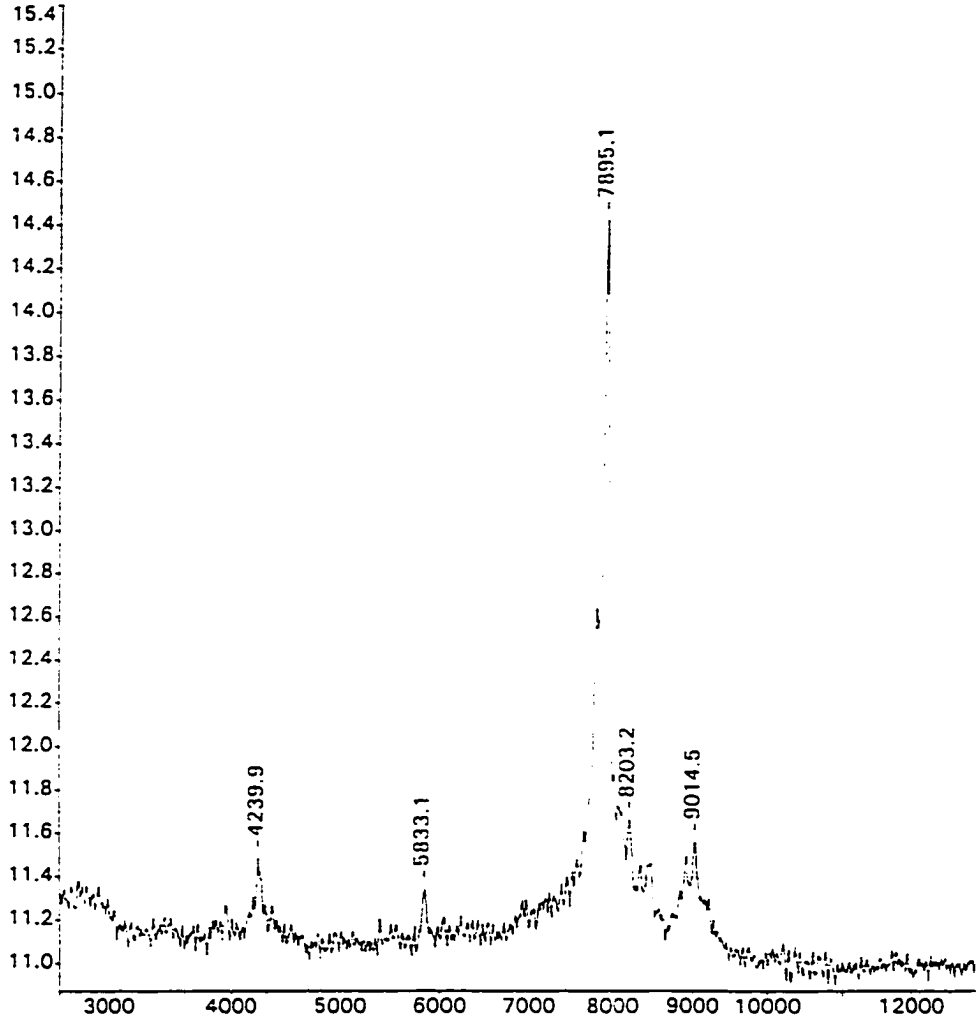

Figure A–2. Mass spectrum of BGTI I

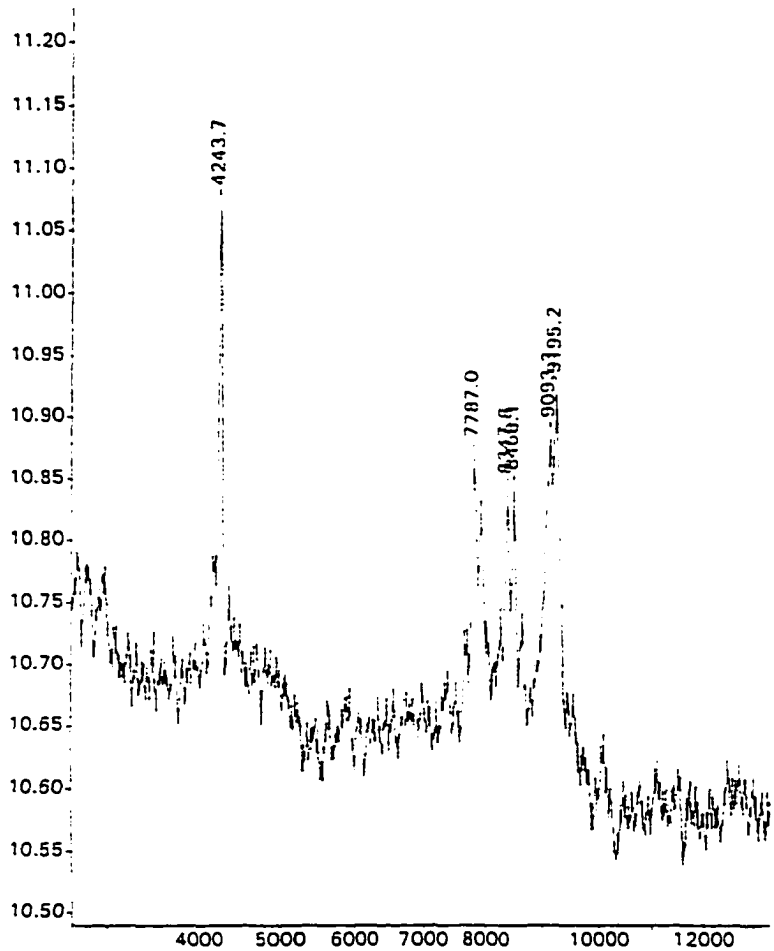

Figure A-3. Mass spectrum of BGTI II

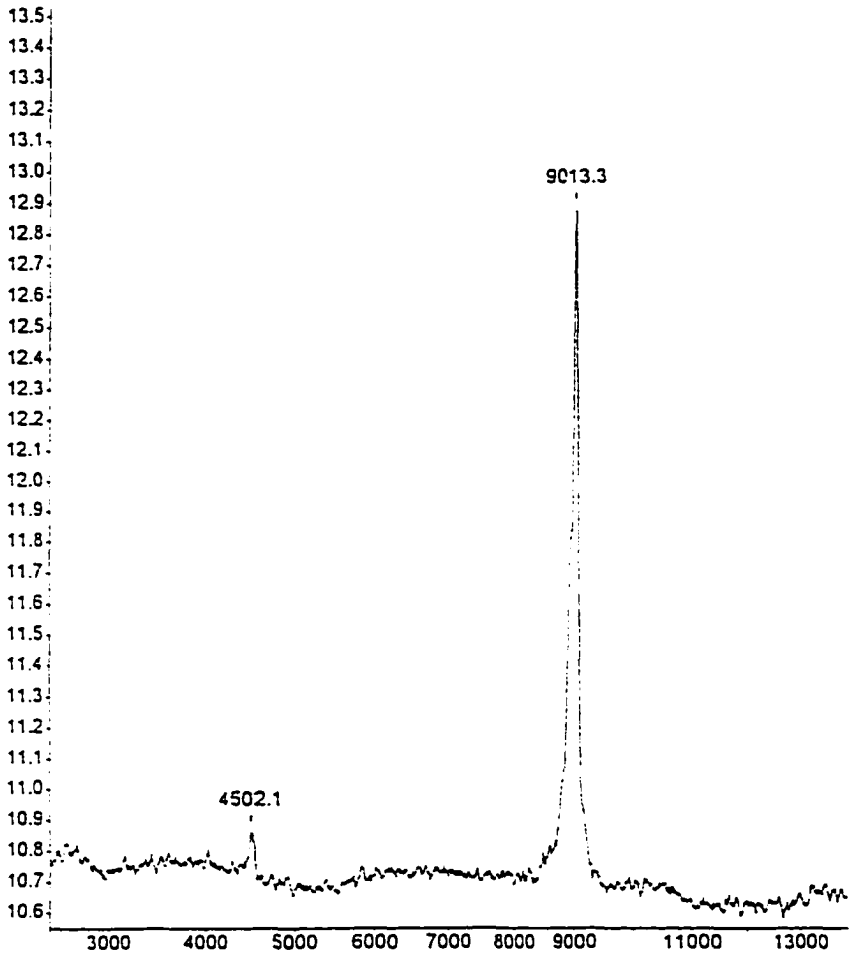

Figure A-4. Mass spectrum of BGTI III.

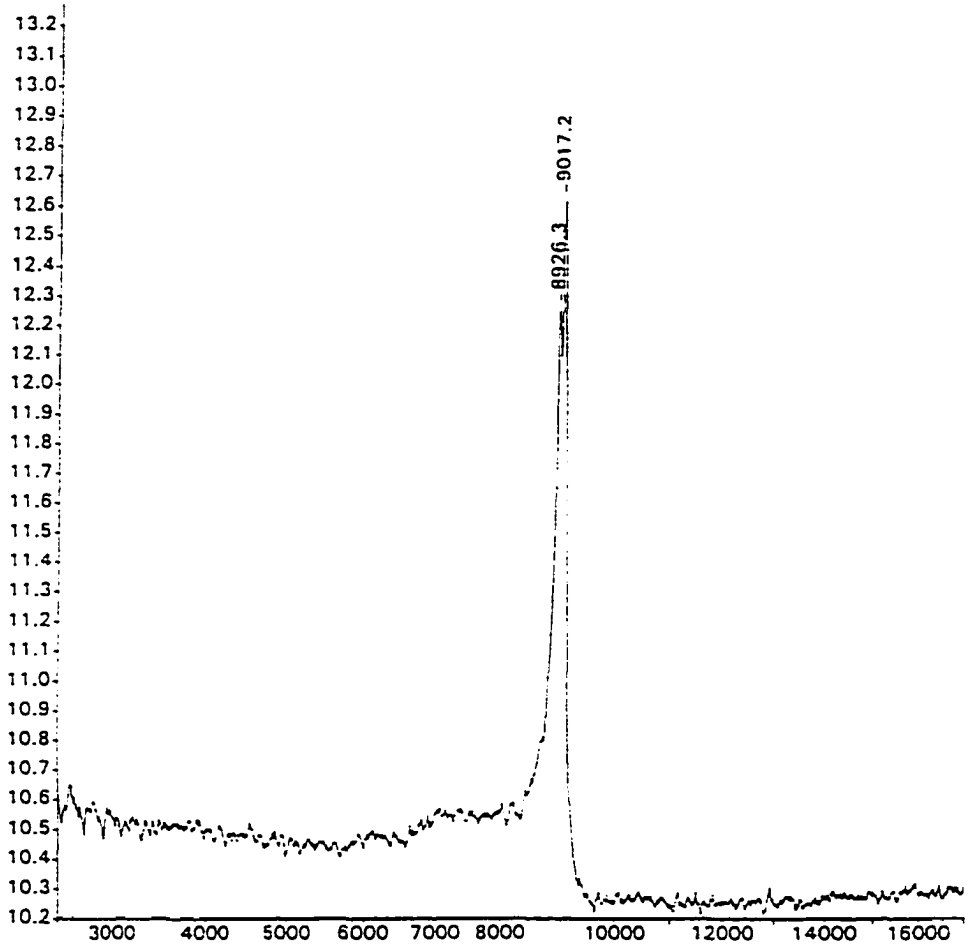

Figure A-5. Mass spectrum of BGTI IV.

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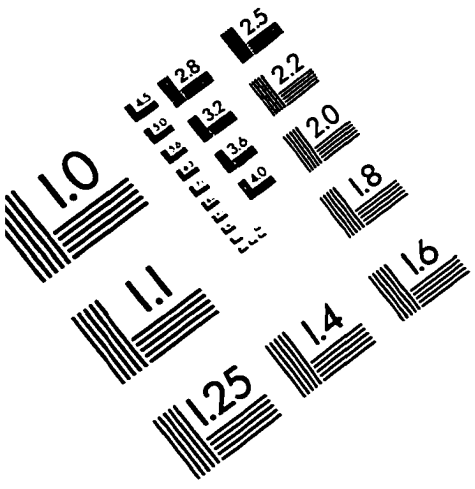
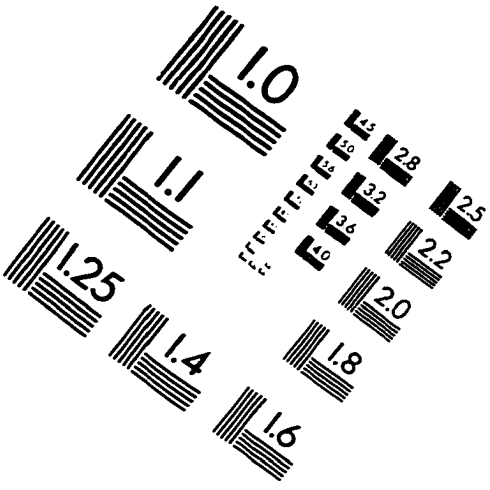
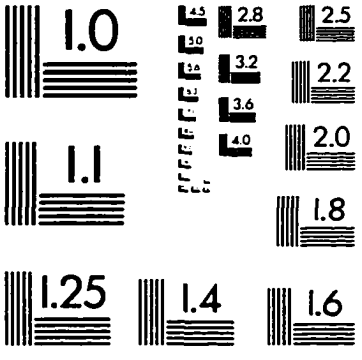
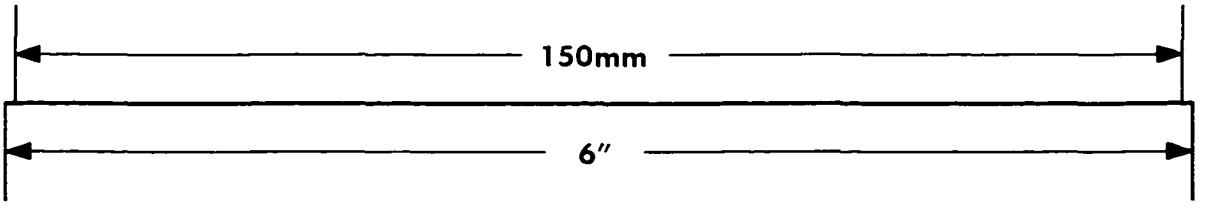
To the Almighty, faithful to the end!

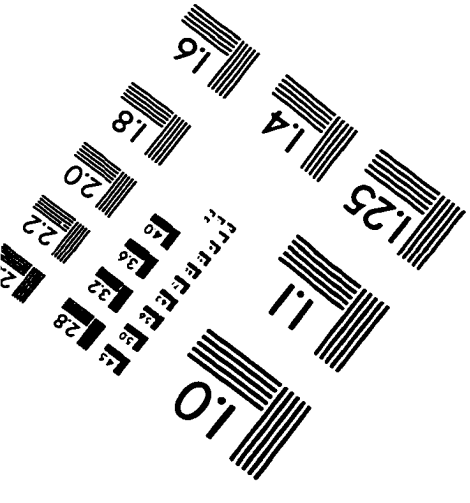
IMAGE EVALUATION TEST TARGET (QA-3)

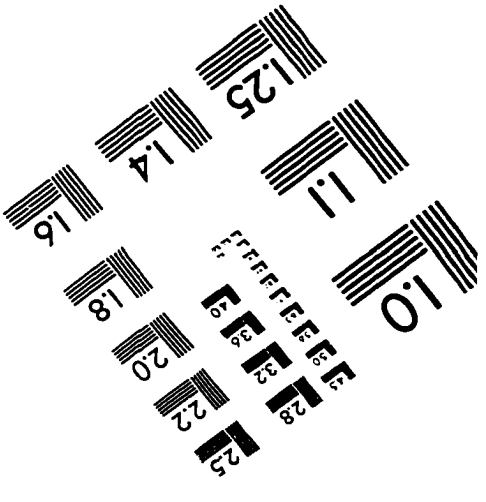












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