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Journal of Chromatography A, 827 (1998) 319–327

JOURNAL OF CHROMATOGRAPHY A

Preparative separation of value-added peptides from rice bran proteins by high-performance liquid chromatography

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Abstract

Peptides from rice bran protein were investigated because of their potential usefulness in industrial food uses. Peptides were generated from defatted rice bran by treatment with a commercial protease to 7.6% peptide bond hydrolysis. Protein hydrolysates were separated into 20 peaks by quaternary methylamine anion-exchange HPLC on a 25 mm×30 cm column with 96% recovery. Out of 12 peptide fractions, the first four contained 37 and 57% of the total protein and amide in the hydrolysate, respectively. Since glutamic acid in peptides is a potent flavor enhancer, these peptides can serve as an excellent source of flavor enhancing ingredients after further deamidation. An HPLC method was developed for the potential commercial scale-up preparation of these functional peptides for food use leading to new value-added products from the under-utilized rice bran.

Keywords: Rice bran; Flavor enhancers; Preparative chromatography; Peptides

1. Introduction

Proteins are key ingredients in many foods as they contribute to the nutritional value, flavor and other important functional properties of food systems [1]. Rice bran contains a substantial amount of protein ranging from 12 to 20% [2] sold primarily as animal feed. Rice bran protein is of high nutritional value [2] and may also be hypoallergenic [3]. However, large portions of rice protein cannot be solubilized by ordinary solvents such as salt, alcohol and acids due to extensive disulfide bonding and aggregation [4]. Proteases have been used to enhance solubilization of rice bran proteins and to obtain a wide range of protein hydrolysates [5]. Protein hydrolysates are widely used as flavor enhancers in foods [1]. Therefore, the isolation of rice peptides may lead to the creation of new value-added products from rice byproducts.

Glutamic acid and its salts have a long history of use in foods to enhance the flavor [6–8]. Monosodium glutamate (MSG) is by far the most widely used glutamate. It is used at a concentration of 0.2–0.8% in a variety of foods such as soups, broth, sauces, gravies, flavoring and spice blends, and in many canned and frozen meats, poultry, vegetables and combination dishes [6]. However, intakes of MSG may cause toxicological effects, such as Chinese Restaurant Syndrome [9–12]. Glutamates as part of a protein are not flavor enhancers [6], but glutamates bound into a peptide structure may have the flavor enhancing properties of the free form [7,8]. Because of the relatively high content of asparagine and glutamine in rice proteins, deamidated peptides and protein hydrolysates can be an excellent source of flavor enhancing ingredients for food applications.

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00657-8

Deamidation of proteins is the hydrolysis of amide groups to acidic groups and is carried out to increase the net negative charges on proteins [7]. Further, the high nutritional value and hypoallergenicity of rice proteins can make these products particularly attractive for infants allergic to milk and/or soy proteins.

The aim of this research was to develop a scheme for producing peptide fractions from rice bran with potential flavor enhancing capabilities to replace MSG and to be used as functional ingredients in foods. This could be accomplished by (1) treating rice bran with protease to optimal levels of protein hydrolysis to maximize recovery and functional properties, and (2) HPLC fractionation of protein hydrolysates to obtain functional peptides for industrial use as flavor enhancers.

2. Experimental

2.1. HPLC unit

A Delta Prep 3000 (Waters, Milford, MA, USA) was used for preparative anion-exchange fractionation and size exclusion analysis executed in this study. Samples were filtered through a 0.45 μm Millex-HV Filter (Millipore, Bedford, MA, USA) before injection. Elution was monitored at 280 nm by a Model 481 Lambda-Max spectrophotometer detector. Data analysis is obtained with PC-based Maxima 810 chromatography software. Fractions were collected using a Foxy Fractionator (ISCO, Lincoln, NE, USA).

2.2. Preparation of defatted rice bran

Defatted rice bran was obtained from the kernels of Bengal variety by milling and defatting with ethyl ether according to the methods of Hamada [4].

2.3. Protein hydrolysis

The method of preparing protein hydrolysates from rice bran was previously described [5]. Defatted rice bran suspension (5.0 g proteins in 250 ml water) was heated to 50°C, the pH adjusted to 8.0 and 0.05 g Alcalase 2.4 L (Novo Nordisk, Franklinton, NC, USA) added to start proteolysis. The extent of hydrolysis was controlled to about an 8% degree of hydrolysis (DH) by the pH-stat method, using an Auto Titrator (Radiometer A/S, Copenhagen, Denmark). During hydrolysis, the bran suspensions were continuously titrated at 50°C to pH 8.0 with 0.25 *M* NaOH. The protease was inactivated by a 10 min heat treatment at 85°C. After proteolysis, the suspensions of rice brans were homogenized in a 1-l Sorval Omni Mixer jar (Omni, Waterburry, CT, USA) using a 20 mm sawtooth blade assembly at 5000 rpm and 20°C for 15 min. The solubilized protein was recovered after three runs of centrifugation at 5000 *g* and 20°C for 20 min. Protein in the bran was washed with 100 ml water during the second and third centrifugation. Combined supernatant solutions were lyophilized and the bran air-dried. The percent protein recovery was calculated as the ratio of protein extracted from bran to the total protein of bran.

2.4. Quaternary methylamine (QM) anion-exchange HPLC

Ion-exchange semipreparative separation of protein hydrolysates was done on a steel column (25 mm I.D.×30 cm) packed with Accell Plus™ QMA medium from Waters. The protein load was 25 mg proteins in 1.0 ml 0.02 *M* Tris–HCl buffer, pH 8.0. Elution by 0.1 *M* Tris–HCl buffer, pH 8.0 and 0–0.25 *M* KCl (from 20 min to 120 min) was completed after 3 h at a flow-rate of 6 ml/min. Anion-exchange separation was also performed using the same conditions and gradient but substituting the 0.1 *M* Tris–HCl buffer (pH 8.0) with 0.1 *M* sodium phosphate buffer (pH 8.0). The freeze-dried sample containing 25 mg protein was dissolved in 1.0 ml 0.02 *M* phosphate buffer, pH 8.0. Anion-exchange separation was also carried out using 0.1 *M* sodium phosphate buffer (pH 8.0) at 25 and 50 mg protein load (in 1 or 2 ml 0.02 *M* phosphate buffer, pH 8.0, respectively) and a flow-rate of 3.0 ml/min for the first 80 min then at 10.0 ml/min for the rest of the 3 h runs.

2.5. Size exclusion HPLC analysis

Protein hydrolysates or some of the anion-ex-

change HPLC pooled peaks were separated by size exclusion HPLC on a 20-mm ID×30 cm Shodex ''Protein WS-2003'', steel column packed with a bonded diol-coated silica gel (exclusion limit= 150 000) from Waters, using Tris–HCl buffer (pH 8.0) as eluent. Two milligrams of protein were injected. Samples were solubilized in 1.0 ml buffer for protein hydrolysates or 1.0 ml water for lyophilized pooled preparations of the first four fractions of anion-exchange HPLC separation. The flow-rate was 2.0 ml/min. Blue dextran and protein markers (Sigma, St. Louis, MO, USA) were used to calibrate the HPLC column. Relative molecular masses (M_r) of standard peptides and proteins were 1600–150 000.

2.6. Analyses of protein hydrolysates and HPLC fractions

Protein content of the rice brans, before and after protease treatment, and lyophilized hydrolysates was determined by the combustion method [13] using a Leco FP-428 Nitrogen Analyzer (Leco, St. Joseph, MI, USA). The nitrogen conversion factor for proteins and protein hydrolysates was 5.95. The protein content of injected and eluted hydrolysates was determined by the macro method of Lowry et al. [14] using DC (Bio-Rad, Hercules, CA, USA) and BCA (Pierce, Rockford, IL, USA) protein assay reagents. Solubilized rice proteins were used as standards. The DH-value of protein hydrolysates was determined by reacting free amino groups with trinitrobenzenesulfonic [5]. The amide contents of protein hydrolysates and HPLC fractions were measured by determining the ammonia released by the sample after total amide hydrolysis with 2 *M* HCl at 100°C, according to the method of Wilcox [15]. In this determination, a correction for free ammonia was applied.

2.7. Statistical analysis

Multifactor analysis of variance of variables (protein hydrolysis, injections, etc..), determined in duplicate, was performed using Statgraphics Plus, a software package from Statgraphics (Rockville, MD, USA).

2.8. Supporting information

Commercial firms are mentioned in this publication solely to provide specific information. Mention of a company does not constitute a guarantee or warranty of its products by the U.S. Department of Agriculture nor an endorsement by the Department over products of other companies not mentioned.

3. Results and discussion

3.1. Rice bran protein hydrolysate

The protein content of the full fat and defatted bran was 12.9 and 15.4%, respectively.

Rice bran protein was hydrolyzed by a commercial food-grade endoprotease (Alcalase) at pH 8 to generate peptides for subsequent preparative separation and characterization. The reaction conditions plus the extent of hydrolysis were controlled by the pH-stat titration method to assure peptide uniformity and reproducibility. Analysis of free amino groups in the protein hydrolysate revealed that 7.6% of peptide bonds in rice bran protein were hydrolyzed, i.e., the %DH was 7.6. The enzymatic treatment facilitated the extraction of 83% of the total protein of rice bran recovered in the protein hydrolysates. These lyophilized preparations contained 27.4% protein and 0.95 millimole of amide groups per gram protein.

3.2. Size exclusion HPLC of protein hydrolysate

A chromatogram of size exclusion HPLC separation of the bran protein hydrolysate is presented in Fig. 1. Protein proportions of the peaks of these chromatograms are shown in Table 1. Protein hydrolysate was fractionated into six major peaks with a 3000–90 000 M_r range for most of hydrolysate. About 71% of protein hydrolysates were medium sized polypeptides (10 000–90 000). Small peptides with less than 3000 were $<$7.2% of the protein hydrolysate. This suggests that peptide bond hydrolysis was so uniform that it precluded the formation of small peptides. Most flavor problems have been attributed to small peptides that are likely to have bitterness.

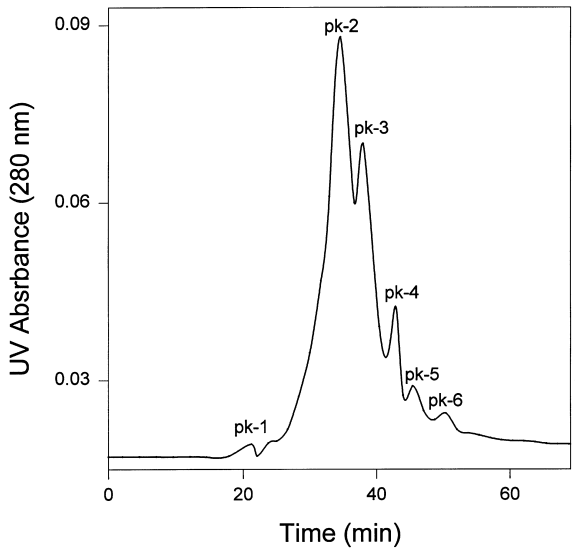

Fig. 1. Size exclusion HPLC of rice bran protein hydrolysate at a flow-rate of 2.0 ml/min using 2 mg proteins.

3.3. Preparative anion-exchange separation of protein hydrolysate

Anion-exchange chromatography was used to develop methodology for large-scale production of flavor-enhancing peptides from rice protein hydrolysates. Protein hydrolysates of rice bran (25 mg protein) were separated into 20 peaks on a preparative QM anion-exchange column using 0.1 *M* Tris–HCl (pH 8.0) and 0–0.25 *M* KCl gradient as the eluent (Fig. 2A). Ten of these peaks are considered major peaks each containing more than 3% of the injected protein hydrolysate, based on % area (Table 2). To evaluate the benefits of this separation in obtaining peptides with different structures, some of them may be more suitable than others in certain applications, fractions were pooled as listed in Table

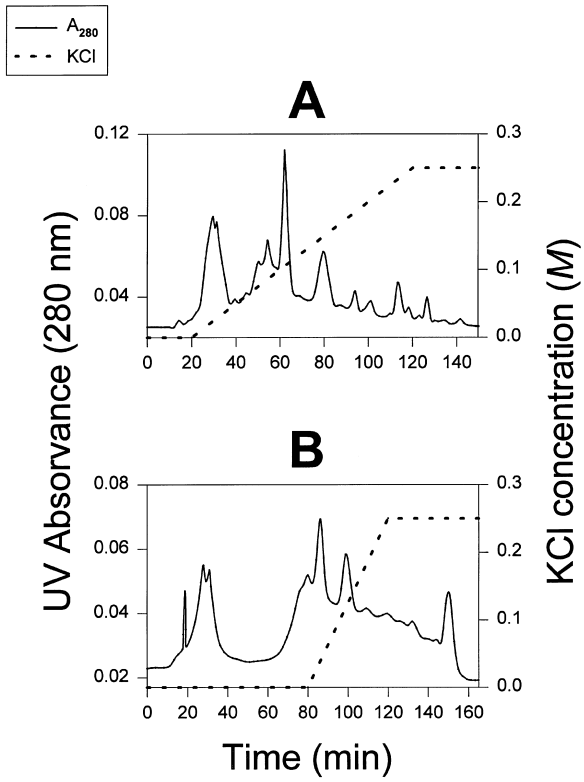

Fig. 2. Anion-exchange separation of protein hydrolysates using KCl gradient and (A) 0.1 *M* Tris–HCl buffer (pH 8.0) and (B) 0.1 *M* phosphate buffer (pH 8.0) at 6.0 ml/min (25 mg protein injection).

Table 1
Molecular weight distribution of rice bran protein hydrolysates by size exclusion chromatography[a]

Peak number	Peak start (min)	Peak end (min)	Retention time (min)	Protein percent			Relative molecular masses
				Area	Reagent[b]	Average	
1	17	24	20	2.9	3.6	3.2	150 000–90 000
2	24	35	34	31.1	32.8	31.9	90 000–50 000
3	35	37	36	11.9	13.1	12.5	50 000–25 000
4	37	41	38	25.9	27.7	26.8	25 000–10 000
5	41	48	43	20.6	16.6	18.6	10 000–3 000
6	48	55	50	6.7	6.1	6.4	3 000–1 000
7	55	65	57	0.9	0.7	0.8	<1 000

[a] Chromatogram is presented in Fig. 1.
[b] Method of Lowry et al. (1951) [14].

Table 2
Fraction pooling of anion-exchange chromatography of rice bran protein hydrolysates[a]

Peak number	Peak start (min)	Peak end (min)	Retention time (min)	Area (%)	Height (%)	Pooled fractions
1	13	16	15	0.3	1.0	None
2	16	31	29	13.5	11.8	F-1
3	31	38	31	9.8	11.2	F-2
4	38	41	40	2.1	3.0	F-3
5	41	46	45	2.9	3.6	F-3
6	46	52	50	7.0	6.9	F-4
7	52	58	54	9.6	9.2	F-5
8	58	67	62	17.9	18.5	F-6
9	67	73	68	3.7	3.3	F-7
10	73	86	80	13.0	8.1	F-8
11	86	90	87	2.2	2.3	F-9
12	90	97	94	3.6	3.9	F-9
13	97	106	101	3.7	2.8	F-10
14	106	117	114	5.2	5.9	F-11
15	117	121	119	1.3	2.2	F-11
16	121	124	123	0.8	1.4	F-12
17	124	131	127	2.1	3.2	F-12
18	131	138	134	0.7	0.8	F-12
19	138	146	142	0.8	0.8	F-12
20	146	150	146	0.1	0.1	None

[a] Chromatogram is presented in Fig. 2A.

2. The protein recovered in the 12 fractions was 95.9% of the total injected protein.

3.4. Characterization of anion-exchange fractions of protein hydrolysate

3.4.1. Protein content

Table 3 presents the protein content of the pooled peptide fractions from the anion-exchange HPLC separation. The peptide fractions varied significantly in their protein with the first four fractions containing 37% of the total protein. Fractions 1, 2, 6 and 8 contained much greater proportions of injected protein hydrolysates.

3.4.2. Amide content

Table 3 also gives the amide content of the pooled peptide peaks from anion-exchange HPLC

Table 3
Protein and amide contents of peptide fractions of rice bran protein obtained by anion-exchange separation

Fractions	Protein (%)	Amide (μmol per fraction)	Amide (μmol per mg protein)
F-1	15.4	3.7	1.1
F-2	9.7	2.7	1.1
F-3	5	2.3	1.8
F-4	7	1.2	0.7
F-5	7.2	1.3	0.5
F-6	18.4	1.1	0.3
F-7	3.2	1	1.1
F-8	11.1	1	0.3
F-9	4.4	1	0.7
F-10	5.8	0.5	0.6
F-11	4.7	0.7	0.4
F-12	4	1	0.9

separation. The peptide fractions varied significantly in their amide content with the first four fractions containing 57% of the total amide. Thus, the peptides of these four fractions can be an excellent source of flavor enhancing ingredients for food applications after further chemical or enzymatic deamidation. Glutamic acid in peptides is a potent flavor enhancer as has been shown by several investigators. For instance, an octapeptide named STEP (savory taste enhancing peptide) containing two glutamic acid residue enhances the meaty flavor of beef [16]. Because of the relatively high content of amide and hence glutamine in these fractions, these peptides, after further deamidation, are expected to serve as outstanding sources of flavor enhancing ingredients for food applications [7].

3.4.3. Size exclusion HPLC

Chromatograms of the size exclusion HPLC separation of the first four fractions of anion-exchange HPLC separation of bran protein hydrolysates are presented in Fig. 3. Size exclusion HPLC gave three peaks for F-1 and four peaks for each of the other three fractions. Protein proportions of the peaks of these chromatograms are presented in Table 4. Most of the peptides of the first fraction of the anion-exchange HPLC separation had M_r of 10 000–25 000. F-2 and F-3 were separated into three major peaks with similar M_r distribution (3000–25 000). F-4 had peptides with the highest M_r (6500–50 000) among all fractions.

3.5. Developing a food-grade anion-exchange procedure for a scale-up

3.5.1. Effect of buffer, flow-rate and protein load on anion-exchange separation

To develop a food-grade anion-exchange procedure for a scale-up of the ion-exchange separation of protein hydrolysates, the 0.1 *M* Tris–HCl buffer (pH 8.0) that is not allowed in food application, was substituted by 0.1 *M* sodium phosphate buffer (pH 8.0). Elution was also completed in 3 h by 0.1 *M* sodium phosphate buffer (pH 8.0) and 0.1–0.25 *M* KCl (from 20 min to 120 min) at a flow-rate of 6 ml/min. Fig. 2B shows the anion separation using phosphate buffers as eluent and same conditions described in Fig. 2A for the separation with Tris–

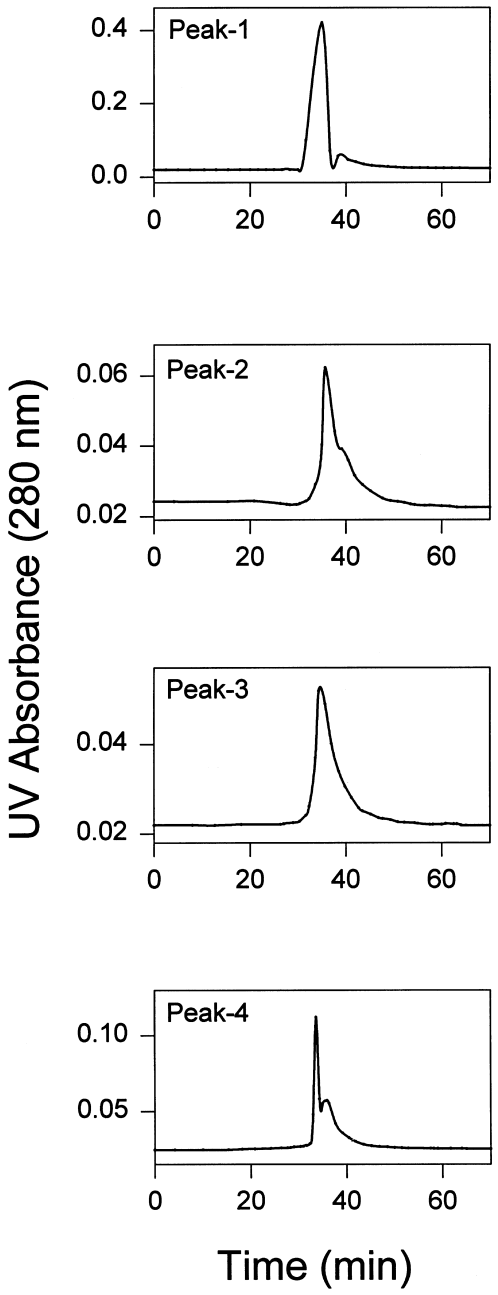

Fig. 3. Size exclusion HPLC of the first four fractions from anion-exchange chromatography of rice bran protein hydrolysate at a flow-rate of 2.0 ml using 2 mg proteins.

Table 4
Molecular weight distribution of the anion-exchange HPLC first four fractions determined by size exclusion HPLC

Fraction[a]	%Protein (peak area)				
	50 000–25 000	25 000–10 000	10 000–6500	6500–3000	<3000
F-1	0	65.2	13.6	19.7	0
F-2	0	34.3	25.1	34.8	5.8
F-3	0	32.1	21.5	40.6	5.9
F-4	7.7	53.1	38.7	0	1.2

[a] Identity of fractions is described in Table 2.

HCl buffer. This chromatography also gave as many peaks of which more than half were considered as major peaks. The first five peaks, with potential for constituting four fractions similar to those pooled for Tris buffer runs, accounted for 32% of the total protein. Since this separation is very close to the separation using Tris buffer, it can substitute for the latter. Nevertheless, the protein recovered in these peaks composing F-3 and F-4 was smaller in phosphate buffer separation.

Flow-rate was changed from 6 ml/min to 3 ml/min until the first four peaks were eluted (80 min) and to 3.0–10.0 ml/min until the end of the run. The reduction of the flow-rate was chosen to improve resolution to affect the separation of the first four peaks containing amide-rich peptides. The QM anion-exchange separation was also carried out using 0.1 *M* sodium phosphate buffer (pH 8.0) at a 25 mg protein load (Fig. 4). Reducing flow-rate was ineffective in completely separating the first four peaks. However, separation of the first four peaks was improved dramatically by decreasing the flow-rate. Complete resolution of these peaks is not needed here: although glutamic acid is the major amino acid in the peptides that influence flavor, other amino acids contribute to the final flavor of peptides. Flow-rate is the operational variable that most influences time. As separation efficiency (α) becomes smaller, the flow-rates may have to be decreased to maintain the desired degree of resolution and solute recovery [17].

The load was increased from 25 mg to 50 mg at the same flow-rate used with the 25-mg load, i.e., at 3 ml/min until the first four peaks were eluted (80 min) and to 3.0–10.0 ml/min until the end of the run. The chromatogram of this separation was identical to that of 25 mg loads, except the absorbance at 280 nm, which was doubled (Fig. 4). It seems that increasing protein load did not affect the chromatogram peaks likely because of the excess plate count available. Sample loads are key parameters that influence throughput since they affect α, column efficiency, retention time and even peak shape [17]. Also, as sample load is increased, the effective plate count decreases.

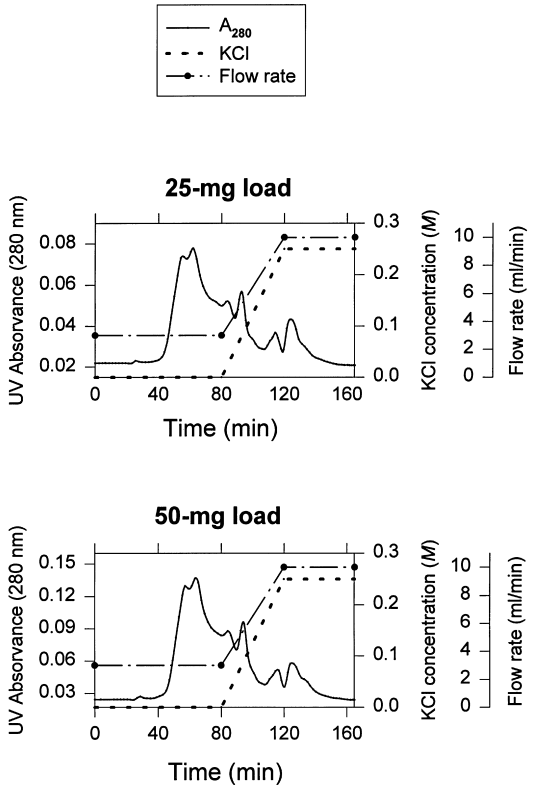

Fig. 4. Anion-exchange chromatography of rice bran protein hydrolysate (25 and 50 mg protein loads) using 0.1 *M* phosphate buffer (pH 8.0) and KCl gradient at a flow-rate of 3–10 ml/min.

3.5.2. Procedures to produce peptides with flavor enhancing capabilities

Processes to produce flavor enhancer peptides by protein hydrolysis and subsequent chromatographic separation must be cost effective. Because of the relatively low value of food ingredients produced in high bulk, compared for instance, to pharmaceutical materials, process HPLC must be inexpensive relative to total costs. Flavor enhancers are used in a relatively small concentration to affect dramatic flavor enhancement of end products. Therefore, it is most likely that the cost of enzymic modification and HPLC processes relative to the value of the food ingredient will be quite economical. The ion-exchange separation is attractive for preparative separation of proteins. This is because it is a very powerful tool and its columns can be easily self-packed and the support medium can be effectively regenerated for continuous use [18].

Ion-exchange chromatography is preferred to other separation techniques in large-scale production of peptides and proteins. It is widely used for downstream processing of significant proteins [19]. This separation has the potential of being scaled-up to a process-scale. Scale-up of an ion-exchange process is usually achieved by increasing the column diameter while maintaining the column bed height and linear flow-rate constant [19]. Loads of preparative chromatographic separation are usually scaled-up a number of times equal to the ratio of their cross-sectional areas. Ideally, runs are carried out at the same linear velocity to maintain an equivalent time for the small and large-scale separations [20].

Deamidation is needed to convert glutamine residues to glutamic acid residues in the isolated peptides. An enzymatic approach to peptide deamidation is preferred to chemical approaches because of the mild conditions, safety, speed and selectivity of enzymatic reactions [7]. Peptidoglutaminase (PGase) hydrolyzes the γ-amides of L-glutamine residues in peptides and proteins. However, modification of peptides using PGase may have one major obstacle: PGase is not a food-grade enzyme. Although PGase is used for protein modification in a small amount in an enzyme to substrate ratio of 1:3000 [21], it must be removed after deamidation until it is granted FDA approval. Favorably, this research on molecular size characterization of the isolated peptide fractions showed that a clean separation of the PGase from the modified peptides is possible. Preparative size exclusion HPLC with exclusion limits of 1000–150 000, such as the column used here for analytical size exclusion HPLC, can separate cleanly PGase after being used in deamidation from deamidated peptide fractions. The peptide fractions show M_r of $<$3000 to 25 000, compared with M_r of 90 000 and 110 000 and 180 000 and 220 000 for the two PGases I and II in dissociated and undissociated forms, respectively [22]. Despite the inadequacy of speed for size exclusion chromatography of proteins, it is suitable for our purpose and for scaled-up process due to attainable purity and excellent resolution.

4. Conclusions

A preparative separation scheme is described for isolating glutamine-rich peptides with flavor enhancing potential from rice bran protein hydrolysates. This chromatographic method has the potential for scale-up in the commercial preparation of functional peptides for industrial use leading to new, high value-added products from an under-utilized byproduct of the rice industry.

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