a value of 0.33 g/mL to a value of 0.44 g/mL at an enzyme level of 100 mg.

This study indicated that limited proteolysis did not reduce the free or bound gossypol content of cottonseed flour to safe levels. However, it improved some functional properties such as nitrogen solubility in water, emulsifying capacity, and foam capacity.

Registry No. Protease, 9001-92-7; gossypol, 303-45-7.

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Received for review July 8, 1982. Accepted November 3, 1982.

Rapeseed Protein Isolates: Effect of Processing on Yield and Composition of Protein

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Countercurrent extractions of defatted rapeseed meal, alternately at pH 9.5 and pH 12.0 in the successive stages, ensured highest dissolution of meal protein (~92%) and phytic acid (~70%). The resulting extract provided, upon consecutive precipitations at pH 6.0 and 3.6, two protein isolates containing ~60% and ~12%, respectively, of meal protein and distinctly different levels of phytic acid (9.8% and 4.3%, respectively). As much as ~70% of phytic acid, but only ~14% of protein, contained in the meal could be removed by extraction at pH 4.0. The resulting meal residue provided, upon extraction at pH 11.0 and subsequent precipitation of the protein from the extract at pH 4.7, a protein isolate containing only 0.2% phytic acid. The color of the protein isolates could be considerably improved by the use of sodium bisulfite in the solvent for protein extraction. Rapeseed protein isolates containing about 10% phytic acid exhibited a portein efficiency ratio of 2.2, as compared to a value of 2.5 for casein.

Seeds of rape, *Brassica napus*, and related cruciferous oilseed crops, such as *Brassica campestris*, *Brassica juncea*, *Brassica carinata*, *Brassica nigra*, and *Sinapis alba*, are a rich source of edible protein (Ohlson and Anjou, 1979) having favorable nutritional properties (El Nockrashy et al., 1975; Mukherjee et al., 1979; Jones, 1979). Technological processes that have become known for the isolation of such seed proteins are based essentially on dissolution of the protein from the defatted seed meal and subsequent recovery of the protein isolate by precipitation (Sosulski and Bakal, 1969; Owen et al., 1971; Girault, 1973; Kodagoda et al., 1973; Thompson et al., 1976; Gillberg and Törnell, 1976a; El Nockrashy et al., 1977). Current interest in large-scale production of these proteins for use in food prompted us to investigate the effect of various processing parameters on the yield and composition of rapeseed protein isolates. The results reported here form the basis for layout, design, and operation of a pilot plant that has recently been installed for the production of protein isolates from cruciferous oilseeds.

EXPERIMENTAL SECTION

Materials. All Chemicals and reagents used were of analytical grade. Technical-grade hexane, purified by distillation, was used for extraction.

Seeds of *B. napus*, cultivar Erglu, harvested in 1979, were used throughout. The seeds were crushed, the lipids were extracted with hexane in Soxhlet apparatus, and hexane was removed from the defatted meal at 40–50 °C in an oven with forced air circulation. Finally, the meal was finely ground to pass a 0.2-mm screen. The rapeseed meal thus obtained contained 41.0% protein; nonprotein nitrogen constituted 10.0% of the total meal nitrogen.

Analytical Methods. Nitrogen was determined by digestion of the samples according to a semimicro Kjeldahl procedure (Association of Official Agricultural Chemists, 1970) followed by measurement of ammonium ions in the digest using an Orion Research Microprocessor Ion Analyzer/901. Protein content was calculated as percent nitrogen × 6.25. Nonprotein nitrogen in solid samples was determined by extraction of the samples with 10% trichloroacetic acid and measurement of nitrogen in the extract. The liquid samples were treated with trichloroacetic acid at a concentration of 10% in the final mixture, protein precipitated was separated by centrifugation, and nonprotein nitrogen in the extract was determined.

Phytic acid was extracted from the samples according to Wheeler and Ferrel (1971); however, 0.4 M perchloric acid was used as solvent. Phytic acid was recovered as sodium phytate, via ferric phytate, and phosphorus content of the sodium phytate solution was determined with molybdenum blue reagent (Bartlett, 1959). On the basis of phosphorus content, phytic acid was calculated as *myo*-inositol hexaphosphate.

The color of the protein isolates was measured as follows. The sample, 25 mg, was dissolved in a solution consisting

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0021-8561/83/1431-0358$01.50/0

Table I. Effect of pH on Yield of Protein by Three Successive Extractions of Rapeseed Meal and Subsequent Precipitations[a]

pH during extraction	protein extracted,[b] %	protein precipitated[c] at pH 6.0, %	protein precipitated[c] at pH 3.6, %	protein precipitated[c] consecutively at pH 6.0 and 3.6, %
8.0	56	36	59	60
9.0	66	42	55	65
10.0	69	49	58	68
11.0	79	51		71

[a] Experimental conditions: meal:solvent = 1:30 (w/v); temperature = 30 °C; time of each extraction = 20 min; extract was separated from the meal residue or protein precipitate by centrifugation at 15000*g* for 10 min. [b] Nitrogen in extract as percentage of total meal nitrogen. [c] Nitrogen in precipitate as percentage of nitrogen in extract.

of 8 mL of 6 M urea and 2 mL of 1 N sodium hydroxide by heating in a boiling water bath for 1 h. After cooling and centrifuging, in order to separate any undissolved particles, we diluted the solution with 5 volumes of water and measured it spectrophotometrically. The absorbance at 350 nm was chosen as a measure of color intensity.

Preparation of Protein Isolates. Extractions of the rapeseed meal, 5- or 10-g portions, with aqueous solvents were carried out in centrifuge tubes by using a homogenizer (Ultra-Turrax, Janke & Kunkel, D-7813 Staufen, i.Br., Federal Republic of Germany) at the highest speed under the conditions specified in Tables I–V. The extracts were separated by centrifuging, and nitrogen content in aliquots thereof was determined. The meal was extracted, either successively with the fresh solvent or according to a countercurrent procedure described elsewhere (El Nockrashy et al., 1977).

Protein was precipitated from the extract by adjusting the pH to definite values, and the precipitate was separated by centrifuging. The protein isolate, thus obtained, was freeze-dried prior to analysis. Recovery of protein was calculated from nitrogen content of the extract and that of the supernatant after precipitation of protein (El Nockrashy et al., 1977). Precipitation of protein was carried out, either at a single pH or consecutively at two different pHs; e.g., the supernatant after precipitation at pH 6.0 was adjusted to pH 3.6, and the protein recovered at each pH was determined.

Nutritional Evaluation of Protein. Semisynthetic diets containing rapeseed meal, rapeseed protein isolates, or casein were fed to male weanling rats for a period of 4 weeks, and protein efficiency ratio was determined from feed consumption and weight gain of the animals (Derse, 1960).

RESULTS AND DISCUSSION

In order to optimize the yield and improve the quality of rapeseed protein isolates, we have investigated various processing parameters involved in the extraction of protein from the meal with aqueous solvents and precipitation of the protein from such extracts. The parameters studied include pH and mode of extraction as well as precipitation, salt concentration during precipitation, and the type and amount of additive used in the extracting solvent.

Effect of pH and Mode of Extraction on Dissolution of Protein from Meal. Solubility of rapeseed proteins has been shown to vary widely with pH of the extracting solvent (Quinn and Jones, 1976; Radwan and Lu, 1976; Thompson et al., 1976; Gillberg and Törnell 1976b). Our results show, both in successive extractions (Table I) and in countercurrent extractions (Table II), a substantial increase in the extent of dissolution of meal protein with increasing pH during extraction.

Table II. Effect of pH and Number of Extraction Stages on Dissolution of Protein from Rapeseed Meal by Countercurrent Extraction[a]

pH during extractions	no. of extraction stages	protein extracted,[b] %
9.5	3	72
9.5	4	80
11.0	3	82
11.0	4	87
11.7	4	88

[a] Experimental conditions: meal:solvent = 1:25 (w/v); temperature = 30 °C; time of each extraction = 10 min; extract was separated by centrifugation at 5000*g* for 15 min. [b] Nitrogen in extract as percentage of total meal nitrogen.

In several experiments (data not shown), large fluctuations of pH in individual extraction steps were observed when countercurrent extractions of the meal were carried out with 0.02 N sodium hydroxide without additional adjustment of pH. Thus, in a four-stage countercurrent extraction pH dropped to values as low as 5–6 in several extraction steps and the dissolution of only 60% of meal protein occurred. Similarly, a three-stage countercurrent extraction with 0.02 N sodium hydroxide without maintaining a high pH in all the extraction steps resulted in the dissolution of only 43% of meal protein. Apparently, the acidic constituents of the meal are responsible for the reduction of pH of the solvent.

In sharp contrast, four-stage countercurrent extractions at constant pH of 9.5 or 11.0 in each extraction step resulted in the dissolution of 80% and 87%, respectively, of meal nitrogen (Table II). Obviously, a relatively high pH must be maintained throughout the countercurrent extractions in order to accomplish extensive dissolution of meal protein.

A comparison of the data given in Tables I and II shows that countercurrent extractions, as compared to successive extractions, result in higher dissolution of meal protein at lower solvent requirement. As expected, increasing the number of stages in countercurrent extraction distinctly raises the extent of dissolution of meal protein (Table II).

Effect of pH during Extraction and Precipitation on Yield of Protein Isolates. We found that pH during extraction as well as precipitation has pronounced effect on the yield of protein isolates. Thus, in spite of extensive dissolution of meal protein at certain pH, the resulting extracts did not always provide protein isolates in high yields. For example, countercurrent extraction at pH 11.7 resulted in the dissolution of 88% meal nitrogen (Table II), yet from this extract only about 65% of meal protein could be recovered by consecutive precipitations at pH 6.0 and 3.6. It appeared that the amount of phytic acid extracted together with the meal protein was one of the crucial factors that determined the yield of protein isolates. Gillberg and Törnell (1976b) have demonstrated that the solubility of protein and phytic acid of rapeseed meal is largely dependent on pH of the extracting solvent. The same authors have shown that the presence of phytic acid in rapeseed protein extracts facilitates precipitation of the protein due to formation of insoluble phytate–protein complexes. Apparently, a moderate yield of protein isolate from extracts obtained at pH 11.7 ought to be attributed to the occurrence of least amounts of phytic acid in the extract, since a minimum solubility of phytic acid has been reported in this pH region (Gillberg and Törnell, 1976b).

Table III. Effect of Salt Concentration on Precipitation of Protein from Alkaline Extracts of Rapeseed Meal

method of extraction	dilution of the extract (v + v)	acid for precipitation	pH during precipitation	yield of protein,[a] %
four-stage countercurrent according to conditions given in Table III	1 + 0	hydrochloric (6 N)	4.7	67.7
	1 + 1	hydrochloric (6 N)	4.7	71.0
	1 + 3	hydrochloric (6 N)	4.7	74.9
	1 + 0	trichloroacetic[b]	1-2	95.0
four successive extractions with 1 N sodium hydroxide according to conditions given in Table I	1 + 0	hydrochloric (6 N)	4.7	11.3
	1 + 0	hydrochloric (6 N) + trichloroacetic[b]	1-2	50.0

[a] Nitrogen in precipitate as percentage of nitrogen in the extract. [b] Trichloroacetic acid was added to give a concentration of 10% in the final volume of the extract.

In an effort to increase the yield of protein isolates, extractions were carried out in such a manner as to facilitate maximum dissolution of meal protein as well as phytic acid. Four-stage countercurrent extractions of the meal, alternately at pH 9.5 (high phytic acid solubility) and at pH 12.0 (high protein solubility) in the successive stages, resulted in the dissolution of about 92% meal protein. Subsequently, as much as 72% of the meal protein could be recovered as protein isolates by consecutive precipitations at pH 6.0 and 3.6. In comparison, single precipitations either at pH 6.0 or at pH 4.7 resulted in yields of 59.9% and 69.9% of protein isolates, respectively. These results taken together with the data given in Table I show that irrespective of whether the meal protein is extracted by successive extractions or countercurrent extractions, the yield of protein isolate is always higher when consecutive precipitations are carried out at pH 6.0 and pH 3.6 rather than single precipitations at pH 6.0 or pH 3.6 or at an intermediate pH 4.7.

Effect of Salt Concentration of Protein Extract on Yield of Protein Isolates. Salt concentration of the protein extract, resulting from neutralization of excess alkali by an acid, was also found to affect the yield of rapeseed protein isolates. Extracts of the meal protein, obtained either by four-stage countercurrent extractions at alternating pH of 9.5 and 12.0 in the successive stages or by four successive extractions with 1 N sodium hydroxide, were diluted to definite volumes, and the yields of protein precipitated at different pH were determined. The results given in Table III show that the yield of protein isolate obtained by precipitation either at pH 4.7 or at a pH between 1 and 2 distinctly increases with decreased salt concentration.

Effect of Processing on Phytic Acid Content of Protein Isolates. The conditions of extraction that favored maximum dissolution of meal protein and phytic acid, and, consequently, a high yield of protein isolates, were found to result in enrichment of phytic acid in the protein isolates (Table IV). Obviously, most of the phytic acid contained in the extract was precipitated with the protein as an insoluble complex. It is interesting to note from the data given in Table IV that the protein precipitated at pH 6.0 contains a much higher level of phytic acid than the protein subsequently precipitated at pH 3.6.

Phytic acid is known to reduce the bioavailability of certain elements (Smith and Rackis, 1957), and hence, the presence of fairly large proportions of phytic acid in protein isolates would require additional dietary supplementation of such elements (Shah et al., 1979). Therefore, an attempt was made to prepare rapeseed protein isolates having a low phytic acid content.

The differences in the solubility of phytic acid and protein contained in rapeseed meal at various pH values (Gillberg and Törnell, 1976b) formed the basis for removal

Table IV. Phytic Acid Content of Rapeseed Meal and Protein Isolates

sample	phytic acid content, %
rapeseed meal	3.6
rapeseed protein isolate I[a]	9.8
rapeseed protein isolate II[a]	4.3
rapeseed protein isolate III[b]	0.2
soybean protein isolate[c]	0.1

[a] Rapeseed protein isolates I and II were prepared by four-stage countercurrent extraction of the meal, alternately at pH 9.5 and pH 12.0, followed by consecutive precipitations at pH 6.0 and 3.6, respectively. [b] Rapeseed protein isolate III was prepared from extract B according to the scheme outlined in Figure 1. [c] Soybean protein isolate was a commercial product.

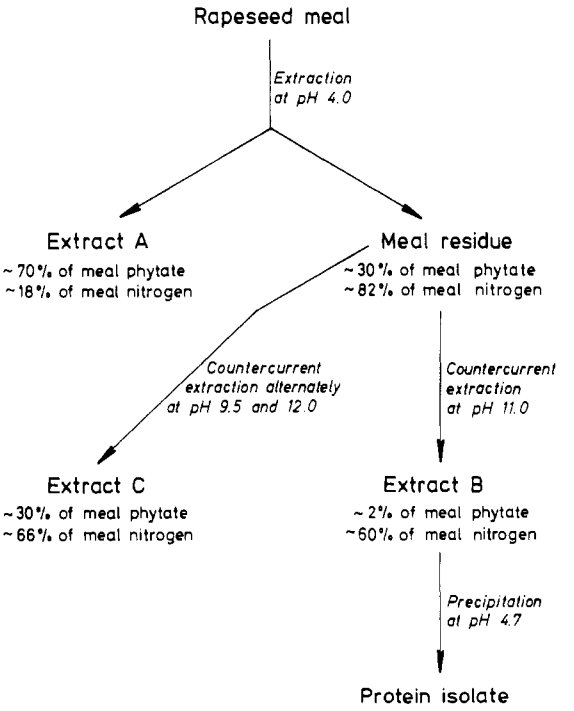

Figure 1. Scheme for the preparation of a low-phytate rapeseed protein isolate.

of phytic acid from the meal and preparation of a low-phytate protein isolate, as outlined in Figure 1. A single extraction of the meal at pH 4.0 and a ratio meal:solvent = 1:25 (w/v) resulted in dissolution of ~70% of phytic acid, ~14% of protein nitrogen, and ~4% of nonprotein nitrogen contained in the meal. A second extraction of the meal at pH 4.0 did not result in further removal of any appreciable amounts of phytic acid which indicates that the residual amounts of phytic acid were rigidly bound to protein. The meal residue, upon countercurrent extraction

Table V. Effect of Additives in Extracting Solvent on Color of Protein Isolates[a]

pH during extraction	additive	concn of additive,[b] %	protein extracted,[c] %	protein precipitated at pH 4.7,[c] %	protein content of isolate, %	yield of protein,[c] %	absorbance of isolate at 350 nm
7.5	none		53	44	93	23	0.45
11.0	none		83	69	92	57	0.60
7.5	poly(vinylpyrrolidone)	1	53	44	94	23	0.39
7.5	poly(vinylpyrrolidone)	5	53	44	95	23	0.38
7.5	poly(vinylpyrrolidone)	10	53	44	97	23	0.32
11.0	poly(vinylpyrrolidone)	1	83	69	92	57	0.58
11.0	poly(vinylpyrrolidone)	5	83	69	92	57	0.55
11.0	poly(vinylpyrrolidone)	10	83	69	92	57	0.48
11.0	sodium bisulfite	0.1	83	62	87	52	0.50
11.0	sodium bisulfite	0.5	79	45	86	36	0.36
11.0	sodium bisulfite	1.0	76	39	85	29	0.32

[a] Experimental conditions: the meal was extracted 5 times, each at meal:solvent = 1:10 (w/v); temperature = 45 °C; time of each extraction = 5 min; extract was separated from meal residue or protein, precipitated at pH 4.7, by centrifugation at 15000*g* for 10 min. [b] Concentration of poly(vinylpyrrolidone) is given as g/100 g of meal; concentration of sodium bisulfite is given as g/100 mL of extracting solvent. [c] Expressed as nitrogen in extract or precipitate as percentage of total meal nitrogen.

at pH 11.0, i.e., the pH at which the phytate–protein complexes are practically insoluble, yielded the extract B from which a protein isolate containing only 0.2% phytic acid was obtained by precipitation at pH 4.7 (Figure 1 and Table IV). The overall yield of low-phytate protein isolate was ~40%.

When the meal residue, after extraction at pH 4.0, was subjected to four-stage countercurrent extraction at pH 9.5 and 12.0 in the alternate stages, as much as 66% of meal nitrogen, originally present, and practically all of the residual phytic acid could be recovered in the extract C (Figure 1). Apparently, the conditions used for extraction resulted in disruption and/or dissolution of phytate–protein complexes that were insoluble at pH 11.0.

Effect of Processing on Color of Protein Isolates. It is well-known that the undesirable greyish brown coloration, often found in protein isolates derived from oilseeds, is mainly due to phenolics which, during processing, readily oxidize to quinonoid substances that become irreversibly bound to protein (Sosulski, 1979). Treatments aimed at improving the color of rapeseed protein isolates involved either removal of phenolic substances by complexation with insoluble poly(vinylpyrrolidone) (Gray, 1978) or inhibition of oxidation of phenolic substances by the use of a reducing agent, such as sodium bisulfite (Edwards et al., 1975).

The effect of various treatments on the color of the protein isolates, monitored by absorbance of the protein solution at 350 nm, is given in Table V. For comparison, a commercial soybean protein isolate exhibited an absorbance of 0.20.

Addition of insoluble poly(vinylpyrrolidone) to the meal prior to extraction of protein, either at pH 7.5 or at pH 11.0, yielded lighter colored protein isolates than in the absence of poly(vinylpyrrolidone) (Table V). Both with and without added poly(vinylpyrrolidone), extraction of the meal protein at pH 7.5 yielded lighter colored protein isolates than extraction at pH 11.0; however, the yield of protein was considerably decreased by lowering the pH of extraction. The yield and purity of protein were practically unaffected by the treatment with poly(vinylpyrrolidone). Nevertheless, the use of poly(vinylpyrrolidone) for the removal of phenolic substances has practical limitations, since its recovery from the meal residue and reuse after regeneration cannot be easily accomplished.

The use of sodium bisulfite to minimize oxidation of phenolic substances during isolation of protein was found to be quite effective in improving the color of the isolates. The results given in Table V show that a substantial reduction in the color intensity of rapeseed protein isolates was achieved when sodium bisulfite was added to the solvent used for extraction of the meal protein. Protein content of the isolate was slightly reduced by the use of sodium bisulfite, but the yield of protein isolate was drastically decreased at high concentrations of sodium bisulfite which ought to be attributed to salt effect, as observed in the experiments given under Table III.

Nutritional Evaluation of Protein. Feeding experiments on rats showed for the rapeseed meal a protein efficiency ratio of 1.9, compared to a value of 2.5 for casein, the reference protein. The protein isolate I (Table IV) exhibited, in spite of a rather high level of phytic acid, a protein efficiency ratio as high as 2.2.

ACKNOWLEDGMENT

F.M.B. and K.D.M. are indebted to Hildegard Benning and Maria-Theresia Keuck for excellent technical assistance.

Registry No. Phytic acid, 83-86-3; poly(vinylpyrrolidone), 9003-39-8; sodium bisulfite, 7631-90-5.

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Received for review June 9, 1982. Accepted December 6, 1982. Financial assistance was provided by Bundesministerium für Forschung und Technologie, Bonn-Bad Godesberg, Federal Republic of Germany.

Water-Soluble Glycoproteins of Tobacco Leaves

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A dialyzed water-soluble extract of defatted tobacco leaves was chromatographed on a DEAE-cellulose column to yield two fractions, A and B, that were rich in carbohydrate and protein. The ratio of carbohydrate to protein in fraction A was about twice that in fraction B. When rechromatographed by gel filtration chromatography with Sepharose 4B, these fractions lost neither of these components. When further rechromatographed on a Sepharose CL-6B column, each of these fractions separated into three subfractions (a_1, a_2, and a_3 and b_1, b_2, and b_3), containing both carbohydrate and protein. The subfractions differed in molecular weight but contained the same sugar moieties (arabinose, galactose, glucose, and rhamnose), although in different mole percentages.

Glycoproteins have only recently come to be generally regarded as components of cell walls in all higher plants (Northcote, 1972) and necessary for their growth (Ridge and Osborne, 1970). Although information concerning the chemical composition of tobacco has increased markedly in recent years, little is known about its glycoproteins. Those that have been examined were shown to be hydroxyproline-rich glycoproteins and were isolated from tobacco cells grown in suspension cultures (intracellular glycoproteins) and from their culture media (extracellular glycoproteins) (Hori and Sato, 1977; Hori and Fujii, 1978, 1980). Also isolated from culture media of tobacco cells were the arabinogalactans of hydroxyproline-rich glycoprotein (Hori et al., 1980; Akiyama and Kato, 1981). Tobacco glycoproteins are important from the standpoint of smoking quality because they contain both amino acid and sugar moieties (Tso, 1972): When tobacco is pyrolyzed, as in cigarettes or other smoking products, its amino acids and sugars form products that may be carcinogenic (Wynder and Hoffmann, 1967; Patterson et al., 1969; Higman et al., 1970). Thus, because plant glycoproteins are important both for their physiological role in plants and for the possible effect of their pyrolysis products, we undertook to examine the water-soluble glycoproteins of tobacco.

MATERIALS AND METHODS

Tobacco Samples. *Nicotiana tabacum* L., cv. Maryland 609, plants were field grown at Beltsville, MD, by conventional cultural practices (McKee, 1978). Maleic hydrazide (MH), the sucker control agent recommended for this area, was applied on plants which had been decapitated after they had reached midbloom. Green leaves were removed at harvest (14 days after MH application) and freeze-dried until just before extraction. The freeze-dried material was ground to pass a 40-mesh screen. Fractions rich in both carbohydrate and protein were isolated and chromatographed by procedures similar to those developed by Hillestad et al. (1977). About 100 g of the dried, ground leaves was extracted sequentially with two chloroform–methanol–water mixtures (10:10:1 and 30:20:1 v/v) and a chloroform–methanol mixture (40:1 v/v), a procedure that removed all the chlorophyll. The leaf residue was then extracted with 2–3 L of water (50 °C), and the extract was evaporated to a small volume at reduced pressure, dialyzed overnight at room temperature against distilled water, and freeze-dried.

Chromatographic Separations. The freeze-dried water extract residue (1.0–1.5 g) was dissolved in buffer A—0.05 M Tris–HCl buffer (pH 6.7)—and applied on a DEAE-cellulose (DE-52) column (5 × 50 cm) that had been preequilibrated with the same buffer. The column was eluted with about 800 mL of buffer A and then with 800 mL of buffer B—0.5 M Tris-HCl buffer (pH 6.7) containing 0.25 M NaCl. Elution was maintained at a rate of 1 drop/8 s, and the eluate was collected at the rate of 17 mL/tube. The tube contents were analyzed spectrophotometrically for total carbohydrate and protein, and those containing high amounts of both these constituents were pooled according to the eluting buffers and called fractions A and B. These fractions were evaporated under reduced pressure, dialyzed overnight against distilled water at room temperature without preservative, and freeze-dried.

Freeze-dried fractions A and B (about 1.0 g each) were then subjected to gel filtration chromatography with a Sepharose 4B column (2 × 25 cm) that had been preequilibrated with 0.025 M Tris-HCl buffer (pH 7.2). This buffer was also used to elute fraction A or B from the column, and the flow rate was 3 drops/min. The eluate was collected at the rate of 6 mL/tube, and the tube contents that were high in both carbohydrate and protein were pooled, dialyzed overnight against distilled water at room temperature with no preservative, and freeze-dried.

Analytical Methods. Total carbohydrate was determined by a phenol–H_2SO_4 procedure described by Dubois et al. (1956), with a mixture of arabinose, galactose, and glucose (5.4:3.6:1) as the standard. Uronic acid content

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