Extraction of Nitrogenous Constituents from the Navy Bean Seed, *Phaseolus Vulgaris*

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The extraction of nitrogenous constituents from bean meal with various solvents has been studied. With four successive extraction periods, 10.40 and 11.47% of the total meal nitrogen was extracted with 70% ethyl alcohol solution and 70% ethyl alcohol solution containing sodium acetate, respectively. By extracting bean meal three times with a sodium chloride solution, about 74% of the total meal nitrogen was extracted. A minimum of 16.6% of the total meal nitrogen was obtained in an extract with a pH of 3.66. An optimum amount of nitrogenous constituents was extracted with an alkaline solvent.

A MAJOR PORTION of the mature dry seeds of the navy bean (*Phaseolus vulgaris*) consists of nitrogenous constituents such as proteins, peptides, amino acids, and amides. With new biochemical techniques, nitrogenous constituents in bean seeds can be studied in more detail. However, prior to the separation and analyses of these constituents, information on the extraction of soluble nitrogenous components should be obtained; consequently, this study has been undertaken. In order to study the extraction of nitrogenous constituents, dry seeds must first be reduced to a fine powder in a grinder or ball mill to break the cell walls and disrupt the cell contents. The size of the meal particles has been shown to influence the extraction of nitrogenous components from various seeds (*5, 9, 10*). The extraction of nitrogenous constituents also may be affected by factors such as meal-solvent ratio (*5, 9*), temperature of the meal-solvent mixture (*9, 11*), and the number of successive extractions (*5*).

In this study, the meal-particle size, extraction temperature, and duration of shaking were controlled, while the influence of chemical composition of the solvent, meal-solvent ratio, and successive extraction periods on the extraction of nitrogenous constituents from navy bean meal were explored.

Materials and Methods

Navy bean seeds of the Michelite variety were ground in a Wiley mill to pass through a 60-mesh screen. Microscopic observations indicated that cell walls were ruptured and the protein matrices were broken into small fragments. The meal sample, used in the ethyl alcohol extraction experiments, contained 8.8% moisture and 4.1% nitrogen on a dry weight basis. The remaining experimentation involved bean meal with 11.8% moisture and 4.1% nitrogen on a dry weight basis.

In studies on the extraction of nonprotein nitrogenous components, each 15-gram portion of bean meal was dispersed with 100 ml. of ethyl alcohol solution, either with or without sodium acetate, in a 200-ml. centrifuge bottle and the mixture was agitated for 3 hours in a Burrell wrist-action shaker at 25°C. The suspension was centrifuged at about 1500 × G at the bottom of the bottle for 15 minutes. The clear yellow centrifugate was poured off and 100 ml. of ethyl alcohol solution were added to the meal residue. The dispersed residue was shaken again for 1 hour and centrifuged for 15 minutes. Subsequent extracts were attained according to the above procedure. Nitrogen in the extracts was determined according to the AOAC Kjeldahl method (*1*) with boric acid in the receiving flasks.

For the extraction of nitrogenous constituents from bean meal by sodium chloride, hydrochloric acid, and sodium hydroxide solutions, each weighed sample of bean meal was placed in a 200-ml. centrifuge bottle and 100 ml. of solvent at 25°C. were added. The dispersed meal mixture was agitated with the aid of a wrist-action shaker for 1 hour at about 25°C. The suspension was centrifuged at about 1400 × G at the bottom of the bottle for 10 minutes to remove the major portion of the insoluble matter. The turbid supernatant, which contained suspended particles, was subjected to further centrifugation at 25,000 × G for 15 minutes. All residues were combined in the centrifuge bottle when further extractions were required. Subsequent extractions were carried out as outlined above, except that the time of agitation per extraction was reduced to 30 minutes. Each centrifugate was brought up to a specific volume and the nitrogen contents of aliquots were determined by the micro-Kjeldahl method (*1*). The pH values of the extracts were determined by a Beckman Model G pH meter.

For the determination of the polysaccharide content of an alkaline extract, the solution was lowered to pH 5 and saturated with ammonium sulfate, to separate the proteins from the polysaccharide fraction. The supernatant with the soluble polysaccharides was dialyzed against running tap water to remove the ammonium sulfate. To the dialyzate, 4 volumes of absolute ethyl alcohol were added and the suspension was allowed to stand for about 16 hours at about 25° C. Subsequently the suspended matter was removed by filtration with Whatman 41H filter paper which had been washed with water and 80% ethyl alcohol, dried at 105° C. for 3 hours, and weighed.

Table I. Extraction of Nitrogenous Constituents from Navy Bean Meal with Ethyl Alcohol Solutions

Extraction Solution	Extraction No.	pH of Extract	Total N Extracted,[b] Mg.	% N Extracted[b]
70% ethyl alcohol solution	1	6.95	32.17	5.75
	2	7.10	14.78	2.64
	3	7.18	6.45	1.15
	4	7.28	4.82	0.86
70% ethyl alcohol solution containing 0.5% sodium acetate	1	7.11	34.46	6.16
	2	7.41	15.66	2.80
	3	7.71	8.29	1.48
	4	7.90	5.76	1.03

[a] Initial weight of bean meal, 15 grams. [b] Mean value for 2 replications.

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For the qualitative detection of carbohydrate, the Molisch test was used. A few drops of 5% 1-naphthol were added to 1 ml. of test solution and the resulting solution was placed above concentrated sulfuric acid. A reddish violet coloration at the interface is indicative of the presence of a carbohydrate.

Results and Discussion

For the extraction of nonprotein nitrogenous constituents from plant tissues, ethyl alcohol solutions have been used extensively (*15*). The use of hot ethyl alcohol solutions for extractions is inadvisable, as glutamine is decomposed at high temperatures (*17*). Consequently, in these studies ethyl alcohol solutions at about 25° C. were employed. Data on the extraction of nonprotein nitrogenous constituents from bean meal with 70% ethyl alcohol solution and 70% ethyl alcohol solution containing sodium acetate are presented in Table I. With four extractions, the total nitrogen extracted was 10.40% for the 70% ethyl alcohol solution ond 11.47% for the 70% ethyl alcohol solution containing sodium acetate. According to the studies of Smith *et al.* (*14*), 8.2% of the total nitrogen of kidney bean meal was solubilized by extracting once with 70% ethyl alcohol solution.

The present study illustrates the importance of repeated extraction of bean meal in order to remove most of the nonprotein nitrogenous matter. Talley, Carter, and Porter (*16*) demonstrated that nine extractions with 70% ethyl alcohol by a batchwise procedure were required to extract amino acids completely from potatoes.

Prolamines were not found in the ethyl alcohol extracts of the bean meal. The results of Smith *et al.* (*14*) concur with this conclusion. Brohult and Sandegren (*3*) have stated that the absence of prolamine is characteristic of dicotyledonous seeds. Some ether-soluble matter was extracted from the residue of evaporated ethyl alcohol extracts. Presumably this matter was composed of phospholipides, as a precipitate was formed upon the addition of an excess of acetone to the ether extract. The presence of phosphatides in navy bean seeds has been reported (*8*). The addition of 10 ml. of 2*M* calcium chloride to the first ethyl alcohol extracts of bean meal caused the formation of a precipitate, which probably consisted of phosphatides and perhaps calcium phytate. With the removal of the precipitate, the nitrogen content of each supernatant was similar to that of the corresponding untreated ethyl alcohol extracts.

Osborne (*12*) has pointed out that salt-soluble globulins are the predominant class of proteins in seeds of *Phaseolus vulgaris*. In this study a 0.5*M* sodium chloride solution was used in an attempt to extract the globulins and albumins from the meal along with the nonprotein nitrogenous constituents. In Table II, the influence of meal-solvent ratio and successive extraction periods on the extraction of nitrogenous constituents is recorded. Most of the salt-soluble nitrogenous constituents were removed from the meal during the first extraction. With three successive extraction periods, the total amount of extracted nitrogen was 74.5% for the 5-gram meal sample and 74.1% for the 15-gram meal sample. In studies on the kidney bean (*Phaseolus vulgaris*), Smith *et al.* (*14*) reported that 76.2% of the total meal nitrogen was extracted from a 1-gram meal sample with 50 ml. of 0.5*M* sodium chloride solution. Considering the complexity of the meal and the manipulations in the extraction procedure, the reproducibility of the results (Table II) can be considered acceptable.

Peterson and Churchill (*13*) and Eichelberger (*6*) found large amounts of polysaccharides other than starch in navy beans. These constituents undoubtedly are bound to some extent to bean proteins. In an attempt to determine the presence of polysaccharides in the first sodium chloride extract of bean meal, the solution was saturated with ammonium sulfate and the precipitated portion was brought down by centrifugation. After the ammonium sulfate had been removed by dialysis, the centrifugate was added to 3 volumes of absolute ethyl alcohol. The fluffy white masses which became apparent upon standing were soluble in water and contained a relatively large amount of carbohydrate as shown by the Molisch test.

As shown in Table III, extracts with pH values ranging from 5.38 to 2.82 possessed small amounts of nitrogenous constituents. Fontaine, Pons, and Irving (*7*) reported that a minimum amount of nitrogenous constituents was present in peanut meal extracts with pH values ranging from 5.8 to 3.8. Detailed studies by these workers have shown that phytic acid in some seed meals reduced the solubility of meal proteins at pH values below the isoelectric points of the proteins. The optimum pH was 3.5 for the formation of the protein–phytic acid complexes in peanut meal extract. As phytic acid has been found in the seeds of *Phaseolus vulgaris* (*2*), the formation of these insoluble complexes may account for the small amount of nitrogenous matter in some of the navy bean meal extracts. However, in the case of the extract with a pH of 5.38, the low extraction value of 24.1% of the total nitrogen

Table II. Effect of Meal-Solvent Ratio and Successive Extraction Periods on Extraction of Nitrogenous Constituents from Navy Bean Meal with 0.5M Sodium Chloride Solution

Initial Meal Weight, G.	Extraction No.	Total N Extracted,[a] Mg.	% N Extracted,[a] Mean SD[b]
5.0	1	118.3	65.5 ± 0.82
	2	12.3	6.8 ± 0.30
	3	4.0	2.2 ± 0.08
15.0	1	325.1	60.0 ± 0.53
	2	58.2	10.7 ± 0.16
	3	18.6	3.4 ± 0.13

[a] Mean value for 4 replications.
[b] Standard deviation.

Table III. Effect of Hydrogen Ion Concentration on the Extraction of Nitrogenous Constituents from Navy Bean Meal[a]

Concn. of Acid or Alkali in Extraction Solution, M.E./100 Ml.	Extraction with HCl or NaOH Solution			Extraction with HCl or NaOH Solution Containing NaCl[b]		
	pH of extract	Total N extracted,[c] mg. N	% N extracted[c]	pH of extract	Total N extracted,[c] mg.	% N extracted[c]
HCl						
0.50	5.38	43.5	24.1	4.98	111.1	61.5
1.00	4.65	33.7	18.7	4.41	107.2	59.4
2.00	3.66	29.9	16.6	3.46	101.9	56.4
3.00	2.82	53.2	29.5	2.76	104.1	57.7
NaOH						
0.50	7.89	135.6	75.0	7.33	123.8	68.6
1.00	9.32	149.9	83.0	9.02	133.3	73.9
2.00	10.62	151.5	83.8	10.00	143.9	79.7

[a] Initial weight of bean meal, 5 grams. [b] 0.5 mole of NaCl per liter of solution. [c] Mean value for 2 replications.

Table IV. Effect of Successive Extraction Periods on Extraction of Nitrogenous Constituents from Navy Bean Meal[a] with an Alkaline Solution[b]

Extraction No.	pH of Extract	Total N Extracted,[c] Mg.	% N Extracted[c]
1	10.62	146.3	81.1
2	11.78	14.3	7.9
3	11.74	2.8	1.6

[a] Initial weight of bean meal, 5 grams.
[b] 0.02*N* NaOH solution.
[c] Mean value for 2 replications.

may be attributed to the intermolecular attraction of proteins in the isoelectric zone, since very little phytic acid reacts with proteins at this pH. If phytic acid combines selectively with specific proteins at definite pH values, perhaps bean proteins may be fractionated by extracting the meal with various acid solvents.

Data in Table III indicate that acid solutions containing sodium chloride were capable of extracting almost as much nitrogenous matter as the sodium chloride solution (Table II) under the same extraction conditions. The ions of sodium chloride in the acid solvent undoubtedly restrict the binding of phytic acid by the meal proteins.

As shown in Table III, the sodium hydroxide solutions were capable of extracting from 75.0 to 83.8% of the total nitrogen of the meal. On the other hand, Smith *et al.* (*14*) reported that 96.4% of the total nitrogen of the kidney bean meal was extracted with 0.01*M* sodium hydroxide solution. Table III indicates that, with the addition of sodium chloride to the alkaline solvents, the solubility of the nitrogenous constituents was reduced. However, as the concentration of sodium hydroxide in the solvent was increased, the insolubilizing effect of sodium chloride was decreased.

Because sodium hydroxide solutions were found to be excellent solvents for nitrogenous constituents of the navy bean, experimentation was conducted to determine the optimum amount of nitrogenous constituents which could be extracted with an alkaline solvent. Table IV shows that most of the alkali-soluble nitrogenous matter in the meal was removed with three successive extractions. The summation of extracted nitrogen values in Table IV yields 90.6%.

Brohult and Sandegren (*3*) have stated that the alkaline limit of stability of seed globulins is between pH 9.5 and 10.5. To determine if some nitrogenous constituents were rendered insoluble by the alkaline treatment of the preceding experiment, meal was extracted first with sodium chloride solvent to remove most of the globulins and albumins, then the residue was extracted with sodium hydroxide solution to solubilize other proteins. As shown in Table V, 88.3% of the total meal nitrogen was extracted with the two extraction solvents. Consequently, the presumption may be discounted that proteins are insolubilized with the alkaline solvent used in the previous experiment. With the two alkali extractions, 11.1% of the total nitrogen in the meal was extracted. Proteins in the alkaline extract would be classified as glutelins according to the classical system. However, the possibility of albumins and globulins in the extract should not be overlooked. Craine and Fahrenholtz (*4*) have indicated that water-soluble proteins exist in corn but are extracted with alkaline buffers of pH 9.

Table V. Extraction of Nitrogenous Constituents from Navy Bean Meal[a] with Sodium Chloride and Alkaline Solutions

Extraction Solution	Extraction No.	pH of Extract	Total N Extracted,[b] Mg.	% N Extracted
NaCl[c]	3 successive extractions (combined)	6.00	139.4	77.2
NaOH[d]	1	11.48	17.1	9.5
	2	11.60	2.9	1.6

[a] Initial weight of navy bean meal, 5 grams. [b] Mean value for 2 replications. [c] 0.5*M* NaCl solution. [d] 0.02*N* NaOH solution.

Some characteristics of an alkaline extract of sodium chloride–treated bean meal were examined. For this study, 15-gram samples of bean meal were extracted four times with sodium chloride solution prior to the alkali extraction. The Molisch test on the sodium hydroxide extract of the sodium chloride–treated bean meal indicated the presence of a large amount of carbohydrates. The amount of alcohol-insoluble polysaccharides in the extract was determined to be 0.393 gram per 100 ml. No precipitation was observed when the alkaline extract was dialyzed. Undoubtedly the polysaccharides in the dialyzate were bound to various types of proteins to render them soluble in water. Upon heating at approximately 100° C. for 15 minutes, the dialyzate (pH 6) remained transparent with no evidence of coagulation. Presumably the polysaccharides acted as protective colloids with the consequence of no protein aggregation. Further work is required to determine any structural alteration of proteins during heating. To determine the pH at which precipitation occurs, a hydrochloric acid solution (0.02*N*) was added slowly with stirring to the dialyzate. At about pH 5.5, the dialyzate became slightly cloudy, but no precipitation was noted after 12 hours. Some matter settled out of the solution between pH 5.2 and 4.1 after 12 hours, but the supernatants were cloudy. The solutions with pH values between 3.8 and 2.3 contained precipitates which settled to produce clear supernatants. Perhaps negatively charged carbohydrates of the solution interacted with positively charged proteins to form insoluble matter at the low pH levels.

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