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JANET REGINA WOZENSKI for the MASTER OF SCIENCE
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Title: PHYTIC ACID (MYOINOSITOL HEXAPHOSPHATE) AND PHYTASE ACTIVITY IN FOUR COTTONSEED PROTEIN PRODUCTS

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Phytic acid, as myoinositol hexaphosphate, (mgm/100gm) found in each product was: 2860 in a glanded flour, 4290 in a glandless flour, 3350 in an air-classified glandless flour, and 2490 in toasted seed kernels. The glandless flour was significantly higher in phytic acid than the glanded flour (5% level), and than the seed kernels (1% level).

The glanded flour was the only product that did not exhibit phytase activity. Inorganic phosphorous released (mgm x 10^{-3} per 50 mgm of product) were: 63.4 for the air-classified glandless flour, 41.4 for the kernels, 38.7 for the glandless flour, and less than 1.0 for the glanded flour. The amount of inorganic phosphate released by the glandless air-classified flour during enzyme activity was significantly higher at the 1% level than the amounts released by either the glandless flour or the toasted kernels. No other significant differences

were found at the 5% level between the amounts of inorganic phosphorous released by the other products demonstrating enzyme activity. The amounts of these cottonseed products recommended for use in recipe formulations are not enough for their phytic acid content to be of nutritional significance. The amount of phytic acid ingested with the kernels when they are used as a snack food may be cause for some concern.

Phytic Acid (Myoinositol Hexaphosphate) and Phytase Activity in Four Cottonseed Protein Products

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Janet Regina Wozenski

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PHYTIC ACID (MYOINOSITOL HEXAPHOSPHATE) AND PHYTASE ACTIVITY IN FOUR COTTONSEED PROTEIN PRODUCTS

INTRODUCTION

Cottonseed is one foodstuff, among many, that has been given considerable attention as an economical source of protein (3, 57, 69). Cotton is a staple crop in both temperate and tropical climates, and can grow where reliable, inexpensive protein products are scarce. The cotton seed has become an increasingly important by-product of the cotton crop. Breeding and processing methods have made it possible to greatly reduce or nearly eliminate the gossypol content of the seed (16, 69). The seed is an important source of polyunsaturated oil (2).

The structure of the cottonseed shows some compartmentalization. The storage proteins in the seed are present in aleuron grains (39, 64). Within the aleuron grain lie smaller discrete areas, called globoid bodies. The globoids in the cottonseed have been shown to contain a total of 14% phosphorous, 60% of which was in the form of myoinositol hexaphosphate (phytic acid) (64), the structure of which is given in Figure 2. Because the globoid bodies are included within the aleuron grain, air-classification concentration of the intact aleuron grains will also concentrate the globoid bodies (69). Use of

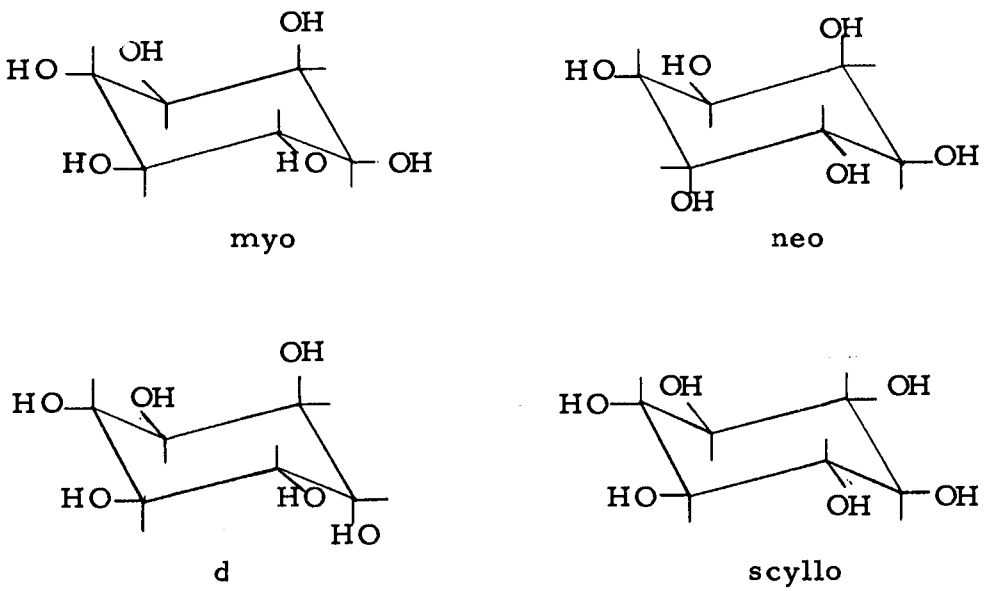

Figure 1. Conformation formulas for inositols (29)

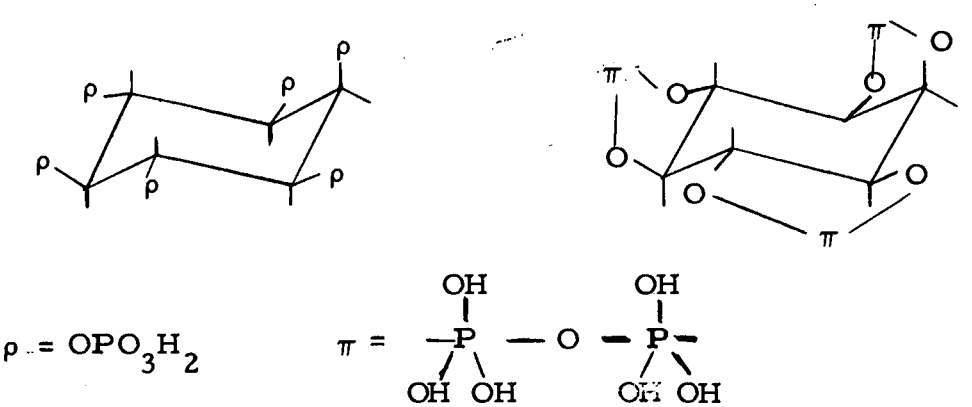

Figure 2. Proposed structures for myoinositol hexaphosphate (51)

cottonseed products for their protein content may result in a simultaneous increase in dietary phytic acid.

Myoinositol hexaphosphoric acid will bind divalent cations, such as Ca^{++}, Fe^{+++}, and Zn^{++}, and has been shown to produce deficiencies of these elements in animals and man (28, 33, 45, 62, 75, 77).

Inositol hexaphosphoric acid will form complexes with proteins at low pH and reduce the solubility of the proteins in the acid pH range (13, 20).

When the phosphate groups are removed from inositol hexaphosphoric acid, it will no longer bind cations (74). Some plants and animals have a comparatively non-specific enzyme, phytase, that will dephosphorylate phytic acid and thereby render it harmless as a cation chelator, as reviewed in Sloane-Stanley (100).

The purpose of this study is to determine both the amount of inositol hexaphosphoric acid and the relative phytase activity in several cottonseed products that are produced primarily for their protein content. This information is of interest as a guide for evaluating the nutritive value of the product as a protein supplement. Since inositol hexaphosphate reacts with cations and proteins, knowledge of the amount of inositol hexaphosphate in a protein concentrate, might help to anticipate or explain some of the functional properties of the product.

A survey of the literature has shown no data published on the amount of inositol hexaphosphate or phytase in the high protein cottonseed products such as those described by Martinez et al. (69).

REVIEW OF LITERATURE

Cottonseed

Structure

The intracellular components of the cottonseed are highly compartmentalized (64, 69, 111). In addition to the normally enclosed cellular structures (e. g., nuclei, mitochrondia), much of the cell's protein, lipid, and phosphorous are each encased in their own packages. The lipid particles are called spherosomes and are defined by a coarse, irregular membrane that is not a unit membrane (69). The protein bodies, or aleuron grains, appear to be bordered by a unit membrane (111). Within the aleuron grains are located smaller discrete areas, called globoid bodies (111). These bodies have been isolated and found to be low in protein, fat, and carbohydrate, but contain 14. 2% phosphorous and about 10% metals (64). Of the total phosphorous, 97. 5% was organic phosphorous, and 60% of the organic phosphorous was identified as myoinositol hexaphosphate. The globoid bodies do not appear to be surrounded by a membrane (111).

Two developments have spurred renewed interest in cottonseed as a food. Breeding has produced glandless strains of cotton, thus completely eliminating the gossypol (57, 69). The second innovation is the Liquid Cyclone Process, which greatly reduces the amount of

gossypol in glanded varieties. Final concentrations of gossypol in the cyclone processed meal are 0.04% or less of free gossypol and less than 0.3% total gossypol (69).

Products Available

Cotton seed protein products range from the meals, which are 40-50% protein (80), to the protein isolates, which are nearly all protein (69). Flours are roughly 50-60% protein, aqueous concentrates 65-75%, air-classified concentrates and Liquid Cyclone Process flour, 70% (69). A nut-like product has been developed from undefatted, glandless cottonseed kernels, that are made edible by toasting, roasting, or frying. The protein content is approximately 33% (57, 18). Meals for feed, the 50-60% food grade flours, and the toasted kernels are available commercially.

The flours are recommended for many bread and cake-like products (104, 105, 106). Their performance in breads has been evaluated, both as a protein supplement to wheat flour (71), and in a low-gluten bread formulation (55, 59). The flour has been shown to be effective in delaying rancidity in raw pastry mixes and in baked pastry (85). Data from the Traders Oil Mill Company (105) on the functional characteristics of their glanded cottonseed flour (Proflo) include moisture binding ability, antioxidant properties, and reduction in fat absorption when Proflo is included in a formula.

The toasted cottonseed kernel product mentioned has been

consumed as a snack food, and incorporated in spreads, candies, cookies, and other bakery items (57).

Evaluation of three different glandless cottonseed flours, by determination of their protein efficiency ratio, showed them to be equal in nutritive value to a casein control (Hopkins, cited in Martinez et al. (69)). A low-cost high protein mix, with 25% cottonseed flour, significantly improved growth rate in rats over that of a rice diet typical of that available to poor Indians (35).

Phytic Acid

Structure

Esters of inositol with less than six phosphate groups are found, though the hexaphosphate is the most common, and the inositol itself may exist in one of several isomeric forms (28, and Figure 1). Phytic acid soil constituents are a more complex mixture than what is usually found in plants (7, 28). Myoinositol is the isomer commonly found in plants (28, 29). In this report, phytic acid will refer to the hexaphosphate of myoinositol.

The structure of phytic acid is a continuing source of debate. The two structures which have received the most support are that proposed by Anderson, and one put forth by Neuberg (8, 28, and Figure 2). The evidence that has accumulated has led to support for either one structure or the other (reviewed in Cosgrove (28)).

More recent papers have lent additional weight to the Anderson model (8, 51). The stoichiometric calculations used in the ferric salt precipitation method for phytic acid determination, to be used in this report, assume the structure of phytic acid to be myoinositol hexaorthophosphate. Hence, the Anderson structure will be used in this paper.

Occurrence

Since 1872, it has been known that the aleuron grains in some plants contained an organic phosphorous compound that existed as a mixed calcium-magnesium salt (28, 39). This compound was later identified as the salt of myoinositol hexaphosphate (5) and generally came to be called phytic acid. The mixed calcium-magnesium salt of the acid was known as phytin. Phytin is a principal storage form of phosphorous in many seeds and grains, and has been found in other plant storage structures, such as potatoes (98, 100). It may contain nearly 90% of the phosphorous present in the seed (reviewed in (28)). Seeds usually have large amounts of organic phosphorous, whereas the vegetative tissues contain predominantly inorganic phosphorous (60). The organic phosphorous in the seeds is usually a phytic acid salt, but in the stems and stalks of the plant, the organic phosphorous is found mainly in nucleic acids (60). The amount of phytin phosphorous in the seed or grain is greatly reduced during germination and later

growth of the plant (19, 38), supporting the concept of phytin as a storage compound for phosphorous in the seed.

The amounts of phytic acid in many plants used for food or for feed have been determined (10, 11, 12, 26, 32, 38, 42, 64, 65, 73, 74, 75, 80, 109). Data on the amounts of phytic acid in cottonseed protein products are scarce. Available data are included in Table 1.

Both cottonseed itself and the feed meals have several times more phytic acid than do wheat or oats, both whole and processed. No data are available for either phytic acid content or phytase activity in food grade cottonseed protein products. The phytase activities of wheat and oats are very different even though their phytic acid contents are similar.

Effect in Food Systems

The metal and protein complexing nature of phytic acid has caused some attention to be focused on it as a component affecting the physical properties of food systems.

Saio et al. (97) has studied the relationships of calcium and phytic acid in the soybean, in order to determine the role of phytic acid during the calcium-initiated coagulation of soybean protein in the making of tofu. He found that increasing the amount of phytic acid present before coagulation of the protein increased the weight

Table 1. Reported phytic acid contents and phytase activity of several seeds

Seed	Phytin or phytic acid mgm/100 gm	% of P as phytin	Time to hydrolysis of 50% of phytic acid	References
Cottonseed minus seed coat, dried				
Deltapine	2301	-[c]	-	(38)
Paymaster	3053	-	-	(38)
Acala	3397	-	-	(38)
Cottonseed meal, 41% protein (solvent extracted)	2700	± 70	-	(80)
50% protein	3300	± 71	-	(80)
Wheat Kernels[d]				
Germ	-	12.9	-	(82)
Endosperm	-	2.2	-	(82)
Aleurone	-	87.1	-	(82)
Hull	-	0	-	(82)
Wheat flour				
100% extraction	-	70	-	(75)
85% extraction	-	55	-	(75)
80% extraction	-	47	-	(75)
Wheat flour various brands	660-1280	-	-	(10)
Wheat				
English	-	-	12 min[a]	(74)
Manitoba	-	-	14 min[a]	(74)
Wheat	-	-	7.1 hr[b]	(74)
Oats, green	-	-	7.1 hr[b]	(74)
Oats, green	-	-	11-15 hr[a]	(74)
Oats, kilned	-	-	30-46 hr[a]	(74)
Oats, hulled	1200	78	-	(80)
Oats, whole and rolled	700	56	-	(80)

[a]phytase present in food as hydrolyzing agent

[b]2 N boiling HCL as hydrolyzing agent

[c]data not supplied

[d]Distribution of phytate phosphorous in the component parts based on air dry weight (82)

of the curd formed, increased the calcium and phosphorous content, and produced a softer than normal gel. Increasing the amount of phytic acid present increased the amount of calcium ions necessary to start the coagulation.

Calcium phytate will remove black spots from maraschino cherries and prevent their further discoloration in the presence of iron (25), and can also delay darkening initiated by copper and aluminum ions (53). Phytic acid, as a polyphosphoric acid, when maintained at the proper pH, prevented the formation of phenol-iron complexes and thereby retarded discoloration in fruits and vegetables. (79, abstracted in Food Science and Technology Abstracts 3:4J350, 1971). Evans et al. (40) showed it to be effective in controlling metallic contamination and oxidative instability of edible oils. Niwa et al. (81, abstracted in Biological Abstracts 49:87502, 1968) demonstrated the pH dependent ability of phytate to protect ascorbic acid from metal ions, even with heating.

Mattson's hypothesis (72) correlating phytic acid and cookability of peas has not been well supported (28, 92, 93). Mattson proposed that phytic acid complexed with calcium and magnesium preventing them from cross-linking the uronic acid groups of pectin, a phenomenon thought to be responsible for the resistance of some peas to softening during cooking. Rosenbaum and Baker (92) could not find a relationship between phytic acid in individual peas and their

cookability. Phytic acid does seem to be correlated with softening of boiled potatoes after cooking, through its action as a calcium precipitant (reported in Cosgrove (28)).

Nutritional Aspects

Concern with the antinutritional properties of phytic acid stems from its ability to combine with metal ions, often rendering the ions metabolically unavailable to the animal and inducing deficiencies. Zinc deficiencies in chickens (61, 77), magnesium deficiency in man (91), and calcium deprivation in many animals and man (110) have been induced or aggravated by high dietary levels of phytate phosphorous (40-50% of dietary phosphorous as phytate phosphorous). Ruminants, such as cows, sheep, and water buffalo, are able to utilize the phytic acid, since it is hydrolyzed (in the small intestine in particular) due to the phytase activity of their microflora (28, 52).

Working with puppies, Mellanby (76) recognized the rachitogenic nature of a diet high in some cereals. Phytic acid was subsequently indicted as the causative factor (76). High phytate salt intake has been linked with a reduction of calcium absorption and retention in humans (28, 73, 90, 110). Fecal calcium losses may exceed calcium intake (45). Cosgrove mentions several studies that noted an increased need for iron in humans on diets high in phosphate and phytate, and low in calcium (28, 43).

There is evidence that some people are able to adapt to a diet high in phytate phosphorous (33, 45). The importance of phytic acid as an antinutritional factor in humans is not clear. Some reviewers emphasize human adaptability to high phytate phosphorous levels, and others concentrate on its toxicity (33, 84, Abstracted in Food Science and Technology Abstracts 2:10C220, 1970).

Any evaluation of a food suspected to have a high phytic acid content must take into consideration the phytase activity as well as the actual amount of phytic acid seen in analysis. Thus, though wheat, rye, oats, and corn contain similar amounts of phytate phosphorous, both rye and wheat contain enough phytase to hydrolyze a large portion of their phytate during the leavening of bread (33), but oats and corn have low levels of phytase, and lose little phytic acid during bread making (74, 87 Abstracted in Biological Abstracts 33:40826, 1959). The type of heat to be used must be considered. Lease (61) found that autoclaving sesame meal was not effective in reducing its phytic acid content.

Complexes

Metal Complexes. Phytic acid forms complexes with metal ions. Vohra, Gray, and Kratzer (108) titrated sodium phytate ($C_6H_6O_{24}P_6Na_{12} \cdot 3H_2O$, FW 977.8) against metal ions, and for each metal obtained an inflection point in the curves when five moles

of the ion had been added. At pH 7.4, sodium phytate formed complexes with these ions, in the following order of stability: $Cu^{++} > Zn^{++} > Ni^{++} > Co^{++} > Mn^{++} > Fe^{+++} > Ca^{++}$. Saio, Koyama, and Watanabe (96) reported that for calcium ions not all the binding sites were independent, since bound calcium exerted an electrostatic repulsion toward more calcium ions.

Protein Complexes. The ability of myoinositol hexaphosphate and some of the lower phosphate esters to form insoluble protein complexes is well known (27, 42, 100). The phytic acid protein coacervate can be formed on either side of the isoelectric point of the protein, but is very labile on the alkaline side, particularly above pH 8, and also with heating (95). The complex is more stable on the acid side of the isoelectric point and may persist down to pH 2 (28). As the acidity increases, particularly below pH 5, the basic groups of the protein are progressively freed from intramolecular bonding and are available to form salt linkages with phytic acid (13, Abstracted in Biological Abstracts 31:20085, 1957). Studies with native and deaminated human serum albumin showed that phytic acid combined "first with the terminal ϵ amino group and α amino group of lysine, then with histidine groups, and finally with arginine guanidino groups" (14, p. 1399). For hen ovalbumin, the order of combination was, first the arginine groups, then lysine, and then histidine (15).

Bourdillon (20) extracted a protein-phytic acid complex from bean seed (Phaseolus vulgaris) and calculated that the aggregate contained 4.5 molecules of phytic acid per protein molecule. The size and tertiary structure of the protein affect phytic acid complexing. Hill and Tyler (49) demonstrated reduction in the solubility of casein with the addition of phytate, but found that no complex was formed with hydrolyzed casein.

Addition of calcium or magnesium ions to protein phytic acid mixtures reduces the amount of phytic acid protein aggregation (13, 49). Phytic acid, in turn, will increase the amount of calcium associated with soybean protein (97). The net effect of the complex is to reduce the pH range of dispersibility of the protein. With a combination of dialysis and use of cation exchange resin, Smith and Rackis (101) separated phytic acid from the protein in a water extract of soybean meal. Later, Saio, Koyama, and Watanabe (96) expressed concern about the possibility of denaturing the protein under the conditions used by Smith and Rackis.

Lipid Complexes. Investigations of the phosphatide fractions of wheat, soybean, corn, flaxseed, peanuts, sunflower seeds, and cottonseeds have revealed a complex sphingolipid (23). This lipid, which has been named lipophytin, comprises about 5% of the crude phosphatides, and is high in phosphorous for inositol-containing

lipids (23). The main metals present in this complex have been found to be calcium and magnesium (28). Ion-exchange resin phosphate fractions yielded mainly myoinositol hexaphosphate, with the penta- and tetraphosphate esters also present (28).

Dephosphorylation

Dephosphorylation of phytic acid can occur either by acid hydrolysis, or by enzymic action (reviewed in (28, 100)).

Enzymic Dephosphorylation. The name given to the phytic acid phosphatase enzyme is phytase. Phytase has been prepared from a variety of plant seeds, but usually not to a very high degree of purity (28, 100). The phytase activity in seeds increases greatly during germination, concomitant with the decrease in phytic acid (19, 44).

Phytase is a relatively non-specific enzyme. Some preparations are active with glycerophosphate and some with isopropyl phosphate (31, 100). Thus, the enzymes isolated exhibit non-specific acid phosphatase, as well as phytase activity (28, 44, 67). Davies and Motzok (34) demonstrated alkaline phosphatase activity in chick intestinal mucosa. Some acid phosphatases have been isolated which will dephosphorylate only the lower phosphate esters of phytic acid, and mono- and triphosphoinositols (31), and not the hexaphosphate, while some phosphatases from a phytase isolation will not attack any

phosphate ester of inositol (reviewed in Sloane-Stanley (100)). Gibbons and Norris (44) used the technique of DEAE cellulose chromatography to separate acid phosphatase from a phytase preparation, purify the phosphatase, and demonstrate that it had no phytase activity. After separation of phytase from phosphatases by gel filtration, Mandal, Burman, and Biswas (67) estimated the molecular weight of phytase to be between 100, 000 and 160, 000.

It has often been demonstrated that there is little or no correlation between phytase activity and general phosphatase or phosphotransferase activity in "different species, strains, or tissues, or in the same seeds at different stages of germination, or in a given enzyme preparation at different stages of fractionation" (100, p. 261). Although an organism containing phytic acid usually has some phytase activity, this is not always true (28, 74). (Refer to Table 1.) Thus, wheat and oats, both having similar contents of phytic acid, have greatly different amounts of phytase activity. Ergle and Guinn (38) have demonstrated the dephosphorylation of phytic acid during germination of cottonseeds. Data regarding retention of this phytase type enzyme activity in the food grade cottonseed protein concentrates has not been found.

Phytase seems to hydrolyze the phosphate groups in definite steps; preferentially attacking the myoinositol hexaphosphate, and removing the phosphate groups in such a manner as to keep the

remaining phosphates as far apart as possible (100). Phytases prepared from wheat attack myoinositol esters much more rapidly than the other isomers of inositol, the decreasing order of activity for the isomers being myo, neo, d, and scyllo (29).

K_m values for various phytase preparations have been reported from 0.3 mM to 10 mM, optimum pH from 2.2 to 7.8, with most plant phytases between 4.5 and 5.5, and optimum temperature from 45 to 56°C (as reviewed by Sloane-Stanley (100)).

Phytase inhibition can be caused by Cu^{++}, Zn^{++}, and Fe^{+++}, through their action as phytic acid precipitants, and in some reports, by inorganic phosphate (34, 67), and by phytic acid itself (as reflected in K_m values as low as 0.05 mM) (34, 100). Ca^{++}, Mg^{++}, CN^-, SCN^-, and oxalate can function as activators under the proper experimental conditions, such as pH (19, 34, 100). Under other conditions, Ca^{++} and Mg^{++} can become phytase inhibitors (47, 100).

Acidic Dephosphorylation. Dephosphorylation of phytic acid with acid yields a more complex mixture of reaction products than does phytase. Two different isomers of myoinositol pentaphosphate are produced with acid hydrolysis (8, 28), whereas enzymic hydrolysis yields only one pentaphosphate (27). Fleury (41) found that with sulfuric acid (40-50 gm/100 ml, 8 hr at 120°C) maximum hydrolysis

of inositol hexaphosphate occurred at pH 3.0. Desjobert (36) was able to obtain myoinositol monophosphate at pH 7.5 with acetic acid, only after 240 hr at 100°C.

Lorenzola, Torazzo and Anselmetti (63, Abstracted in Food Science and Technology Abstracts 2:11M988, 1970) tried various combinations of acids, ion exchange columns, and irradiation, in an effort to find the best conditions for the hydrolysis of phytin extracted from rice husks. They obtained 80% hydrolysis by first passing the calcium and magnesium phytate through a cationic resin, then adding HCl to the effluent, followed by 10 Mrad irradiation.

Available Methods of Analysis

Measurement of Dephosphorylation

Analysis of phytase activity may consist of monitoring the amount of inorganic phosphate freed during the course of the hydrolysis either by chemical determination (31, 32, 41, 44), or by determining the change in specific conductivity during the course of the reaction (7).

Since it was not known whether the cottonseed products would exhibit phytase activity, the method of Peers (86) was chosen for use on the basis of the similarity of wheat and wheat flour used in his study to the cottonseed products to be analyzed. An acetate buffer system similar to the one used by Peers (86) was also used by Gibbons and Norris (44), Courtois and Pérez (32), and Cosgrove (29).

Extraction

The problem in extracting phytic acid is to remove the compound without hydrolyzing the phosphate groups. The conditions necessary for hydrolysis are quite rigorous, as discussed earlier (refer to Table 1). Extraction procedures found in the literature are considerably milder than conditions necessary for hydrolysis: 3-6% trichloroacetic acid (TCA) (42, 109); or 0.5 N HCl (28, 66) for approximately an hour at room temperature. Trichloroacetic acid will precipitate proteins, nucleotides, and phosphoproteins, and extract both inorganic phosphorous and esterified phosphorous. Carbohydrate phosphate esters extracted by the TCA will not be precipitated during the phytic acid determination, therefore, the acid extraction and subsequent iron precipitation will exclude phosphorous compounds other than phytic acid. An isobutyl alcohol method for determining inorganic phosphate in TCA extracts was selected for this study because it can be used in the presence of large amounts of organic phosphorous (89).

Phytic Acid Determination

The common method for analysis of phytic acid involves precipitation of the ferric phytate salt, then direct or indirect measurement of the amount of iron that is bound to the precipitate.

Calculation of the amount of phytic acid is based on an assumed Fe/P ratio (50, 66, 109). The inaccuracy of this method arises from the fact that the ferric ion will also precipitate orthophosphate, lower phosphate esters of inositol, and phosphate esters of other organic compounds, as well as myoinositol hexaphosphate (6). Ferric chloride ($FeCl_3$) is the ferric compound normally used to precipitate the phytic acid. Jackman and Black (50) have reported that ferric sulfate [$Fe_2(SO_4)_3$] and ferric ammonium sulfate ($FeNH_4SO_4$) will precipitate more of the lower phosphate esters of inositol, and thus give higher phytic acid values.

An ion exchange method has been developed which is able to separate 5 of the 6 phosphate esters of inositol and inorganic phosphate (7, 22, 27, 28, 30). It is quicker and probably more reliable than the precipitation method. Marrese, Duell, and Sprague (68) compared the two methods and obtained much higher values for phytic acid with the precipitation method than with the ion exchange method. The method of choice will depend on the information desired. The precipitation method will give a composite estimate of all the inositol phosphoric acid, and the ion exchange method should distinguish between the various inositol esters. The iron precipitation method will be used in this study because it is the more common method used, and results obtained might more easily be compared with previous studies completed by this method.

MATERIALS AND METHODS

Cottonseed Products

Sources and Treatment by Suppliers

Four food grade high protein cottonseed products were analyzed: 1) a glanded flour, 2) a glandless flour, 3) a glandless air-classified flour, and 4) glandless toasted cottonseed kernels. Product data supplied by Traders Oil Company, the Blaine Richards Company, and the Oilseed Products Division of Texas A & M University are summarized in Table 2.

The glanded flour, Proflo, a partially defatted, cooked cottonseed flour, which was then toasted, was supplied by Traders Oil Mill Company, Fort Worth, Texas. This flour is produced from delinted and decorticated cottonseeds which are screened, rolled, and heated. Oil is expressed and the cooked meats are then reheated to obtain the shade desired, usually varying from light to dark brown (107).

The other products analyzed were provided by Dr. Carl M. Cater of the Food Protein Research and Development Center of Texas A & M University, College Station, Texas. The cottonseed kernels are distributed by Blaine Richards and Company, Inc., Lake Success, New York. The glandless cottonseed was rolled to flakes

Table 2. Product composition data available from suppliers of the cottonseed products analyzed [d] (in weight %)

Product	Moisture and volatiles	Oil	Protein (N x 6.25)	Free gossypol	Total gossypol	Crude fiber	Ash	Total sugars
Glanded flour[a]	3.0	5.0	55.0	0.045	0.60	2.53	7.1	- [e]
Glandless flour[c]	11.62	1.00	56.0	0.043	0.043	2.1	7.1	12.4
Glandless air-classified flour[c]	10.75	1.01	61.0	0.030	0.030	1.8	7.7	10.5
Toasted kernels[b]	5.7	38.5	33.0	- [e]	0.056	1.5	- [e]	- [e]

[a] Data and product from Traders Oil Mill Company, Fort Worth, Texas (103).

[b] Data from Blaine Richards and Company, Lake Success, New York (18).

[c] Data and product from Dr. Carl M. Cater, Texas A&M University, College Station, Texas (59).

[d] Amino acid composition given in Appendix Table 3.

[e] Data not available.

of 0.010 in thickness and extracted with hexane. The solvent was removed with warm air at 32°C and the flakes ground to pass an 80 mesh sieve (59).

The higher protein glandless air-classified flour was produced by a single cut air classification from the original glandless flour (59). Air classification is strictly a centrifugal purification by means of air currents (112).

The exact manner of preparation of the cottonseed kernels received was not known. The recommended procedure is to toast kernels at atmospheric pressure to an end temperature of 141°C and then lightly salt (57).

Storage

Each of the cottonseed products was thoroughly mixed on arrival, samples were taken, and all products stored at -25°C. The cottonseed kernels to be analyzed were hand ground to a 25 mesh size before storage.

Methods of Analysis

Detailed descriptions of procedures used are included in Appendix II.

The fat extraction method used a benzene-alcohol azeotrope and was essentially that of Pons et al. (89).

Dry weight was determined by the AOAC method (9), under a partial vacuum, with temperatures near 100°C.

Acid extractions and phytic acid determinations were carried out using modifications of the method of Wheeler and Ferrel (109). Trichloroacetic acid was the extracting solvent used. The phytic acid analysis involved precipitation of the ferric phytate salt, conversion of the ferric ion to ferric hydroxide, and then colorimetric measurement of the amount of ferric ion present.

Phytase activity was estimated by the method of Peers (86), with only slight modifications. This procedure colorimetrically measures the amount of phosphorous released during enzyme activity.

Two methods were used for analysis of inorganic phosphorous. The method of Dickman and Bray (37) was used during much of the preliminary work done in this study. Their method is a colorimetric determination of a phosphomolybdic complex. During preliminary studies it was discovered that large amounts of organic phosphate interfered with phosphorous determination by this method. The method of Pons and Guthrie (88), which extracts the phosphomolybdate complex into isobutyl alcohol before colorimetric determination, was then used. The digestion method of Pons et al. (89) was used to determine total inorganic phosphorous by both the Dickman and Bray method (37) and the Pons and Guthrie method (88).

Samples were digested with concentrated sulfuric acid, and organic matter oxidized with hydrogen peroxide.

Statistical analyses included analyses of variances of the phosphate and phytic acid data. Least significant differences were determined between products, between products expressed on different bases for the phytic acid data, and for the amounts of inorganic phosphate released by enzyme action. Standard deviations were calculated for the data on fat, moisture and volatiles, phytic acid, and phosphate determinations. The statistical consultant was Dr. Kenneth Rowe of the Oregon State University Department of Statistics.

RESULTS AND DISCUSSION

Fat-free weight and dry weight of each of the cottonseed products were determined in order to provide these bases for comparisons. The data obtained are given in Table 3. Comparative data provided by the respective product suppliers are given in Table 2.

Moisture and Volatiles

Values obtained here for moisture and volatiles seem to agree fairly well with the data supplied by the manufacturers, taking into account changes in moisture levels possible in storage, before, during, and after transit.

Fat-free Weight

Fat-free weight is here defined as the weight remaining following a benzene-alcohol azeotropic extraction as described in the methods section. Agreement of the fat data obtained in this study with that given by the manufacturers was good for the kernels and the glanded flour. Since the values obtained for the glanded flour and the toasted kernels were comparable with data given by the suppliers, the discrepancies in fat values for the glandless flours may be the result of different methods used. The Texas A & M staff (4) used petroleum ether as a solvent.

Table 3. Moisture and volatiles[a] and fat[b] in four cottonseed protein products

Product	Moisture and volatiles	Fat
	(%)	(%)
Glanded flour[d]	2.80 ± 0.4	5.38 ± 1.2
Glandless flour[c]	10.2 ± 0.9	4.84 ± 0.6
Glandless air-class. flour[c]	13.3 ± 1.0	9.86 ± 0.4
Toasted kernels[c]	3.08 ± 0.1	34.8 ± 3.0

[a]AOAC (9).

[b]Method of Pons et al. (89).

[c]Product supplied by Dr. Carl M. Cater of Texas A & M University, College Station, Texas.

[d]Product supplied by Traders Oil Mill Company, Fort Worth, Texas.

The method used in this study was selected by Pons et al. (89) to maximize phosphatide phosphorous extraction. The extracting solvent was a benzene-alcohol azeotrope.

Phytic Acid

The values obtained for phytic acid, as myoinositol hexaphosphate, are given in Table 4. The amounts of phytic acid found (mgm/100 gm) in the products as received were: 2860 for the glanded flour, 4290 for the glandless flour, 3350 for the air-classified glandless flour, and 2490 for the toasted kernels. Statistical data

Table 4. Phytic acid, as myoinositol hexaphosphate[a], in four cottonseed products (mgm/100 gm)

Product	As received	Defatted[b]	Defatted values on a received weight basis[f]	Dry weight basis[c]
Glanded flour[e]	2860 ± 560	2440 ± 349	2310	2940
Glandless flour[d]	4290 ± 330	4250 ± 534	4050	4780
Air-class. glandless flour[d]	3350 ± 352	3940 ± 891	3560	3860
Toasted kernels[d]	2490 ± 32	4010 ± 481	2700	2570

[a] As determined by an iron precipitation method (109).

[b] Benzene-alcohol azeotrope extraction (89).

[c] AOAC (9).

[d] Received from Dr. Carl M. Cater, Texas A & M University, College Station, Texas.

[e] Received from Traders Oil Company, Fort Worth, Texas.

[f] $\left(\frac{\text{Phytic acid}}{\text{wt. as received}}\right) \left(\frac{\text{wt. remaining after defatted}}{\text{received wt.}}\right)$

for analyses of variance are given in Appendix I, Table 1. Using the LSD test, the glandless flour had a significantly higher phytic acid content than either the glanded flour (5% level), or the toasted kernels (1% level). Differences between the other products were not significant at the 5% level.

This difference may be explained by the heat treatment used in the production of the glanded flour and the kernels, during which some of the original phytic acid may be destroyed by enzyme hydrolysis. The relatively lower amount of phytic acid found in the toasted kernels as received was not accounted for by the high fat content of the kernels. The amount of phytic acid (mgm/100 gm) in the toasted kernels in the defatted product (4010) when recalculated to account for the defatting process, is 2700, which is not significantly different (LSD test) from the phytic acid value obtained on the product as received (2490). The phytic acid values found for the kernels and for the glandless flour were significantly different when compared on a received weight basis (1% level) and when compared on the basis of the defatted values corrected for weight loss (5% level). This further supports the idea that the different levels of phytic acid found in the glandless flour and in the kernels are due to the heat treatment of the kernels, and not to their fat content.

Literature values for phytic acid in cottonseeds and cottonseed products are given in Table 1. The only data available for

comparison of phytic acid values in cottonseed products of similar protein content is the 50% protein cottonseed meal reported by Nelson, Ferrara, and Storer (80) and given in Table 1. Nelson's value of 3300 mgm/100 gm is somewhat lower than the value of 4290 found in this study for the glandless flour (56% protein), but slightly higher than the 2860 mgm/100 gm for the glanded flour (55% protein, Tables 2 and 4).

The phytic acid values for all the cottonseed products in both Tables 1 and 4 are not much lower than the values reported for the dried, decorticated seeds in Table 1. In wheat, phytic acid appears in a higher concentration in the bran than in the whole grain (10, 80, 82). The concentration of phytic acid would therefore be expected to be lowered during the refining of wheat. As mentioned in the Review of Literature, the structure of the cottonseed is somewhat compartmentalized. The globoid bodies containing the phytic acid are enclosed within the aleurone grains, which have a protein storing function. The phytic acid in the cottonseed, therefore, would not be expected, on the basis of structure, to be lost during the production of the refined cottonseed products, particularly when protein content is to be maximized.

Martinez et al. (69) states that in dry air classification of the glandless cottonseed flour, the aleurone grains remain intact, and that concentration of these protein bodies also results in concentration

of the phytic acid-containing globoid bodies. The results obtained in this study do not support this statement. Phytic acid values for the glandless flour (56% protein) were slightly higher (not significant at the 5% level) than for the glandless air-classified flour (61% protein). The proteins in the glandless cottonseed flour may have been separated somewhat from the phytic acid present in this flour, and more phytic acid remained with the lower protein portion of the air classification cut.

Varietal differences are shown between the cottonseeds listed in Table 1. The variety of seed from which cottonseed products are made would be somewhat expected to influence the amount of phytic acid in the end product. Watson GL-16 glandless cottonseed was used to produce both the glandless flours analyzed in this study (24). It is not known what varieties of seeds were used for the glanded flour and the toasted kernels.

Cosgrove (28) and Carter et al. (23) had reviewed the inclusion of a calcium-magnesium phytate salt in the phosphatide fraction in corn. Phytic acid determinations were done in this study on fat-free samples for comparison with the phytic acid values found for the products as received. If the phytic acid values for the defatted samples on a received weight basis (i. e., defatted phytic acid values corrected for the amount of fat lost, Table 4, footnote f) were substantially lower than the phytic acid values obtained in the products

as received, then it would seem that some of the phytic acid was associated with the fraction removed during the defatting process. The phytic acid values (mgm/100 gm) found (Table 4) were: for the glanded flour, 2860 as received, and 2310 defatted products on a received weight bases; for the glandless flour, 4290, and 4050, respectively; 3350, and 3560, for the air-classified glandless flour; and 2490, and 2700 for the toasted kernels. For each product, there were no significant differences at the 5% level (LSD test) between the product analyzed as received, and when the phytic acid value determined on the defatted samples were corrected for loss in weight during the extraction. (Statistical data for the analyses of variance are given in Appendix I, Table 2.) Therefore, it did not seem that there was an appreciable amount of phytic acid associated with the fat fraction in the cottonseed products examined. Although the methods used here are much less sensitive than those used by Carter et al. (23) and Cosgrove (28), these results are as expected, considering the small amounts of myoinositol phosphate compounds that they found in the lipophytin fraction.

To evaluate the importance of the phytic acid which would be contributed by the cottonseed products to the diet, it is necessary to consider the amount of cottonseed protein product recommended for incorporation in various recipes as a protein supplement or product improver. Amounts generally recommended are on the order of 1%

of the total formula weight of the recipe (59, 104, 106). Cosgrove (28) and Foy et al. (43) reported an increased need for iron when phytic acid phosphorous was as high as 40% of the dietary phosphorous. A level of 40% of the RDA for phosphorous as phytic acid phosphorous has been arbitrarily chosen here for illustrative purposes, as an unsafe level of daily phytic acid intake (Table 5).

Statistically significant differences between the phytic acid contents of the cottonseed products as reported above thus would seem to have little relationship to nutritionally significant amounts of phytic acid ingested, when such low levels of the cottonseed products analyzed are used in food formulations. Using a formulation for "High Protein Peanut Butter Crisps" (106) and any of the four cottonseed products as 3.2% of the formula weight, ingesting our defined unsafe level of phytic acid would necessitate eating a minimum of 80 ten gm cookies per day.

Of the products, only the toasted kernels would be likely to be ingested as purchased. The amount of these nuts needed to reach the hypothetical 40% level is small (7-1/2 tablespoons for a 14-18 year old male). Efforts could be made to explore ways to lower the amount of phytic acid in the toasted kernels, or to encourage their incorporation in food systems that would promote phytase activity, rather than their use as a snack food.

Table 5. Amount of four cottonseed products that would supply 40% of the RDA[d] for phosphorous as phytic acid phosphorous[e] for normal adults and adolescent males

Product	Grams		Cups[a]	
	RDA level gm			
	0.8	1.4	0.8	1.4
Glanded flour[b]	40	70	0.42	0.74
Glandless flour[c]	26	46	0.22	0.40
Air-classified glandless flour[c]	34	59	0.71	1.23
Toasted kernels[c]	46	80	0.28	0.50

[a] 8 oz cup, sifted volume for flours, volume of whole kernels.

[b] Product supplied by Traders Oil Mill Company, Fort Worth, Texas.

[c] Product supplied by Dr. Carl M. Cater, Texas A & M University, College Station, Texas.

[d] Recommended dietary allowance per day, 1968 values of the Food and Nutrition Board of the National Research Council of the United States (0.8 gm for normal adults, 1.4 gm for 14-18 year old male).

[e] Phytic acid as determined in this study by the method of Wheeler and Ferrel (109) and reported as myoinositol hexaphosphate.

Estimation of Phytase Activity

The enzyme activity exhibited in the glandless products was termed phytase activity. The specific enzymes responsible for release of the phosphorous are not known. The actual amount of phytic acid destroyed was not measured by a phytic acid determination, since the acetate buffer used to estimate enzyme activity would have interfered with the iron precipitation method for phytic acid determination. This buffer system was selected to give good enzyme activity (86). Actual enzyme activity in a particular food product would need to be measured in a food system of interest. In the system used, of all the products tested, only the glanded flour did not exhibit phytase activity (Table 6). The amounts of inorganic phosphate released (mgm x 10^{-3} per 50 mgm of product) were: for the glandless air-classified flour 63.4; for the glandless flour 38.7; for the toasted kernels 41.4; and for the glanded flour, -1.0. Literature values for comparison of phytase activity in cottonseed protein products have not been found. Tests for least significant differences indicated that the amount of inorganic phosphorous released by the air-classified glandless flour during the incubation period was significantly different at the 1% level from the amount released by the glandless flour, and from the amount released by the toasted kernels. There was no significant difference at the 5% level in the

Table 6. Changes in inorganic phosphate[a] and estimation of phytic acid,[e] as myoinositol hexaphosphate, loss in four cottonseed products with moist heat treatment[b].

Product	Inorganic phosphorous mgm x 10^{-3}/50 mgm product			Estimated phytic acid lost during heating
	Before heating	After heating	Change after heating	
				%
Glanded flour[c]	50 ± 1.2	49 ± 2.4	-1.0	0
Glandless flour[d]	50 ± 2.8	89 ± 4.9	+38.7	6
Glandless air-class. flour[d]	28 ± 2.8	92 ± 5.7	+63.4	13
Toasted kernels[d]	22 ± 2.8	63 ± 3.8	+41.4	12

[a]Method of Pons and Guthrie (88).

[b]Method of Peers (86) set for 45°C holding temperature.

[c]Product supplied by Traders Oil Company, Fort Worth, Texas.

[d]Product supplied by Dr. Carl M. Cater, Texas A & M University, College Station, Texas.

[e]Total phytic acid as reported in Table 4.

amounts of inorganic phosphorous released by the toasted kernels and the glandless flour.

The practical differences between the amount of enzyme activity exhibited by the three glandless products lessen when considered in terms of the proportion of phytic acid destroyed (Table 6). Using the phytic acid values reported in this study, and the amount of phosphorous released by each product during incubation, the amount of phytic acid lost due to enzyme activity was estimated to be: 6% for the glandless flour, 0% for the glanded flour, 13% for the glandless air-classified flour, and 12% for the toasted kernels. For this estimation, the assumptions were made that the loss of six inorganic phosphate groups represented the destruction of one molecule of myoinositol hexaphosphate, and that all the phytic acid present before moist heat treatment was the hexaphosphate ester of phytic acid. Since it is not necessary to completely dephosphorylate the phytic acid to render it incapable of metal chelation, the assumptions made above make the figures given for loss of phytic acid conservative.

The lack of enzyme activity in the glanded flour is thought possibly to be due to enzyme inactivation during the moist heat treatment used in the manufacture of this product. Exact times and temperature used in the process are not known. The information could not be secured through inquiries made of Traders Oil Mill

Company, and it has been indicated by Dr. Carl M. Cater of Texas A & M University, that the information is also not available to him (24).

Some differences in enzyme activity may have been due to different substrate concentrations of phytic acid phosphorous, but in this study release of phosphorous could not be correlated with the original substrate concentration of phytic acid phosphorous. It might be of interest to separate and further characterize the enzyme or enzymes responsible for release of the phosphorous in the system used, in terms of optimum pH, temperature, and substrate concentrations. Inhibition and activation by various ions could be explored.

SUMMARY

Phytic acid was measured and phytase activity was estimated in four food grade cottonseed protein products.

The phytic acid contents in mgm/100 gm of the cottonseed products as received were: 2860 for a glanded flour, 4290 for a glandless flour, 3350 for an air-classified glandless flour, and 2490 for toasted cottonseed kernels. For comparative purposes, data is also reported on defatted and dry weight bases. Between the products analyzed, the only significant differences found were that the glandless flour was significantly higher in phytic acid than the glanded flour at the 5% level and higher than the toasted kernels at the 1% level. The only product that seems cause for nutritional concern, as consumed, is the toasted kernels.

Only the glanded flour did not demonstrate phytase activity. The amounts of inorganic phosphate released (mgm x 10^{-3} per 50 mgm of product) were: for the glandless air-classified flour 63.4; for the glandless flour 38.7; for the toasted kernels 41.4; and for the glanded flour -1.0. The amount of phosphate released during incubation by the glandless air-classified flour differed significantly at the 1% level from the amounts released by the glandless flour and the amounts released by the toasted kernels. For the products with enzyme activity the amount of phytic acid lost was

estimated to be between 6 and 13% of the initial phytic acid present.

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APPENDIX

Appendix Table 1. Statistical analyses of the variance of phytic acid values between cottonseed products and between the products as received and after defatting

Source of variation	Degrees of freedom	Mean square[a]	F value[b]
Products	3	3, 737, 303	6. 69*
Defatting	1	1, 373, 653	2. 46
Products x defatting	3	1, 432, 595	2. 56
Extractions	8	558, 616	4. 19**
Analysis x extractions	16	133, 316	

[a] Phytic acid as myoinositol hexaphosphate mgm/100 gm.

[b] * indicates significance at the 0. 05 level.
** indicates significance at the 0. 01 level.

Appendix Table 2. Statistical analyses of variance of phytic acid values between cottonseed products on received weight basis, between defatted products on a received weight basis, and between the two bases of expression for each product analyzed

Source of variation	Degrees of freedom	Mean square[a]	F value[b]
Products R[c]	3	2,439,842	5.45*
Products D[d]	3	2,613,390	5.83
R x D			
Means of all products	1	109,278	1.04
Glanded flour	1	599,512	1.34
Glandless flour	1	122,512	0.27
Air-classified glandless flour	1	84,050	0.19
Toasted kernels	1	30,012	0.07
Extraction error	8	447,991	4.25**
Analysis error	16	105,378	

[a] Phytic acid, as myoinositol hexaphosphate, mgm/100 gm.

[b] * indicates significance at the 0.05 level.
** indicates significance at the 0.01 level.

[c] Received weight basis.

[d] Defatted values on a received weight basis.

Appendix Table 3. Amino acid data provided by the suppliers of the cottonseed products analyzed (gm/16 gmN)

Amino acids	Glanded flour[c]	Glandless flour[d]	Glandless air-classified flour[d]	Toasted kernels[e]
Lysine	4.49	4.2	4.0	4.1
Available lysine	-[a]	3.5[f]	3.6[f]	-[a]
Tryptophan	1.02	1.5[f]	1.5[f]	1.2
Phenylalanine	5.87	5.3	5.3	4.7
Methionine	1.52	1.6	1.6	1.6
Threonine	3.27	3.2	3.1	4.7
Leucine	6.10	5.7	5.7	6.6
Isoleucine	3.32	3.1	3.0	3.7
Valine	4.54	4.4	4.4	5.3
Cystine	1.52[b]	2.5[g]	2.5[g]	-[a]
Aspartic acid	9.68	8.7	8.8	-
Serine	4.49	4.0	4.2	-
Glutamic acid	21.47	19.7	20.0	-
Proline	3.91	3.8	3.9	-
Glycine	3.74	4.0	4.0	-
Alanine	3.89	3.7	3.7	-
Tyrosine	3.38	3.0	3.3	-
Histidine	3.01	2.8	2.8	-
Arginine	12.67	11.4	11.3	-

[a] Data not available.

[b] 1/2 cystine.

[c] Product and data supplied by Traders Oil Mill Company, Fort Worth, Texas, analyzed by Moore et al. technique (78).

[d] Product and data supplied by Dr. Carl M. Cater, Texas A & M University, College Station, Texas. The method used was that of Spackman et al. (102) except where otherwise noted (footnotes f and g).

[e] Data from Blaine Richards & Co., Inc., Lake Success, New York, method unknown (18).

[f] Tryptophan determined by Dr. Cater's associates according to the method of Kohler and Palter (54).

[g] Cystine determined by Dr. Cater's lab using a modification of the method of Schram et al. (99).

APPENDIX II

Details of Procedures Used

All glassware was cleaned with concentrated nitric acid (HNO_3) and rinsed with distilled water. Distilled water was used in all solutions.

Fat Extraction

The method used was modified from Pons et al., (89). Into an asbestos thimble (44 x 94 mm) were weighed 10-20 gm of a sample and the sample was secured with a mat of glass wool. Approximately 200 ml of a benzene-alcohol azeotrope were used in a Soxhlet extractor. The benzene-alcohol mixture was 32.4% by weight absolute ethyl alcohol and 67.6% by weight benzene. The extraction was continued at a rapid rate for a minimum of 4 hr, or until the solvent surrounding the thimble appeared clear. The extracted cottonseed products were allowed to air dry at room temperature to a constant weight. A minimum of three samples was used for fat determination for each product. Portions of the extracted samples were used for phytic acid analysis.

Dry Weight

The method was modified from that given by the AOAC (9). About 5 gm of a well-mixed sample were weighed accurately into a covered glass dish which had been previously dried at 98-100°C, cooled in a desiccator over $CaSO_4$, and weighed soon after reaching room temperature. The cover was loosened and the sample heated at 98-100°C for five hours in a partial vacuum. A sulfuric acid gas-drying bottle was connected with the oven to admit dry air when releasing the vacuum. The samples were covered immediately upon removal from the oven and desiccated over $CaSO_4$ to a constant weight. The weight of the residue was reported as dry weight and the loss in weight as moisture and volatiles. A minimum of three samples was used for dry weight determinations on each product.

Acid Extraction

The method was modified from Wheeler and Ferrel (109). A 2 gm sample with a maximum particle size of 2 mm and estimated to contain 30-80 mgm of phytate phosphorous was weighed into a 250 ml erlenmeyer flask. To this was added 5 ml of 95% ethanol, and the flask vibrated methanically to disperse the mixture. The sample was extracted for 90 min with 12.3% trichloroacetic acid (TCA), introduced in three additions at half hour intervals. The

total volume of the extracting solution at 90 min was 90 ml. The flasks were vibrated mechanically for 5 sec every 10 min. At the end of the extraction period, the mixture was centrifuged at 1350 x g for 30 min. Aliquots of the centrifuged supernatant samples were taken for phytic acid determination. This extraction procedure was also used for the determination of total and inorganic phosphorous in the cottonseed products and in the barium phytate standard.

Phytic Acid Determination

The method for the phytic acid determination was modified from Wheeler and Ferrel (109). To aliquots from the TCA extraction were added 10 ml of ferric chloride ($FeCl_3$) solution in 12.3% TCA by rapidly blowing out the pipette and immediately mixing the tube. The quantity of $FeCl_3$ added was such that the molar ratio of Fe/P in the sample was approximately 4/3. The tubes containing the samples were then heated in a boiling water bath for 45 min. After 30 min of heating, 2 pipette drops of 3% sodium sulfate in 3% TCA were added. The samples were cooled in ice water for 10 min before being centrifuged at 1350 x g for 45 min at room temperature. The supernatants were discarded and the precipitates were washed twice by adding 25 ml of 3% TCA, followed by 10 min of heating in a boiling water bath and centrifuging at 1350 x g for 45 min. The washing

procedure was repeated once with water. The precipitate was dispersed in a few milliliters of water and 3 ml of 1.5 N sodium hydroxide (NaOH) were added with mixing. The volume in each sample was brought to approximately 30 ml with water, and the tubes were then heated for 30 min in a boiling water bath. The suspensions were filtered hot through Whatman #42 ashless paper (9.0 cm), and the precipitates washed with three 10 ml portions of hot water. Forty milliliters of hot 3.2 N nitric acid (HNO_3) were poured through the filter paper and collected in a 100 ml volumetric flask. The filter paper was included in the flask. The funnel was rinsed with distilled water and the flask and contents heated for 30 min in a boiling water bath. The flasks were cooled, the contents diluted to volume, and then centrifuged for 10 min at 1350 x g. Portions of the supernatants were used for spectrophotometric analysis for iron. Appropriate aliquots were put into 25 ml volumetric flasks. To this were added 5 ml of 1.5 M potassium thiocyanate (KSCN), the flasks diluted to volume, and absorbance read with a Spectronic 20 at 480 nm against distilled water, 60 sec after addition of the KSCN. Blank values were obtained and subtracted from sample absorbances. Concentrations of ferric iron were determined from a standard curve prepared with $FeCl_3$.

On the basis of extensive preliminary investigation and efforts to reduce variability, and after consulting with a statistician, the

data reported are the results of two samplings from each product, one extraction from each sampling, and two analyses on each extraction.

The phytic acid standard was purchased as crystalline sodium phytate from Sigma Corporation (St. Louis, Mo.). Cosgrove (30) had examined this product by ion exchange chromatography and found that it contained no inorganic orthophosphate or phosphate esters of inositol other than myoinositol hexaphosphate.

After preliminary analyses with the Sigma sodium phytate gave low yields, the sodium phytate was purified as done by Wheeler and Ferrel (109). The sodium phytate was alternately precipitated as ferric phytate (Fe_4IP_6) with concentrated $FeCl_3$, and then precipitated as barium phytate (Ba_4IP_6) with saturated $BaCl_2$. The double precipitation cycle was repeated twice and the final barium phytate was washed with water, then methanol, and dried for 5 hours under partial vacuum at 80°C.

Purity was determined by measuring orthophosphate by the method of Pons and Guthrie (88) on a TCA solution of the dried barium phytate both before and after digestion by the method of Pons et al. (89) as described below. According to Johnson and Tate (51), the method used for drying the purified barium phytate would have resulted in a trihydrate. Hence, the molecular weight used to calculate purity was 1196. On this basis, phosphorous was calculated to be 15.55% of the weight of the barium phytate, and was measured

to be 12.16%, for a purity of 78.2%. Inorganic phosphorous was 6.82% of the total phosphorous.

A standard curve was obtained by performing the TCA extraction and subsequent phytic acid determination on weighed samples of the purified barium phytate. The standard curve was linear and the phytic acid observed was 97% of that expected on the basis of the 78.2% purity. Recovery of barium phytate added to a cottonseed product was 98.8%.

Preliminary experiments using $FeNH_4SO_4$ did not indicate the presence of lower esters of phytic acid. Variability was reduced during preliminary studies by raising the TCA level used for extraction prior to determination from 3% to 12.3%, and by closely approximating a 4/3 Fe/P ratio in the initial iron phytate precipitate.

Estimation of Phytase Activity

This method is essentially that of Peers (86). Into 25 ml erlenmeyer flasks were weighed 50 mgm of a cottonseed product. To this were added 10 ml of a 0.1 M acetate buffer solution that was 0.002 M $MgSO_4$, pH 5.0 and prewarmed to 45°C. (The final substrate concentration was estimated to be 2.77×10^{-3} M phytate phosphorous for the glanded flour, 3.39×10^{-3} M for the air-classified flour, 3.02×10^{-3} M for the toasted kernels, and 4.68×10^{-3} M for the glandless flour.) After the buffer was added, a 4 ml

sample was immediately removed and added to 2 ml of 12.3% TCA. The remaining solution in the flask was incubated in a water bath at 45° C for 4 hr. Another 4 ml sample was taken from the suspension solution at the end of the 4 hr period and also added to 2 ml of 12.3% TCA. The acid treated samples were filtered through Whatman #5 filter paper and the filtrates used for determination of orthophosphate phosphorous by the method of Pons and Guthrie (88). In a similar manner, 4 ml of acetate buffer were added to 2 ml of 12.3% TCA, filtered, and the resulting solution used for blank values in the orthophosphate determinations.

Following preliminary experiments and on the advice given by the statistician, the phosphate data reported for estimations of phytase activity are the results of experiments on two samples from each product. One sample from each product was analyzed per day in a randomized daily order. Three buffered incubations were done on each day's product. One sample was taken from each incubating flask at two different time intervals. Two analyses for inorganic phosphorous were done on each time sample.

Inorganic Phosphorous Methods

Dickman and Bray Method for Inorganic Phosphorous. The Dickman and Bray method (37) was used to measure total

orthophosphate on the TCA extracts of the cottonseed products. Prior to phosphate determination, the extraction supernatants were digested by the Pons et al. (89) method, and then passed through a Jones reductor (17, 21). Dickman and Bray (37) found that interference with color development by the presence of 15 to 30 ppm of ferric iron in the final dilute solution could be avoided by reduction to the ferrous ion. Higher iron concentrations needed to be accounted for by using approximately the same concentration of ferrous iron in the phosphate standards as in the solutions used to develop a standard curve. The Traders Oil Mill Company Product Data (105) listed the iron content of the glanded flour as 87 ppm, so use of a Jones reductor seemed advisable. Use of the reductor did lower the apparent amount of phosphate.

A 15 cm column of amalgamated zinc on a glass wool pad was placed in a 50 ml buret. The tip of the buret was put through one hole of a two-holed rubber stopper, and the second hole was connected to a water pump. Between samples the column was washed with 25 ml of approximately 1 N HCl, followed by 200 ml of distilled water. Flow rate through the column was approximately 100 ml per min. The solutions passing through the column were collected in erlenmeyer flasks attached to the stopper.

The pH of the solutions was adjusted to 2.5 ± 0.1 with HCl or NH_4OH. An aliquot of the sample was placed in a 50 ml graduated

cylinder and diluted to 35 ml with distilled water. To this were added with mixing, 10 ml of a hydrochloric acid-molybdate reagent (3.5 N HCl and 0.015 gm of ammonium molybdate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$] per ml). Immediately, 5 ml of a dilute stannous chloride ($SnCl_2$) reagent were added and the entire contents mixed thoroughly. (The stock $SnCl_2$ solution was 10 gm of $SnCl_2$ in 25 ml of concentrated H_2SO_4. The dilute $SnCl_2$ solution was made by diluting 3 ml of the stock solution up to a liter with water.) Absorbance was read 60 sec after the addition of the dilute $SnCl_2$ with a Spectronic 20 at 440 nm. A standard curve was prepared with aliquots of a potassium dihydrogen phosphate solution that had been passed through the Jones reductor and had also had the pH adjusted. The KH_2PO_4 was dried to a constant weight and 2.194 gm were made up to one liter with distilled water for a stock solution of 500 ppm phosphorous.

Isobutyl Method for Inorganic Phosphorous

The following method was used to measure orthophosphate in the solutions during the course of the enzyme activity experiments, to determine the purity of the barium phytate standard, and to estimate inorganic phosphorous in acid extracts of the cottonseed products. The method was only slightly modified from that of Pons and Guthrie (88). An aliquot of an extraction supernatant or filtrate containing a

maximum of 0.010 mgm of inorganic phosphorous (usually 1-5 ml) was pipetted into a 125 ml separatory funnel. To this were added 5 ml of molybdate-sulfuric acid reagent [5% $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 4 N H_2SO_4 and filtered through Whatman #2 paper]. The volume was brought up to 20 ml with distilled water and the contents swirled. Then 10 ml of isobutyl alcohol (b.p. 107.5-108.5) were added and the funnel shaken for 2 min. The aqueous layer was discarded and the isobutyl layer was washed twice by shaking for 30 sec each with two 10 ml portions of approximately 1 N H_2SO_4. Following the washings, 15 ml of dilute $SnCl_2$ were added. ($SnCl_2$ stock solution was prepared by dissolving 10 gm of stannous chloride hexahydrate in 25 ml of concentrated HCl. Dilute $SnCl_2$ was made by diluting 1 ml of stock solution to 200 ml with approximately 1 N H_2SO_4 just before use.) The contents were shaken for 1 min and the aqueous layer discarded. The isobutyl alcohol layer was transferred quantitatively to a 10 ml volumetric flask and made up to volume with 95% ethyl alcohol. Absorbance was read with a spectrophotometer at 730 nm at 40 min from the time of addition of the $SnCl_2$.

A standard curve was prepared by performing the analysis as described on a standard phosphate solution prepared by recrystallizing analytical grade potassium dihydrogen phosphate (KH_2PO_4) three times from distilled water, drying at 110°C and storing over concentrated H_2SO_4.

A stock solution of 1.0 mgm phosphorous per ml was prepared by dissolving 4.3929 gm of the dry phosphate in water and 200 ml of approximately 1 N H_2SO_4. Three drops of 0.1 M potassium permanganate ($KMnO_4$) were added as a preservative, and the volume was brought to one liter with water. The standard curve determinations included 1 ml of the acetate buffer-TCA solution used as blanks in the estimation of phytase activity.

In this method it was found to be unnecessary to use a Jones reductor for samples thought to contain a large amount of ferric iron (37). The amount of orthophosphate analyzed by this method remained the same whether or not the reductor was used.

Digestion for Total Phosphorous. Total phosphorous was measured as total orthophosphate following an oxidative digestion of the samples. This procedure was performed on acid extracts of the cottonseed products and on the repurified barium phytate standard. This method was taken from Pons et al. (89). A sample was weighed or pipetted into a 30 ml Kjeldahl flask. Solid material did not weigh more than 0.167 gm and liquid extracts did not contain more than 0.5 mgm of phosphorous. To the sample were added 1 ml of reagent grade concentrated H_2SO_4, and two 6 mm glass beads. The flasks were heated until the organic matter charred and a homogeneous

solution was obtained. The flasks were then cooled until the fuming stopped, and 3 macro-pipette drops of 30% hydrogen peroxide were cautiously added down the neck of the flask. The solutions were heated until colorless. More hydrogen peroxide was added if necessary. Heating was continued for 10 min after the last addition of peroxide. The flasks were cooled, 6 ml of water were added, and the flasks were then heated in a boiling water bath for 10 min to remove any remaining peroxide and to complete conversion of the phosphorous to the ortho form. The flasks were again cooled, and the contents transferred quantitatively to 100 ml volumetric flasks, prior to phosphorous determination.