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Sephadex G-25 and Charcoal Separation of Mouse Growth Inhibitors in Soybeans from Trypsin Inhibitors

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SEPHADEX G-25 AND CHARCOAL SEPARATION OF MOUSE GROWTH INHIBITORS IN SOYBEANS FROM TRYPSIN INHIBITORS

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Rising costs have changed man's ideals and eating habits. Plant ingredients, because of their relatively low costs, have been increasingly used as a substitute for animal proteins. If these plant ingredients could be directly utilized by man, this would be a more efficient use of energy and nutrients. One plant source currently used in such a manner is soybeans, which is a prime source of protein for human and livestock nutrition. However, soybeans contain several factors that are detrimental to man and livestock. Heating of soybeans counteracts these factors, since the undesirable factors are heat labile. However, heat alters the protein's properties making them unsuitable for many food products and calf milk replacers. Consequently, soybean processors currently use little or no heat in the production of soybean "isolates" and "concentrates." In their process, they discard, as waste, the soybean whey fraction. This fraction contains the toxic factors, but also usable protein and carbohydrate material. Such a practice wastes 20% of the soybean protein in addition to adding to today's ever pressing pollution problem.

The purpose of this research project was to further isolate and purify a small molecular weight growth inhibitor from soybeans and to gain needed knowledge about its properties. Once this growth

inhibitor is identified, appropriate methods of inactivating or removing the inhibitor may be developed, so that much, if not all of the soybean whey proteins may be used beneficially for mankind.

LITERATURE REVIEW

More than 50 years have passed since Osborne and Mendel (47) reported the improved growth-promoting property of cooked versus raw soybeans. The explanation for this fact still remains unclear. Numerous studies and improved technology have only increased the complexity of the problem. Many factors have been implicated as the cause for the growth inhibition associated with raw soybeans, and these will be discussed in the following pages.

Trypsin inhibitors. The finding (22) of a trypsin inhibitor in soybeans appeared to explain the cause of the growth depression found when feeding raw soybeans. Westfall et al. (64) further substantiated this finding, when they found that the activity of this inhibitor was destroyed by autoclaving. This concept was supported when a crude trypsin inhibitor preparation was added to a heated soybean ration and reduced growth rates in chicks (23) and rats (30) resulted. Because of these results, trypsin inhibitors have received the majority of the attention in investigations designed to find the cause of growth depression by raw soybeans.

Kunitz (31) first isolated a crystalline globulin protein from raw soybeans which forms, instantaneously, an irreversible stoichiometric compound with trypsin (20, 35, 57). When this trypsin inhibitor is denatured, it can be readily digested by pepsin (32). Even the undenatured form is slowly digested by pepsin (28). To date nine trypsin inhibitors have been isolated from soybeans (40). These

inhibitors vary in size from 8,000 (16) to 24,000 molecular weight (32) and in other physical and chemical properties. Commercially available soybean trypsin inhibitors are usually the Kunitz inhibitor (31).

Crystalline soybean trypsin inhibitor, when added to chick (17, 19) and rat (19, 21) diets, depressed growth but never to the extent found when raw soybean meal (RSBM) was fed. When the soybean whey fraction was separated into two fractions, one fraction high in trypsin inhibitor activity and the other high in hemagglutinating activity, the combination of the two fractions inhibited chick growth rates to a greater extent than either fraction alone (17). The soybean whey fraction contains trypsin inhibitors, hemagglutinins and unidentified components (14, 50, 51). Adding a potent trypsin inhibitor, p-aminobenzamidine, to the drinking water of rats caused growth depression, but reduced feed intake probably caused most of the growth depression (18).

Borchers and Ackerson (9) and Brambila (10) proposed that the depressed growth assessed to trypsin inhibitors could be compensated by adding trypsin to the ration and thus tie up the soybean trypsin inhibitors with exogenous trypsin. However, the addition of trypsin to RSBM diets fed to rats (9) and chicks (10) could not overcome the growth-depressing effect of RSBM.

Another approach was based on the hypothesis that if feeding RSBM caused interference of the enzymatic digestion in the small intestine and therefore caused growth depression, supplementing amino

acids should correct the growth depression (44). Some researchers (2, 7, 8, 9) felt they could raise the biological value of raw soybeans by supplementing the sulfur-containing amino acids instead of feeding intact proteins. This appeared to be a reasonable assumption because soybeans are low in methionine (59), while trypsin contains a relatively high level of cystine (3) which is usually synthesized from methionine. However, methionine supplementation of RSBM diets never produced growth rates equal to those achieved on autoclaved soybean meal diets (3, 8). Additional support for these results can be gathered from the research (24) that the supplementation of a RSBM diet with 11 amino acids required by chicks did not improve growth rates. Replacement of the RSBM by heated soybean meal resulted in substantially increased weight gains. Borchers (7) first found that supplementation of amino acids could alleviate the growth depression found when RSBM was fed, but later concluded that amino acid supplementation could produce only 75% the growth rate of the rats fed heated soybean meal diets (8). The addition of amino acids to the diet should have compensated for all the growth depression if trypsin inhibitors were the sole cause of growth depression.

The ability of the animal to secrete extra enzymes as compensation for trypsin inhibitors in the diet (56) also indicates that trypsin inhibition was not the growth depressant. Even with 95% of the pancreas removed, there was no reduction in nitrogen digestion and absorption in rats as measured by fecal excretion (56). Protein digestion was only reduced when 99.5% of the pancreas was removed.

Schingoethe et al. (54), using a Sephadex G-50 column, separated a small molecular weight growth inhibitor from trypsin inhibitors. Half of the growth inhibition attributed to the soybean whey fraction was caused by the small molecular inhibitor, but caused no pancreas enlargement.

Finally, germinated soybeans had a protein efficiency ratio almost equal to that of heated meal (15), but much higher than raw soybeans. The higher protein efficiency value with germinated soybeans occurred even though there was no reduction in trypsin inhibitor concentration (13).

Hemagglutinins. Besides the antitryptic factor, hemagglutinins in raw soybeans have been incriminated as the growth depression factor. Hemagglutinating agents have been known to be present in plants since the 1880's (39). Hemagglutinin extracts from different seeds agglutinate the red blood cells from some species of animals but not the cells from other species (4, 34, 42). Liener and co-workers (41) had difficulty correlating growth inhibition and trypsin inhibitor activity when feeding a diet containing a protein hydrolysate along with an antitryptic factor. They suggested the presence of some substance other than the antitryptic factor which adversely affects growth. Further investigation by Liener and Pallansch (42) resulted in the isolation of a homogeneous protein high in hemagglutinin activity. Additional purification indicated it had a molecular weight of 96,000 and contained 6-10% glucosamine (61).

Evidence that the hemagglutinins may be responsible for the growth-inhibiting properties of soybeans has been minimal. Intraperitoneal injections of hemagglutinin preparations were lethal to young rats (37). However, physiologically this information is questionable because hemagglutinins are readily inactivated by peptic digestion when as few as 12% of the peptide bonds are split (38, 6). This should result in complete or almost complete inactivation prior to entering the small intestine. Also it is unlikely that an intact protein of 96,000 molecular weight, even if it survived gastric digestion, could be absorbed from the gut. Wada et al. (61) found that as they increased the hemagglutinin activity during the purification procedure there was only a slight increase in toxicity. When the hemagglutinin-containing fraction of soybean whey was separated from trypsin inhibitors and small molecular weight growth inhibitors by ion exclusion chromatography, very little growth inhibitor activity was found in the hemagglutinin fraction (54).

Saponins. Saponins are glycosides which occur in a variety of plants. They are characterized by bitter taste, foaming in aqueous solutions and hemolyzing red blood cells. Upon complete hydrolysis they yield sapogenins and sugars. Proteolytic activity of trypsin was inhibited by high levels of soybean saponin (26).

Since saponins interfered with proteolytic activity and caused hemolysis of red blood cells, it was quite possible that saponins were the cause of the poor nutritive value of RSBM. Birk et al. (5) found that heat treatment had no effect on the hemolytic activity of

saponins. Since heat treatment of soybean meal alleviates the growth depression, saponins must not be the growth-depressing factor. The antiproteolytic activity was caused from a nonspecific reaction of saponins with protein and was readily counteracted by the presence of dietary proteins (26).

Pancreatic enlargement. Feeding RSBM diets cause growth depression along with pancreas enlargement in chicks (11, 46, 53), rats (43, 48) and mice (54). Chernick et al. (11) found that chicks fed a RSBM diet had enlarged pancreases and suggested this was caused by the increased demand for trypsin which was met by increased secretion by the pancreas. The overall concentration of the proteolytic enzymes in the pancreases of the chicks fed raw soybeans was unchanged, but with increased size of the organ the total activity was increased.

This increase in size of pancreases has been attributed to levels of fat in diet (45), a factor in soybean hulls (58) and also trypsin inhibitors (43, 49). The pancreas enlargement produced by feeding raw soybeans has been suggested to be associated with growth depression, but there is no definite proof of the cause and effect. Kakade and co-workers (27) tested 104 varieties of soybeans and found a negative correlation ($r = -.77$) between pancreas size and protein efficiency ration when feeding raw soybean diets to rats. Schingoethe and Thomas (55), when feeding rats diets containing soybean trypsin inhibitors, found growth depression with only two of the four diets although all four diets caused pancreas enlargement.

The addition of soybean hulls to a purified ration caused pancreas enlargement with no growth depression (58). Also, a growth inhibitor has been separated from soybeans that did not cause pancreas enlargement (54).

Loss of endogenous nitrogen. Because of increased enzyme production in animals fed a raw soybean diet, Lyman and Lepkovsky (43) felt that this could result in a large loss of endogenous nitrogen. The loss of nitrogen via this route could possibly account for some of the growth depression attributed to raw soybeans. This suggestion was further substantiated when rats fed heated meal had lower proteolytic activity in their feces than rats fed unheated meal (36). Other researchers (12, 21, 29, 55) found increased proteolytic activity and increased trichloroacetic acid-insoluble protein in the intestinal contents of rats fed RSBM or diets containing soybean trypsin inhibitors.

However, Kwong et al. (33) doubt whether this endogenous loss of nitrogen accounts for the growth depression found when feeding raw soybeans. They found no decrease in the percentage of nitrogen absorbed in rats fed a diet of unheated flakes starting at 25% and increasing to 75% of the total diet. Others (55) observed that despite the increased intestinal proteolysis on four trypsin inhibitor-containing diets, rat growth rates were reduced on only two of the diets.

Amino acid deficiency. Little agreement is found as to the extent amino acid supplementation will improve the nutritive value

of RSBM diets. Adding methionine to a RSBM diet did not completely compensate for the growth depression (2, 8). Borchers (7) claimed that adding methionine to a RSBM diet could completely counteract the growth depression. However, the weight gains (4.0 g/day) of these rats were much less than gains (6.5 g/day) of rats fed by Barnes et al. (2). They found that added methionine could account for only 75% of the growth depression attributed to RSBM. The differences in growth rates could be attributed to the protein levels in the diets. Borchers fed a low protein diet (15%) compared to the diets (20% to 35% protein) fed by Barnes et al. (2). The lower protein diets may have been deficient in methionine, and the added methionine would show more response in the lower protein diet. In later studies, Borchers (8) could attain only 75% of the growth of rats fed the heated soybean diet. Barnes et al. (2) suggested that supplemental methionine would increase growth rates of rats receiving low levels of heated or unheated soybean preparations because methionine is the most limiting amino acid in soybeans. However, high levels of heated soybean meal provided the needed methionine and supplemental methionine showed no increased response.

Supplementation of eight amino acids to a RSBM diet equaled the growth rate of the heated soybean meal diet (7). Again, because of the lower protein content in the diet, the growth rates were not as high as those attained by the rats fed by Barnes et al. (2). However, there is no agreement among different investigators as to whether the addition of amino acids would make the protein present

in raw soybean meal equivalent biologically to that of heated meal, or simply substitute for the protein's deficiencies.

MATERIALS AND METHODS

Preparation of soybean meal fractions. Raw soybean meal (RSBM) was prepared by grinding and hexane-extracting soybeans (Corsoy variety) as previously described by Schingoethe et al. (54).

Heated raw soybean meal (HRSBM) was prepared by autoclaving raw meal according to procedures outlined by Renner and Hill (52). This procedure was modified slightly, in that the meal was autoclaved at 110 C (15 lb steam pressure) for 15 minutes. After autoclaving the meal, it was air dried at room temperature and finely ground.

One hundred grams of RSBM was extracted with one liter of distilled water for two hours at room temperature. During the extraction, the RSBM was slowly agitated with a magnetic stirrer and then centrifuged (5,000 revolutions per minute for 10 minutes) to remove the water insoluble portion. After pouring off the supernatant, the meal was re-extracted with 500 ml of distilled water for one hour and centrifuged. The resulting supernatants were combined, acidified to pH 4.4 with 6N HCl (remove the acid insoluble proteins) and centrifuged. After lyophilizing[1], the pH 4.4 supernatant (pH 4.4-S) was stored for later use.

Ion exclusion chromatography. Ion exclusion chromatography was employed in an attempt to further purify and separate the growth inhibitor (s). Experimentation with Sephadex G-25, G-15 and G-10

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[1]Virtis Research Equipment, New York.

columns indicated that the G-25 column gave the most desirable separation. With an exclusion limit of 5,000 molecular weight (MW) for proteins, the trypsin inhibitors of 8,000 MW (16) to 24,000 MW (31) and hemagglutinins of 96,000 MW (61) would not be retarded, and would elute with the void volume. The growth inhibitor (s) isolated by Schingoethe et al. (54) would be slightly retarded.

Lyophilized pH 4.4-S (2.25g) was redissolved in 75 ml of distilled water and applied to the Sephadex G-25 column (5.7 x 107 cm), which was monitored by a recording spectrophotometer at 280 nm. The pH 4.4-S was eluted with distilled water at 23 ml/minute. The effluent was collected in 23 ml aliquots and every other tube assayed for trypsin inhibitor activity (25), protein (62, 63) and carbohydrate concentration (1). Approximately 20 runs were required to recover enough material for one mouse growth assay.

Charcoal fractionation. Figure 1 shows the fractionation scheme that was employed using activated charcoal.[1] The pH 4.4-S was used as the starting material and mixed thoroughly with the activated charcoal prior to filtering. The resulting filtrate (char filtrate) was saved. The charcoal and adsorbed material was then washed with pH 11.5 NaOH, pH 8.0 NaOH and pH 2.5 HCl and each of the resulting filtrates were saved. Volumes of filtrates were approximately equal to the amount of liquid that was used to wash the charcoal. Samples were taken from

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[1] Mallinckrodt Chemical Works, St. Louis, Missouri.

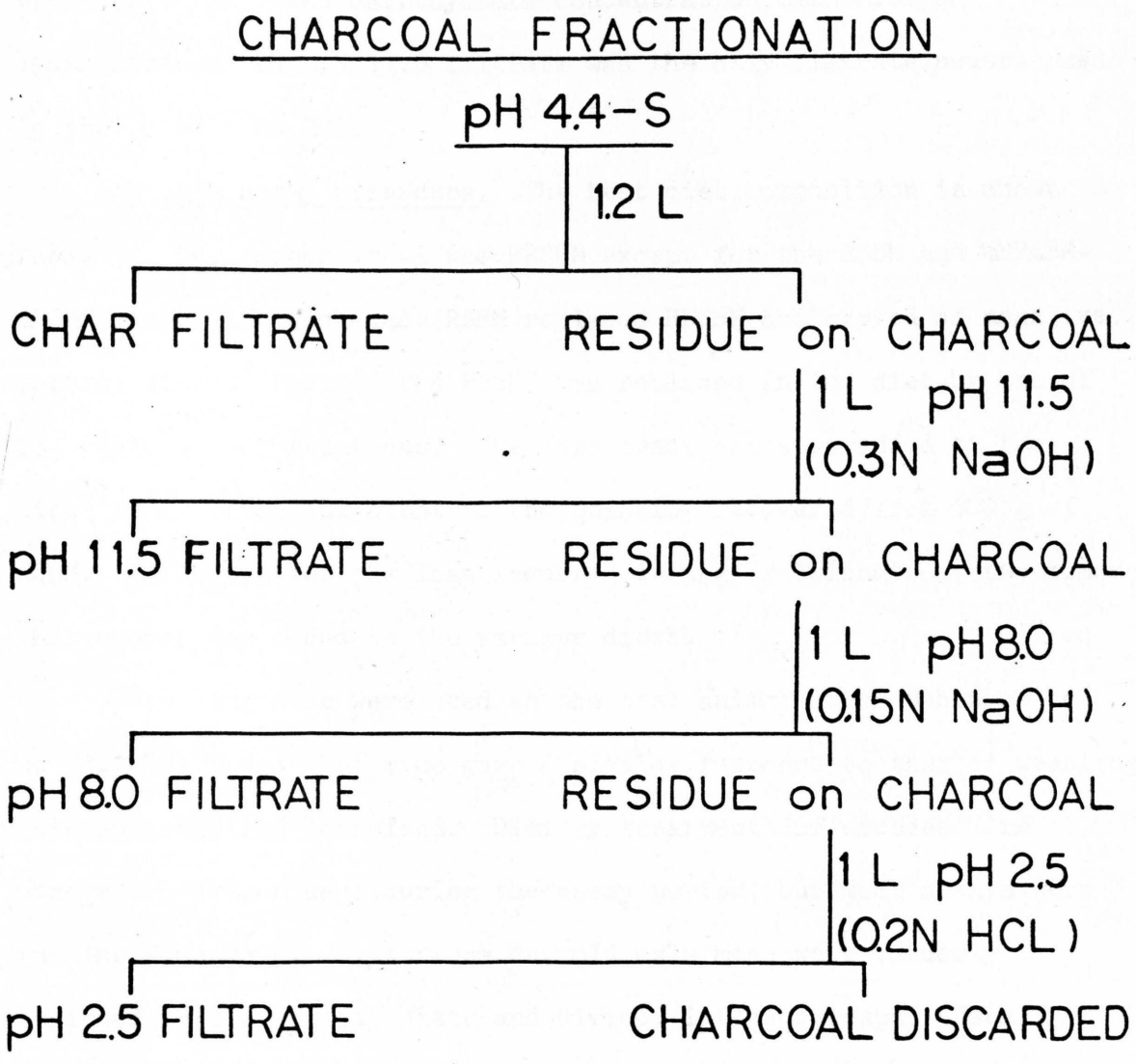

Figure 1. Fractionation of the pH 4.4 supernatant using activated charcoal as a crude ion exchange bed.

each of the filtrates and analyzed for trypsin inhibitor activity (25), protein (62, 63) and carbohydrate concentration (1) prior to lyophilizing. The pH 11.5 filtrate was the only filtrate neutralized to pH 7.0 with 6N HCl.

Growth assay procedure. The test diet composition is shown in table 1. The soybean meal was HRSBM except for the RSBM and ½HRSBM-½RSBM diets, in which case RSBM replaced HRSBM and served as negative control diets. Part of the HRSBM was replaced in the diet by one of the various test fractions. The test fractions were added to the diets in amounts equivalent to the quantity recovered from 100 g of RSBM. To adjust for the loss incurred during fractionation, two times this amount was added to the various diets.

Weanling mice were used as the test animal since Schingoethe et al. (54) found that mice gave a similar response to that of weanling rats but required less feed. Dietary treatment differences were observed at three days during the assay period, but most assays were run for five days. Twenty-one day old male mice were randomly assigned to the various diets and divided into subgroups. Usually eight to ten mice were used per treatment with four to five mice in each wire meshed cage. Beginning, three-day and terminating weights were recorded. On the fifth day the mice were sacrificed and pancreases removed and weighed. Feed intake for each treatment subgroup was determined by weighing the feed fed along with estimating feed in the feeders at termination of the experiment. A positive

Table 1. Composition of Diets Fed to Mice

Ingredient	Amount
	g
Salt mix[1]	4.0
Vitamin mix[2]	2.2
Corn oil	5.0
a - Cellulose[3]	1.5
Glucose[4]	37.3
HRSBM[5,6]	50-X
Soybean meal test fraction[6]	X
	100.0

[1]Wesson modification of Osborne-Mendel Formula, Nutritional Biochemicals Corporation, Cleveland, [2]Ohio.
Vitamin diet fortification mixture, Nutritional [3]Biochemicals Corporation, Cleveland, Ohio.
Nutritional Biochemicals Corporation, Cleveland, [4]Ohio.
Dextrose, J. T. Baker Chemical Corporation, [5]Phillipsburg, New Jersey.
Soybean meal is HRSBM in all cases except for [6]raw soybean meal fraction.
Soybean meal fractions replaced part of the HRSBM.

control (HRSBM) along with two negative controls (RSBM or ½HRSBM-½RSBM and pH 4.4-S) were fed during each mouse growth assay to serve as controls for that particular trial.

Growth inhibitor (GI) activity was calculated by the following formulas as prepared by Schingoethe et al. (54):

1) Total GI activity (units) =

$$\frac{(\text{wt gain}_{\text{HRSBM}} - \text{wt gain}_{\text{test}})\ (\text{feed intake}_{\text{test}})}{(\text{wt gain}_{\text{HRSBM}})\ (\text{feed intake}_{\text{HRSBM}})} \times 100$$

2) Specific GI activity (units/g) =

$$100 \times \frac{(\text{wt gain}_{\text{HRSBM}} - \text{wt gain}_{\text{test}})}{(\text{wt gain}_{\text{HRSBM}})} \times \frac{1}{(\text{feed intake}_{\text{HRSBM}})} \times \frac{1}{(\text{xg test})}$$

One unit of GI activity equals 1% reduction in growth rate compared to the positive control. Specific GI activity takes into account the amount of test fraction in the diet. Differences in feed intake were negligible except for the RSBM, ½HRSBM-½RSBM and pH 4.4-S diets (Appendix tables 1-7).

Enzyme inhibitor assay. Trypsin inhibitor activities of soybean meal and various fractions were determined by the measurement of the inhibition of hydrolysis of p-toluenesulfonyl-L-arginine methyl ester (TAME)[1] by trypsin (25). The inhibitor samples were diluted to insure that the assay mixture was not saturated by inhibitor.

Carbohydrate concentration determination. Carbohydrate concentration was determined as outlined by Badin et al. (1) at 520 nm.

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[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

Statistical analysis. The data from the various mouse growth assays were analyzed individually according to the procedures by Steele and Torrie (60). The statistical significance between the means was analyzed by Duncan's new multiple range test (60).

RESULTS AND DISCUSSION

Separation on Sephadex G-25 column. Figure 2 illustrates the elution pattern of the pH 4.4-S on the Sephadex G-25 column. Protein values were calculated by the methods of Waddell (62) and Warburg and Christian (63). Since absorbencies at 280 nm are proportional to protein concentration, for expediency, the column runs were monitored at this reading. On the basis of protein determinations, the effluent was divided into five fractions and designated as fractions I, II, III, IV and V, respectively.

Fraction I (Fig. 2) contained all of the trypsin inhibitors. Hemagglutinins were assumed to be present in fraction I (61), although hemagglutinin assays were not conducted. Chymotrypsin inhibitors presumably were also located in this fraction (54), since most trypsin inhibitors also inhibit chymotrypsin. However, the fractions were not analyzed for chymotrypsin inhibitor activity. Carbohydrates were eluted in the same area of fractions II and III (Fig. 2) and, thus, were not separated from proteins or with one distinct protein fraction by this method. Small molecular weight material was located in fractions IV and V (Fig. 2).

Growth inhibitor assay results of feeding the various pH 4.4-S fractions separated on the Sephadex G-25 column are shown in table 2. Weight gains (g/day) of mice fed fractions I and II were significantly different from the HRSBM diet ($P<0.05$), but were not significantly

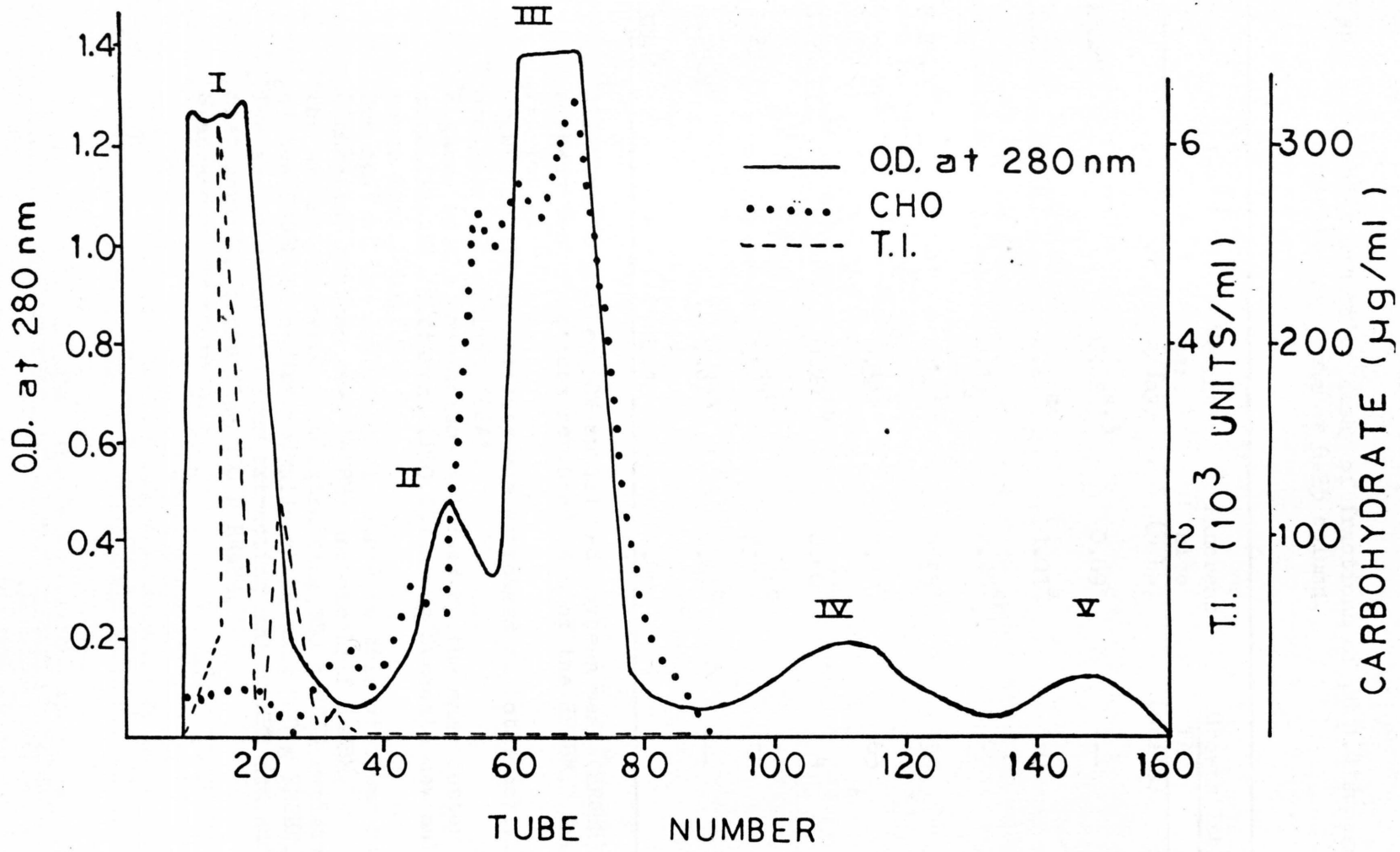

Figure 2. Elution pattern of pH 4.4 supernatant on a Sephadex G-25 column. Column dimensions: 5.7 X 107 cm. Sample: 2.25g dissolved in 75 ml distilled water. Eluting buffer distilled water. Flow rate: 23 ml/min. O.D. at 280 nm (——), trypsin inhibitor activity (- - -), carbohydrate concentration (. . .).

Table 2. Growth inhibitor assay of fractions of pH 4.4 supernatant separated on Sephadex G-25 column.

Test fraction[1]	Weight gain (g/day)	Pancreas size (% bw)	Growth Inhibitor[2] TA (units)	Growth Inhibitor[2] SA (units/g)
HRSBM	0.82[a,3]	0.69[c]	---	---
½HRSBM - ½RSBM[4]	-0.24[d]	1.01[a]	130	97
pH 4.4 - S[5]	0.19[c]	0.95[ab]	77	145
G-25-I[6]	0.42[bc]	0.95[ab]	50	860
G-25-II[6]	0.30[c]	0.77[b]	63	190
G-25-III[6]	0.79[ab]	0.60[c]	4	30
G-25-IV[6]	0.84[a]	0.64[c]	---	---
G-25-V[6]	0.87[a]	0.67[c]	---	---
SEM[7]	0.12	0.06		

[1] All diets contained 50% autoclaved soybean meal (HRSBM); soybean test fractions replaced part of the HRSBM. Diets were
[2] fed for five days.
[2] Growth inhibitor activity was expressed as total activity (TA)
[3] and specific activity (SA).
[3] Figures in the same column followed by the same letter are not significantly different ($P>0.05$) using Duncan's new multiple
[4] range test (60).
[4] One half of the soybean meal source in this diet was raw
[5] (unheated) soybean meal (RSBM) and one half HRSBM.
[5] The amount recovered from extracting 100 g RSBM replaced part
[6] of the HRSBM in a diet normally containing 100 g HRSBM.
[6] The amount recovered from extracting 200 g RSBM and added to a
[7] diet normally containing 100 g HRSBM.
[7] Standard error of mean.

different ($P>0.05$) than the pH 4.4-S diet. Fractions III, IV and V were not significantly different from the HRSBM diet ($P>0.05$).

Growth inhibition due to the fraction I diet could possibly be attributed to trypsin inhibitors or some other proteinaseous material with a molecular weight greater than 5,000 but less than 30,000. This seems to be a reasonable assumption since Schingoethe et al. (54) found little or no GI activity in the fraction-containing proteins with molecular weights greater than 30,000. However, they found relatively the same amount of GI activity in their trypsin inhibitor-containing fraction as was observed with fraction I. Also, crystalline trypsin inhibitor does not cause the extreme growth depression found when feeding RSBM (17, 19, 21). Fraction I had a very high specific GI activity (860 units/g) and caused pancreatic enlargement, 0.95% compared to 0.69% for the HRSBM fed mice, when expressed as %-body weight. Total GI activity was divided into fractions I and II, with fraction II containing slightly more than half of the total GI activity. The lower specific GI activity associated with fraction II was attributed to the extraneous material present in this fraction. A trace of GI activity was found in fraction III, which may have resulted from incomplete separation from fraction II. Fraction IV and V did not cause growth depression or pancreas enlargement.

The retention of the growth inhibitor located in peak II on the Sephadex G-25 column indicated a small molecular weight material.

Known molecular weight substances were eluted on the same Sephadex G-25 column (previously used for the separation of the pH 4.4-S) in an attempt to estimate the molecular weight of the growth inhibitor located in peak II. Comparing the elution pattern of these known molecular weight substances to the elution pattern of the pH 4.4-S (Fig. 2), soybean trypsin inhibitor[1] (24,000 MW) eluted off the same as peak I. Glucagon[2] (3,400 MW) eluted off between peaks I and II, whereas tryptophan[3] (200 MW) was eluted on the declining side of peak III. Plotting log of molecular weight versus elution volume indicated a molecular weight of 1,200 for peak II, 500 for peak III and molecular weights similar to those of smaller amino acids for peaks IV and V.

The data of four mouse growth assays (see Appendix tables 1-3 for actual data), in which the various G-25 fractions were fed, are summarized in table 3. Growth depression was divided between fractions I and II, with fraction I causing pancreas enlargement as compared to the HRSBM diet. Fraction III had a higher GI activity than observed in table 2. This was probably due to poor separation from fraction II in initial column runs. Although total GI activity was about the

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[1] Soybean trypsin inhibitor 5 X crystallized, Nutritional Biochemical Corporation, Cleveland, Ohio.
[2] Nutritional Biochemical Corporation, Cleveland, Ohio.
[3] Eastman Organic Chemicals, Rochester, New York.

Table 3. Growth Inhibitor and Pancreas Enlargement Activities of G-25 separated fractions of pH 4.4 supernatant. (See Appendix tables 1-3 for actual data.)

Test fraction[1]	Weight gain (g/day)	Growth Inhibitor[2] TA (units)	Growth Inhibitor[2] SA (units/g)	Relative[3] pancreas size (%)
HRSBM	0.69	---	---	100
½HRSBM - ½RSBM[4]	-0.14	120	85	143
pH 4.4 - S[5]	-0.10	114	200	131
G-25-I[6]	0.23	67	913	144
G-25-II[6]	0.16	77	334	106
G-25-III[6]	0.45	35	170	100
G-25-IV[6]	0.78	---	---	98
G-25-V[6]	0.79	---	---	101

[1] See footnote 1, table 2.
[2] See footnote 2, table 2.
[3] $\frac{\text{Pancreas wt. as \% body wt. on test diet}}{\text{Pancreas wt. as \% body wt. on HRSBM}} \times 100$
[4] See footnote 4, table 2.
[5] See footnote 5, table 2.
[6] See footnote 6, table 2.

same in fractions I and II, fraction I had a much higher specific GI activity. Protein fraction II had a lower specific GI activity than fraction I because of the high carbohydrate concentration present with the protein fraction. Fractions IV and V showed no GI activity, and fractions II, III, IV and V did not cause pancreas enlargement.

Growth inhibition associated with fraction I was not separated from the trypsin inhibitors. However, until this fraction is further purified the growth depression may or may not be attributed to the trypsin inhibitors. Fraction II caused growth depression, was free of any trypsin inhibitors and did not cause pancreatic enlargement. Sharper separation between the trypsin inhibitors and small molecular weight growth inhibitor, than that reported by Schingoethe et al. (54), was accomplished by using the Sephadex G-25 column. However, since the specific GI activity attributed to this fraction was not as high as fraction I, more purification is needed to ascertain whether the carbohydrate material or other unidentified materials are contaminants or a cause of the growth inhibition. Because of the clear separation between fractions I and II, it was concluded that these were two different growth inhibitors.

Separation by activated charcoal. In an attempt to separate the carbohydrate material from the protein fractions, a fractionation scheme (Fig. 1) was devised using activated charcoal as a crude ion exchange bed. If this system would separate the growth inhibitor from carbohydrates or from other materials, it could be readily applied on a commercial basis.

After lyophilizing the various filtrates that resulted from charcoal fractionation, they were redissolved in distilled water and applied to the same Sephadex G-25 column that was used for the pH 4.4-S separation. This was done as a means of desalting and also to see if any separation had been achieved by this fractionation scheme.

Figure 3 shows the separation of the pH 4.4-S achieved by the activated charcoal fractionation. In comparing figures 2 and 3, peak I (Fig. 2) was located primarily in the charcoal filtrate (scan no. 1, Fig. 3), which also had trypsin inhibitor activity and contained almost all of the carbohydrate material that was recovered. The latter may be more clearly shown in table 4. The pH 11.5 filtrate (scan no. 2, Fig. 3) contained a small amount of peak I (Fig. 2) and predominantly peaks II and III (Fig. 2). The pH 11.5 filtrate contained small amounts of trypsin inhibitor activity and carbohydrate material (table 4). Peak II (Fig. 2) was the most abundant in the pH 8.0 filtrate (scan no. 3, Fig. 3) with no trypsin inhibitor activity and a very small amount of carbohydrate material (table 4). Scan 4 (Fig. 3) showed that a very minute amount of protein was recovered in the pH 2.5 filtrate. The protein present was primarily peak II (Fig. 2) components. Also, the carbohydrate concentration was low, with no trypsin inhibitor activity (table 4) being found.

Table 4 also shows the dry matter recovered in the various freeze dried filtrates. Because of the added NaOH and HCl, the dry matter recovered from all of the filtrates was higher than that

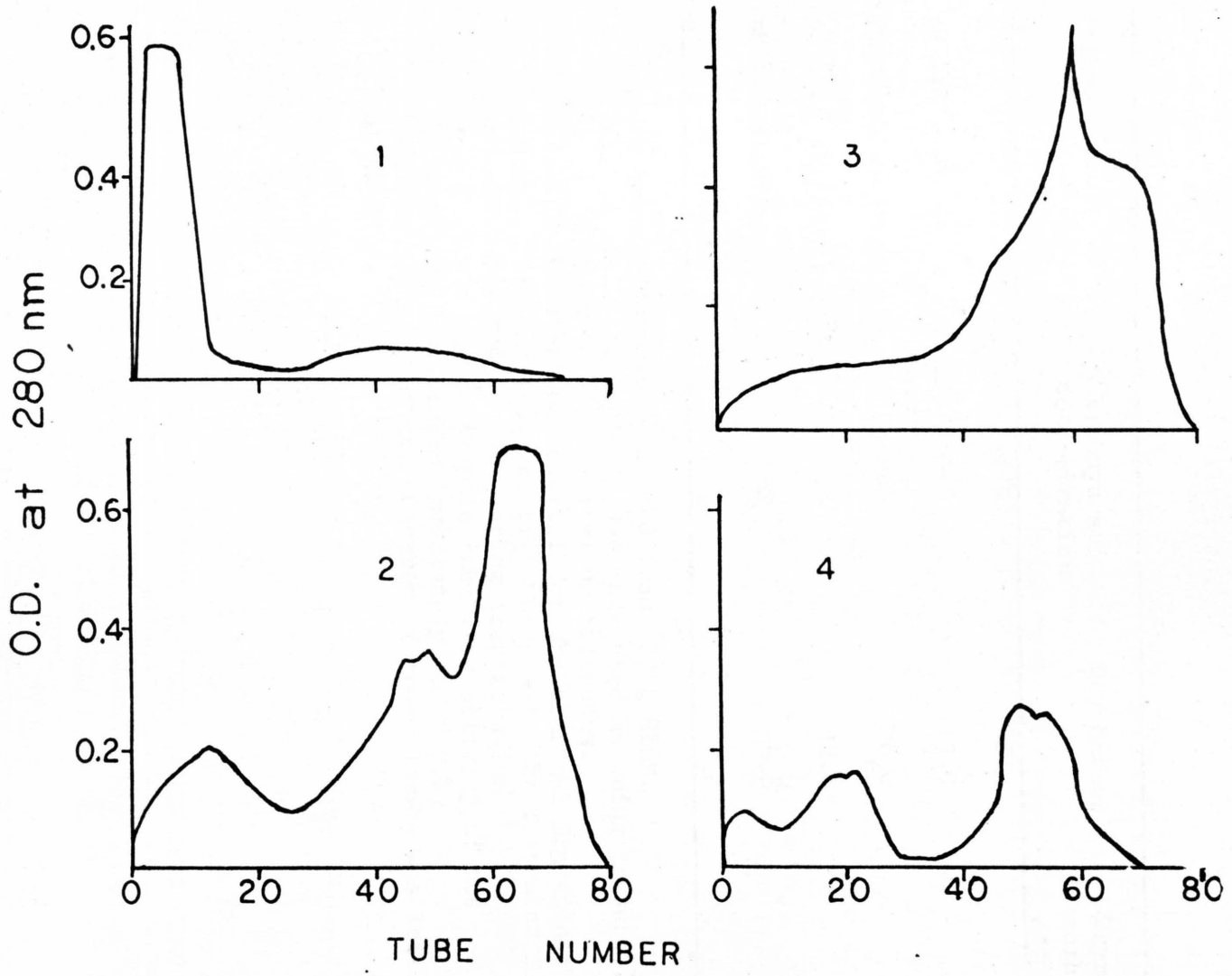

Figure 3. Chromatography on Sephadex G-25 of charcoal filtrate fractions of pH 4.4 supernatant. Charcoal filtrate, 1; pH 11.5 filtrate, 2; pH 8.0 filtrate, 3; pH 2.5 filtrate, 4.

Table 4. Carbohydrate concentration, dry matter recovered and trypsin inhibitor activity in the various charcoal separated pH 4.4-S filtrates.

Filtrate	Carbohydrate concentration	Dry Matter[1]	Trypsin[2] inhibitor activity
	(µg/ml)	(g)	
pH 4.4 - S[3]	965	21.0	+
Charcoal filtrate	460	6.0	+
pH 11.5 filtrate[4]	58	12.8	±
pH 8.0 filtrate	32	4.8	-
pH 2.5 filtrate	22	3.0	-

[1] Dry matter that was recovered from 100 g RSBM.
[2] Trypsin inhibitor activity was indicated as being present (+) or not present (-) in the various filtrates.
[3] This was the starting material for the charcoal fractionation scheme. This was analyzed to compare with the filtrates for amounts recovered or lost during fractionation.
[4] This filtrate showed trypsin inhibitor activity if the filtrate was neutralized immediately to pH 7.0. Denaturation of proteins occurred if the filtrate was left at pH 11.5.

normally recovered from the freeze dried pH 4.4-S. Salt concentrations in the pH 11.5 and pH 8.0 filtrates were 22% and 18%, respectively. After desalting, both contained 11% salt.

Data of four mouse growth assays in which the charcoal filtrates were utilized as the test diets are summarized in table 5 (see Appendix tables 4-7 for actual data). Although no large differences were found between the weight gains of the mice fed the various test diets and the HRSBM diets, the pH 8.0 filtrate had a high GI activity with no pancreas enlargement. Since elution on Sephadex G-25 (Fig. 3) indicated that the pH 8.0 filtrate contained predominantly fractions II and III and no fraction I, growth inhibition was probably due to fraction II. However, because of the extreme pH changes, the proteins were denatured and thus decreased the growth inhibitor activity.

Fractions II and III (pH 4.4-S separated on Sephadex G-25 column) were collected together and lyophilized. Thirty-five grams of fractions II and III (amount recovered from 25 column runs) were redissolved in one liter of distilled water and sent through the same fractionation scheme shown in figure 1. The procedure was modified slightly in that much weaker concentrations of base and acid were used. Approximately 23 grams of dry matter were recovered in all of the filtrates.

Growth trial results from feeding the various filtrates of the charcoal fractionation of fractions II and III are shown in table 6 (see Appendix table 4 for actual data). Growth inhibitor activity was

Table 5. Growth assay by mice fed various fractions of pH 4.4 supernatant eluted through Charcoal. (See Appendix tables 4-7 for actual data.)

Test fraction[1]	Growth Inhibitor[2]		Relative[3] pancreas size
	TA (units)	SA (units/g)	(%)
HRSBM	---	---	100
½HRSBM - ½RSBM[4]	120	85	143
pH 4.4 - S[5]	114	201	131
Charcoal filtrate[6]	19	33	104
pH 11.5 filtrate[6]	6	7	98
pH 8.0 filtrate[6]	25	350	97
pH 2.5 filtrate[6]	18	122	93

[1] See footnote 1, table 2.
[2] See footnote 2, table 2.
[3] See footnote 3, table 3.
[4] See footnote 4, table 2.
[5] See footnote 5, table 2.
[6] See footnote 6, table 2.

Table 6. Growth assay by mice fed various fractions of G-25 Fraction II and III eluted through Charcoal. (See Appendix table 4 for actual data.)

Diet[1]	Growth Inhibitor[2]		Relative[3] pancreas size
	TA (units)	SA (units/g)	(%)
RSBM	---	---	100
RSBM - ½RSBM	120	85	143
pH 4.4 - S[4]	114	201	131
G-25 Fraction II and III			
Charcoal filtrate[5]	17	39	96
pH 11.5 filtrate[5]	23	230	89
pH 8.0 filtrate[5]	27	547	92
pH 2.5 filtrate[5]	---	---	104

[1,2] See footnotes 1 and 2, table 2.
[3] See footnote 3, table 3.
[4] See footnote 5, table 1.
[5] The amount recovered from 35 g, freeze dried fractions II and III, sent through charcoal fractionation.

again located in the pH 8.0 filtrate, substantiating results reported in table 5. The pH 11.5 filtrate contained the second highest GI activity with none of the filtrates causing pancreas enlargement.

Although the small molecular weight growth inhibitor was in fractions II and III prior to sending it through the charcoal, very little of the growth depressant was recovered in the charcoal filtrates. Absence of the growth depression was attributed to the extreme pH changes, which caused denaturation of proteins.

SUMMARY AND CONCLUSIONS

Ion exclusion chromatography was employed to further purify and separate a small molecular weight growth inhibitor from soybean trypsin inhibitors. The pH 4.4-S was separated on a Sephadex G-25 column into five fractions. Fraction I contained proteinaceous material greater than 5,000 molecular weight and trypsin inhibitor activity. Diets containing this fraction when fed to mice caused growth depression and pancreas enlargement. Growth depression was caused by fraction II diets, but no pancreas enlargement was noticed. The growth inhibitor present in fraction II was calculated to have a molecular weight of 1,200. The diets containing G-25 fractions III, IV and V caused little or no growth depression to mice and did not cause pancreas enlargement. Because of the clear separation accomplished between fractions I and II, with both causing growth depression and only fraction I causing pancreas enlargement, it was concluded that these are two different growth inhibitors present in the soybean whey fraction. The mechanism of the growth inhibition by these inhibitors may be different.

Animal growth assays are the only positive measurement of the growth depressant in soybeans. However, this method involves a great deal of time in preparation of the fractions to be used in the diets. An attempt was made to batch separate the growth inhibitor from soybeans, utilizing activated charcoal as a crude ion exchange bed.

In comparing this fractionation to the separation via the Sephadex G-25 column, preferential separation was achieved. However, the extreme pH changes employed, denatured the proteins and decreased the growth inhibitor (GI) activity of the various filtrates. Elimination of some of the carbohydrate material as the cause of the growth depression was accomplished by this fractionation. The pH 8.0 filtrate had the highest GI activity of the various filtrates in most of the mouse growth assays. There are three possible explanations for this, and they are:

1) Greater denaturation occurred at the pH extremes.
2) G-25 peak II was the most prevalent in the pH 8.0 filtrate.
3) The growth inhibitor may be active at pH 8.0 and inactive at extreme pH's.

The pH 8.0 filtrate caused growth inhibition even though it was almost void of G-25 peak I. However, it was concluded that because of the denaturation of proteins incurred during the fractionation, this method would possibly be better suited to detoxify the soybean whey fraction than to separate and purify the growth inhibitor.

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APPENDIX

Appendix Table I. Individual weight gains, pancreas size, feed intake and quantity of test fraction in various diets fed to mice. (Mouse growth assay No. 1. Diets fed for five days.)

Test fraction[1]	Weight gain	Pancreas size		Feed intake	Test fraction[2] in diet
	(g/day)	(g)	(% body wt)	(g/day)	(g/100 g diet)
HRSBM	0.78[a,3]	0.11	0.57[c]	4.8	50.0
½HRSBM - ½RSBM	-0.43[c]	0.14	0.92[a]	2.5	25:0
pH 4.4 - S	0.43[b]	0.14	0.77[b]	4.4	7.0
G-25 - I[4]	0.78[a]	0.13	0.68[b]	4.9	0.3
G-25 - II & III	0.56[ab]	0.11	0.64[b]	4.8	4.0
G-25 - IV & V	0.71[a]	0.12	0.63[bc]	4.9	0.1
SEM[5]	0.09		0.04		

[1] See text for complete description of test fractions.
[2] Test fraction is equivalent to the quantity recovered from 100 g RSBM except for HRSBM, ½HRSBM - ½RSBM.
[3] Figures in the same column followed by the same letter are not significantly different (P>0.05) using Duncan's new multiple range test (60).
[4] This fraction was concentrated in a Virtis freeze concentrator and probably denatured the proteins. (Only for this trial.)
[5] Standard error of mean.

Appendix Table II. Individual weight gains, pancreas size, feed intake and quantity of test fraction in various diets fed to mice. (Mouse growth assay No. 2. Diets fed for three days.)

Test fraction[1]	Weight gain	Pancreas size		Feed intake	Test fraction[2] in diet
	(g/day)	(g)	(% body wt)	(g/day)	(g/100 g diet)
HRSBM	0.30[a,3]	0.15	0.69[bc]	7.4	50.0
½HRSBM - ½RSBM	-0.66[cd]	0.16	0.83[ab]	5.1	25.0
pH 4.4 - S	-0.91[d]	0.16	0.84[a]	7.3	10.0
G-25 - I	-0.36[bc]	0.19	0.94[a]	6.3	1.5
G-25 - II	-0.27[b]	0.15	0.69[bc]	5.4	3.8
G-25 - III	0.07[ab]	0.14	0.67[c]	7.3	5.7
SEM[4]	0.09		0.04		

[1] See footnote 1 Appendix Table I.
[2] Test fraction is equivalent to 2 X the quantity recovered from 100 g RSBM except HRSBM, ½HRSBM - ½RSBM, and pH 4.4.
[3,4] See footnotes 3 and 5 Appendix Table I.

Appendix Table III. Individual weight gains, pancreas size, feed intake and quantity of test fraction in various diets fed to mice. (Mouse growth assay No. 3. Diets fed for five days.)

Test fraction[1]	Weight gain	Pancreas size		Feed intake	Test fraction[2] in diet
	(g/day)	(g)	(% body wt)	(g/day)	(g/100 g diet)
HRSBM	0.74[a,3]	0.10	0.59[c]	7.25	50.0
½HRSBM - ½RSBM	-0.18[b]	0.14	0.97[a]	4.8	25.0
G-25 - I	0.50[a]	0.14	0.93[ab]	4.0	1.6
G-25 - II	0.46[a]	0.10	0.65[c]	4.6	3.9
G-25 - III	0.50[a]	0.11	0.68[bc]	5.5	3.5
SEM[4]	0.09		0.09		

[1] See footnote 1 Appendix Table I.
[2] See footnote 2 Appendix Table II.
[3,4] See footnotes 3 and 5 Appendix Table I.

Appendix Table IV. Individual weight gains, pancreas size, feed intake and quantity of test fraction in various diets fed to mice. (Mouse growth assay No. 4. Diets fed for five days.)

Test fraction[1]	Weight gain	Pancreas size		Feed intake	Test fraction[2] in diet
	(g/day)	(g)	(% body wt)	(g/day)	(g/100 g diet)
HRSBM	0.75[a,3]	0.11	0.66[c]	6.3	50.0
½HRSBM - ½RSBM	-0.21[c]	0.16	0.92[a]	5.6	25.0
pH 4.4 - S	-0.48[c]	0.12	0.88[ab]	3.5	10.0
G-25 - I	0.37[a,b]	0.13	0.90[a]	5.6	2.5
Charcoal filtrate	0.24[b]	0.11	0.75[bc]	4.5	8.8
pH 11.5 filtrate[4]	-1.12[d]	----	----	2.2	12.8
pH 8.0 filtrate	0.03[bc]	0.09	0.64[c]	4.6	5.2
pH 2.5 filtrate	0.51[a]	0.11	0.61[d]	6.2	2.9
Charcoal G-25 II & III filtrate	0.63[a]	0.10	0.62[cd]	5.6	6.8
pH 11.5 G-25 II & III filtrate	0.58[a]	0.09	0.58[d]	5.5	1.6
pH 8.0 G-25 II & III filtrate	0.54[a]	0.11	0.61[d]	6.3	0.8
pH 2.5 G-25 II & III filtrate	0.79[a]	0.11	0.67[c]	5.6	0.3
SEM[5]	0.15		0.04		

[1] See footnote 1 Appendix Table I.
[2] See footnote 2 Appendix Table II.
[3] See footnote 3 Appendix Table I.
[4] Mice died before termination of experiment so three day weights were used and pancreases were not weighed. Death may be attributed to high salt concentration.
[5] See footnote 5 Appendix Table I.

Appendix Table V. Individual weight gains, pancreas size, feed intake and quantity of test fraction in various diets fed to mice. (Mouse growth assay No. 5. Diets fed for five days.)

Test fraction[1]	Weight gain	Pancreas size		Feed intake	Test fraction[2] in diet
	(g/day)	(g)	(% body wt)	(g/day)	(g/100 g diet)
HRSBM	0.61[a,3]	0.09	0.67[ab]	5.4	50.0
½HRSBM - ½RSBM	-0.07[b]	0.10	0.86[a]	3.8	25.0
pH 4.4 - S	0.17[b]	0.10	0.81[a]	2.7	10.0
Charcoal filtrate	0.68[a]	0.11	0.75[a]	3.5	6.4
pH 11.5 filtrate[4]	0.65[a]	0.09	0.61[b]	5.7	2.4
pH 8.0 filtrate[4]	0.60[a]	0.09	0.61[b]	3.9	0.4
HRSBM, pair fed[5]	0.57[a]	0.09	0.59[b]	3.5	50.0
SEM[6]	0.12		0.06		

[1] See footnote 1 Appendix Table I.
[2] See footnote 2 Appendix Table II.
[3] See footnote 3 Appendix Table I.
[4] NH_4OH used instead of NaOH and also at a weaker concentration.
[5] Fed HRSBM equal to the charcoal filtrate diet.
[6] See footnote 5 Appendix Table I.

Appendix Table VI. Individual weight gains, pancreas size, feed intake and quantity of test fraction in various diets fed to mice. (Mouse growth assay No. 6. Diets fed for five days.)

Test fraction[1]	Weight gain	Pancreas size		Feed intake	Test fraction[2] in diet
	(g/day)	(g)	(% body wt)	(g/day)	(g/100 g diet)
HRSBM	0.98[a,3]	0.10	0.56[b]	4.9	50.0
½HRSBM - ½RSBM	0.29[cd]	0.15	0.92[a]	4.0	25.0
pH 4.4 - S[4]	0.54[c]	0.14	0.85[a]	4.9	8.0
Charcoal filtrate[5]	0.46[c]	0.08	0.52[b]	4.6	10.8
pH 11.5 filtrate[6]	----	----	----	---	4.0
pH 8.0 filtrate[6]	----	----	----	---	4.4
pH 11.5 filtrate[7]	1.19[a]	0.11	0.63[b]	4.4	1.3
pH 8.0 filtrate[7]	0.86[ab]	0.09	0.57[b]	4.6	0.3
pH 11.5 filtrate[8]	0.99[a]	0.10	0.54[b]	4.3	2.1
pH 8.0 filtrate[8]	0.94[a]	0.82	0.53[b]	3.8	0.3
15 g NaCl[9]	0.61[bc]	0.09	0.58[b]	4.4	6.0
30 g NaCl[9]	0.15[d]	0.07	0.53[b]	4.4	12.0
SEM[10]	0.09		0.04		

[1] See footnote 1 Appendix Table I.
[2] See footnote 2 Appendix Table II.
[3] See footnote 3 Appendix Table I.
[4] Diet was made up to 250 g instead of the usual 200 g in an
[5] attempt and may be reason for higher gain/day.
[6] Added to diet 5 X the normal rate recovered from 100 g RSBM.
[6] $Ba(OH)_2 \cdot 8H_2O$ and $ZnSO_4$ used as basic and acidic reagents.
[7] Mice died of zinc toxicity.
[8] $Ba(OH)_2 \cdot 8H_2O$ and H_2SO_4 used as basic and acidic reagents.
[8] NaOH concentration was much weaker than used previously.
[9] This was a higher level of salt than was present in pH 11.5 and
[10] pH 8.0 filtrates, mouse growth assay No. 4 (Appendix Table IV).
[10] See footnote 5 Appendix Table I.

Appendix Table VII. Individual weight gains, pancreas size, feed intake and quantity of test fraction in various diets fed to mice. (Mouse growth assay No. 7. Diets fed for five days.)

Test fraction[1]	Weight gain	Pancreas size		Feed intake	Test fraction[2] in diet
	(g/day)	(g)	(% body wt)	(g/day)	(g/100 g diet)
HRSBM	1.11[a,3]	0.11	0.55[c]	4.8	50.0
½HRSBM - ½RSBM	0.30[b]	0.16	0.95[a]	4.0	25.0
pH 4.4 - S	0.22[b]	0.12	0.81[b]	4.2	10.0
Charcoal filtrate	0.85[a]	0.10	0.52[c]	5.5	7.4
pH 11.5 filtrate[4]	0.86[a]	0.12	0.61[c]	4.8	6.4
pH 8.0 filtrate[4]	0.97[a]	0.11	0.60[c]	5.3	4.0
pH 11.5 filtrate[5]	1.00[a]	0.09	0.51[c]	4.5	4.5
pH 8.0 filtrate[5]	1.02[a]	0.10	0.53[c]	5.5	2.5
15 g NaCl[6]	0.81[a]	----	----	4.8	6.0
30 g NaCl[6]	0.10[b]	----	----	4.6	12.0
SEM[7]	0.11		0.04		

[1] See footnote 1 Appendix Table I.
[2] See footnote 2 Appendix Table II.
[3] See footnote 3 Appendix Table I.
[4] Test fraction fed is half as much as fed in mouse growth assay No. 4 (Appendix Table IV). Not desalted.
[5] Test fraction fed is half as much as fed in mouse growth assay No. 4 (Appendix Table IV). Desalted by eluting through Sephadex G-25 column.
[6] Pancreases were not weighed because in a previous trial (Appendix Table VI), the pancreases had not been affected by the high salt concentration.
[7] See footnote 5 Appendix Table I.