Production of Peptic Hemoglobin Hydrolysates: Bitterness Demonstration and Characterization

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Hemoglobin could be valorized in enzymatic hydrolysate form aimed at human and animal feeding. Thus, the hydrolysis of hemoglobin by pepsin was studied. A bitter taste in the hydrolysates was encountered, so different separation processes were developed to isolate the bitter fractions according to several characteristics. Therefore, ultrafiltration, reversed phase chromatography, and organic solvent extraction were used. Bitter peptides were concentrated in YM5 membrane (cutoff: 5000) permeate. They were eluted with 30–40% CH_3CN from a C_{18} bonded silica column and selectively extracted by 2-butanol. They were also adsorbed on a Superose 12 gel filtration column, and so they were represented by a chromatographic peak appearing after total column volume. Chromatography on Superose 12 could thus constitute an interesting analytical method for detecting bitterness in hydrolysates.

Keywords: *Hemoglobin; pepsin; hydrolysate; bitter peptides*

INTRODUCTION

Hydrolyzed proteins from a variety of sources find a number of uses in the food industry.

Their production can be achieved using acids or enzymes. However, chemical methods may result in loss, partial destruction, or racemization of essential amino acids (Clegg and McMillan, 1974; Parker and Pawlett, 1986), undesirable side reactions with nonprotein components of the reaction mixture, and formation of toxic byproducts. As proteolytic enzymes are active under mild processing conditions, the nutritional quality of the original protein is retained (Parrado et al., 1993); therefore, enzymatic methods offer a valuable tool for modifying proteins and improving their properties.

Protein hydrolysates can be produced as a basic food ingredient for their desirable functional properties, easy digestibility, high water solubility, resistance to denaturing agents such as heat, high protein content, and potential as flavor enhancers. Several such products are commercially available as a component in dehydrated soups, flavoring condiments in sauce, bouillon, processed foodstuffs, and animal feed (Parker and Pawlett, 1986; Whitaker, 1977).

Dietetic food is an important area that could make use of hydrolysates. The clinical treatment and enteral nutrition of patients with specific disorders of digestion such as cystic fibrosis or of absorption and amino acid metabolism are possible applications of predigested proteins (Clegg and McMillan, 1974; Garbutt, 1993; Parker and Pawlett, 1986). Hydrolysates present two main advantages: the nitrogen source exclusively constituted with free amino acids is not satisfactory in a nutritional sense (Tanimoto et al., 1991) as both rate and extent of absorption of peptides are greater (Webb, 1990); moreover, protein hydrolysates are considerably less expensive than synthetic amino acid mixtures (Clegg and McMillan, 1974).

There has been considerable research activity on hydrolysates from dairy products such as casein (Parker and Pawlett, 1986; Vegarud and Langsrud, 1989) and whey (Perea et al., 1993), from vegetable proteins such as soybean proteins (Adler-Nissen, 1986), gluten (Garbutt, 1993), zein (Tanimoto et al., 1991) and sunflower meal (Parrado et al., 1991, 1993), and even from fish proteins (Fujimaki et al., 1973; Rebeca et al., 1991) and tannery wastes (Montoneri et al., 1994).

Enzymatic protein hydrolysis processes have shown only a slow penetration into the food ingredients industry (Adler-Nissen, 1986). The formation of a bitter taste, observed first by Murray and Baker (1952), is a consistent side effect of the treatment, and bitterness has a negative hedonic response that makes the product unpalatable and even organoleptically unacceptable (Clegg and McMillan, 1974).

Since Carr et al. (1956), it has been well-known that bitter peptides occurred during the hydrolysis and resulted from the degradation of the protein substrate itself (Adler-Nissen, 1986).

Several studies have been made to ascertain the cause of bitterness and to provide methods for avoiding or overcoming it.

Bitter-tasting peptides exhibit a high content of hydrophobic amino acids (Matoba and Hata, 1972). Several of them have been isolated and analyzed. In 1971, Ney established the so-called Q rule (Ney, 1979). It predicts whether a peptide is bitter or not with its Q value, which represents the average hydrophobicity of the side chain of the amino acids involved. Ney's rule, valid only for peptides up to molecular weight approximately 6000, because larger peptides are not bitter (Ney, 1979), can be applied to the majority of bitter peptides studied (206) by Guigoz and Solms (1976).

Ney also postulated that the tendency of a protein to form bitter peptides could be predicted from its amino acid composition and the calculation of its Q value. Adler-Nissen (1986) refuted this rule extension because the hydrophobicity distribution of the peptides was neglected. Furthermore, the bitterness of a protein hydrolysate cannot be easily predicted because of the numerous and complex factors that influence it. Obvi-

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ously, the control of the level of bitterness is to a large extent dependent on the control of the proteolytic reaction itself (Adler-Nissen, 1986).

In the search for novel cheap proteins, slaughterhouse blood has been little considered (Stachowicz et al., 1977), although blood proteins have high nutritional value and are cost-competitive and available in large quantities.

Hemoglobin, the major component, presents several advantages: it is a very well-defined and constant protein source that is easy to obtain in pure state, and its high content of histidine (3 times that in casein hydrolysates), an essential amino acid for infants, makes it a favorable choice for nutritional applications in pediatrics compared to other protein hydrolysates (Piot et al., 1988).

Moreover, proteolysis can remedy the principal hemoglobin drawback, its dark brown color, using for instance Alcalase Novo (Hald-Christensen, 1979) and pepsin (Piot et al., 1988). In these cases, the bitterness of the obtained hydrolysates (Stachowicz et al., 1977) has been sometimes mentioned.

The present paper deals with hemoglobin hydrolysis by pepsin in relation to its bitterness. A complete study of the production and analysis of peptic hemoglobin hydrolysate is shown. Different separation methods lead to the isolation and characterization of bitter fractions.

MATERIALS AND METHODS

Materials. Bovine hemoglobin was obtained from Serva Feinbiochemica. It was undenatured erythrocyte powder.

Pepsin (2500 FIP-U/g) was purchased from Merck. The concentration defined in this paper is expressed by mass unit of enzyme commercial powder.

All reagents were of analytical grade.

All chromatographic methods were performed on a fast protein liquid chromatography (FPLC) system (Pharmacia LKB). Absorbance was detected on line at 226 nm (Uvicord SII LKB detector).

Hydrolysates Preparation. Hemoglobin (50 g/L) was dispersed in 60 mM HCl and centrifuged for 10 min at 12000*g* to eliminate the insoluble part (8% w/w) composed of erythrocyte walls. Only the supernatant was used afterward.

The hydrolysis reaction was carried out at pH 3 and 37 °C and controlled by a pH-stat (Metrohm apparatus) by adding 0.05 N HCl, because at pH 3 the pH increased during proteolysis. Pepsin was added to obtain variable enzyme–substrate ratios (E/S). The reaction was stopped by adding NaOH to pH 7. The hydrolysates were freeze-dried.

Moreover, the hydrolysate solutions were centrifuged (5 min at 12000*g*) before analysis or treatment to eliminate the insoluble part (low degraded hemoglobin and heme) that appeared during the increase of pH.

Hydrolysates Characterization. *Degree of Hydrolysis (DH) Determination.* The trinitrobenzenesulfonic acid (TNBS) method described by Adler-Nissen (1979) was used.

The protein hydrolysate, dissolved at 40 g/L in water, was diluted in 1% SDS to obtain 0.25–2.5 mM amino groups. L-Leucine solutions (0–2 mM) were used as standard.

The molar concentration of primary amino groups, expressed in leucine amino equivalents, in the hydrolysate, was determined.

Transformation to DH (percent) was carried out by dividing this result by the concentration obtained with a total acid hydrolysate of initial hemoglobin.

Gel Filtration. A prepacked column of Superose 12 (HR 10/30) (Pharmacia LKB) was eluted with 20 mM ammonium acetate buffer at pH 7, containing 0.15 M NaCl, at a flow rate of 0.5 mL/min.

The total packed bed volume (V_T) was 22 mL. The void volume and the mobile phase volume [determined with acetone (58 g/mol)] of the column were, respectively, 6 and 18 mL. Bovine serum albumin (MW 67 000), chymotrypsinogen A (MW 25 000), ribonuclease A (MW 13 700), and vitamin B_{12} (MW 1355) were used as molecular weight markers.

Fifty microliters of hydrolysate powder sample (25 g/L) was applied to the column.

Analytical Reversed Phase Chromatography. The separation was performed on a Pep RPC column (HR 5/5) (Pharmacia LKB) eluted at 0.7 mL/min with gradients of H_2O–0.1% trifluoroacetic acid (TFA) as eluent A and CH_3CN–0.1% TFA as eluent B. After a 10 min step of 10% B, a linear gradient from 10% to 40% B in 90 min was applied. One hundred percent during 10 min and then 10% B during 10 min cleaned and re-equilibrated the column after each analysis.

Fifty microliters of hydrolysate powder sample (25 g/L) applied to the column.

Sensory Analysis. The bitterness of the hydrolysates and the different obtained fractions was estimed in aqueous solution.

The hydrolysates exhibited a great unpleasant odor which could mask the bitter taste, as gustative and retronasal olfactive sensations were generally confused. So air was blown into the nose during the degustation, to suppress retronasal olfaction, thus preventing odors from going up the nose.

The degustation was performed with 200 μL of sample put with a syringe onto the base of the tongue, where bitterness is especially perceptible (Pfaffman et al., 1971).

The bitterness intensity was scored by comparison with a quinine sulfate concentration scale and expressed as an isointensity quinine concentration. A stimulus and a standard solution were compared, and the concentration of the standard solution was reduced or increased according to the subject's response, until a balance was reached. This sensory test, inspired by the "up-and-down" method of Dixon (Massey and Dixon, 1960), was performed with pair comparison and forced choice. The reliability of the subject throughout the experiment was controlled with quinine solution of known concentration, which was determined with about 8% average error.

All of the solutions tasted had to be beforehand neutralized and desalted, so that sour and salt tastes did not interfere with bitterness. The sample concentration for tasting was 25 g/L for the whole hydrolysate and equivalent to 25 g/L of initial hydrolysate for the different fractions.

Hydrolysates Fractionation Processes. The hydrolysate of DH 13% was chosen for all fractionation treatments as it exhibited great bitterness.

The evaluation of each process was made by sampling dry matter or freeze-dried powder and expressed by weight percentage from the initial hydrolysate.

Each fraction was freeze-dried and analyzed like the hydrolysate (see Hydrolysates Characterization), except DH determination, at a concentration equivalent to 25 g/L of initial hydrolysate.

Ultrafiltration Process. The hydrolysate was dissolved in water at a concentration of 20 g/L and then centrifuged.

The hydrolysate was fractionated into four parts by successive treatments in stirred ultrafiltration cell of 50 mL (Amicon Grace), with ultrafiltration membranes YM10, YM5, and YC05 purchased from Amicon Grace; cutsoff were, respectively, 10 000, 5000, and 500. The YM10 permeate was used for ultrafiltration with the YM5 membrane, and the YM5 permeate was used for ultrafiltration with the YC05 membrane. The ultrafiltration operation was run until rates of 99% in volume for YM10 and YM5 and 90% for YC05 were reached. A rate of 90% in volume meant that the sample was concentrated from 50 to 5 mL, and then the retentate was readjusted to 50 mL and concentrated again from 50 to 5 mL.

Organic Solvent Extraction. The hydrolysate was dissolved in water at a concentration of 50 g/L and then centrifuged.

According to the water miscibility of the solvent, two different procedures were accomplished. If the solvent was not water miscible, two successive extractions were made by vigorously mixing, in equal volume, hydrolysate aqueous solution and solvent, during 10 min, and by centrifuging (10 min at 1100*g*), to separate the two phases. In the case of miscible solvents, 1 volume of hydrolysate aqueous solution was mixed during 10 min with 3 volumes of pure organic solvent; such a partial precipitation by 75% of solvent was

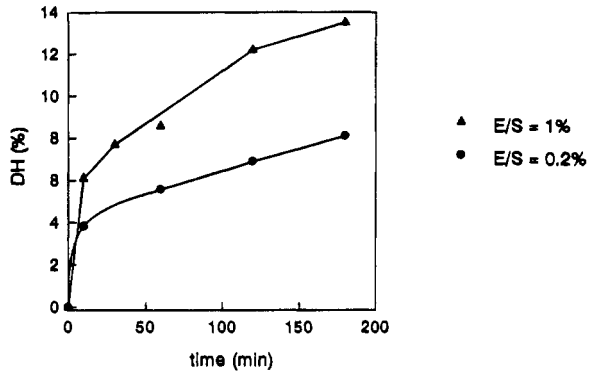

Figure 1. Peptic hydrolysis curves. Hemoglobin (50 g/L) was digested by pepsin at pH 3 and 37 °C.

completed by a centrifugation which separated supernatent and insoluble matter.

The solvent was eliminated by vacuum evaporation, and the fractions were freeze-dried.

Preparative Reversed Phase Chromatography. A glass column (10 × 100 mm) packed with Lichroprep RP18 silica (15–25 m) (Merck) was eluted at 4 mL/min with a gradients of H_2O–0.1% TFA as eluent A and CH_3CN–0.1% TFA as eluent B. After a 10 min step of 20% B, a linear gradient from 20% to 55% B in 70 min was applied. One hundred percent B for 10 min and then 10% B for 10 min cleaned and re-equilibrated the column after each injection.

Twenty milligrams of hydrolysate dissolved with 1 mL of 20% eluent B was injected. Fractions were collected according to the solvent rate by which they had been eluted: 0–20%, 20–30%, 30–40%....

The solvent was eliminated by vacuum evaporation, and the fractions were freeze-dried.

RESULTS AND DISCUSSION

Production and Analysis of Peptic Hemoglobin Hydrolysates. Several hydrolysates were obtained depending upon the reaction time and E/S ratio as shown in Figure 1. Surprisingly, the evolutions between DH and hydrolysis time are not linear even for low enzyme/substrate ratios. First, DH rapidly rises with time but, later on, flattens out. This may be due to the changing nature of the substrate and the molar dilution effect of the pepsin because gradually with hydrolysis, substrate molarity increases but the splitting site number decreases. A "fast reaction" followed by a "slow reaction" was pointed out by Adler-Nissen for various substrates. As it happens in our case, he had also noticed that the rate of splitting peptide bonds appears to increase more than proportionally to E/S and explained this behavior by the hypothesis of "narrowing specificity" or by the formation of inhibitory peptides during the hydrolysis (Adler-Nissen, 1986).

The gel permeation profiles of hemoglobin and different hydrolysates are presented in Figure 2. Although hemoglobin purity was confirmed by SDS–PAGE electrophoresis, its chromatogram exhibits several peaks. In fact, with regard to the molecular weight column calibration, the peaks corresponded to several hemoglobin forms: tetra-, di-, and monomeric forms (respectively, peaks 1, 2, and 3). Indeed, the hemoglobin chains are partially dissociated at pH 3 and there are few tetrameric forms.

We notice that tetrameric hemoglobin was more slowly digested by pepsin in comparison with the other forms. There are few differences between the two hydrolysates with DH 6% and 13%. The same peaks, numbered 1–10, are obtained by chromatography on Superose 12.

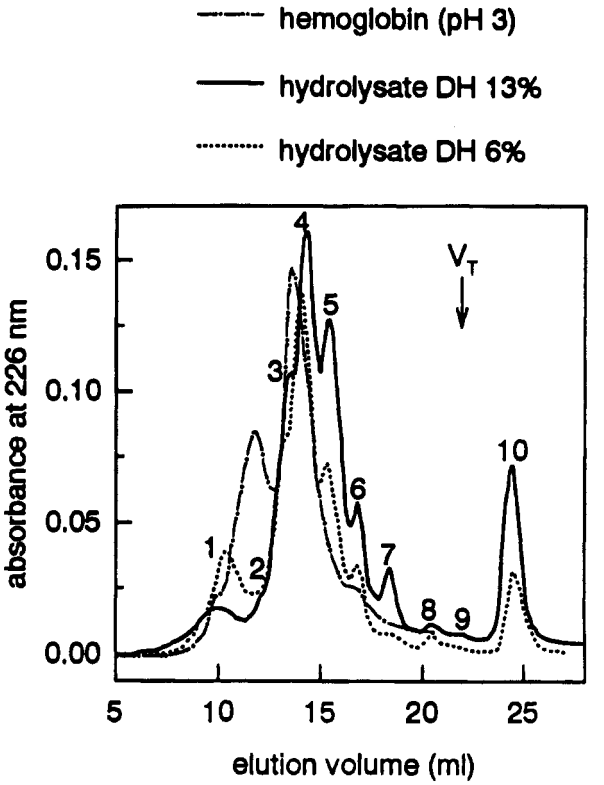

Figure 2. Superose 12 gel filtration chromatograms of peptic hemoglobin hydrolysates. Superose 12 (HR 10 × 30) column (Pharmacia LKB) was eluted with 20 mM ammonium acetate buffer at pH 7, containing 0.15 M NaCl, at a flow rate of 0.5 mL/min. The total column volume (V_T) was 22 mL. Hydrolysate samples were injected at 25 g/L (50 μL). Hemoglobin was injected at a lower concentration to agree with the same OD scale and to make comparison easier. Absorbance was detected on line at 226 nm.

A high peak (10) appears after the total packed bed volume: it corresponds to peptides interacting specifically with the gel, so this technique is not suitable for determining molecular weights of all the hydrolysate compounds. However, the Superose 12 column offers several advantages. Its fractionation range is broad (1000–300 000) and allows us to analyse the whole hydrolysate. Obviously, molecular weights cannot be exactly determined, but their distribution evolution can be monitored. This simple pattern of hydrolysate would allow us, unlike the Pep RPC chromatogram (Figure 3), to easily compare different hydrolysates and fractions.

The hydrolysate composition complexity is revealed by analytical reversed phase chromatography performed on a Pep RPC column (Figure 3). There are numerous peptidic fragments, and some of them seem to be in the majority. The whole hydrolysate is eluted with only 40% CH_3CN.

Peptic hemoglobin hydrolysates exhibit a distinct bitter taste and unpleasant odor, which are completely different from intact hemoglobin. The bitter taste is not due to heme or its derivatives, as a decolored hemoglobin hydrolysate has the same flavor. As presented in Figure 4, bitter taste appears from the start of proteolysis and increases with DH (from 6% to 13%). Average quinine detection threshold value for the panelists is 3.5 mg/L (4.5 μmol/L) in these tasting conditions. The hydrolysates' bitterness is very strong, as isointensity quinine concentration is scored between 35 and 82 mg/L (45 and 105 μmol/L), that is to say 10–25 times the quinine detection threshold value.

Hydrolysate Fractionation in Accordance with Bitterness. As peptic hemoglobin hydrolysate bitter-

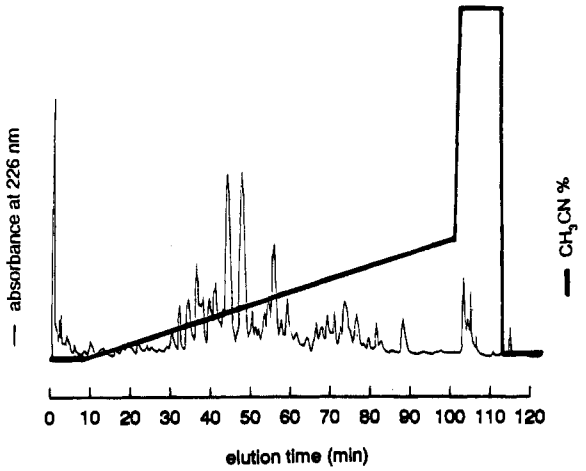

Figure 3. Analytical reversed phase chromatographic profile of a peptic hemoglobin hydrolysate of DH = 13%. Pep RPC column (HR 5/5) (Pharmacia LKB) was eluted at 0.7 mL/min with gradients of H_2O–0.1% TFA as eluent A and CH_3CN–0.1% TFA as eluent B. After a 10 min step of 10% B, a linear gradient from 10% to 40% B in 90 min was applied. One hundred percent B during 10 min and then 10% B during 10 min cleaned and re-equilibrated the column after each analysis. Absorbance was detected on line at 226 nm. Fifty microliters of hydrolysate (25 g/L) was applied.

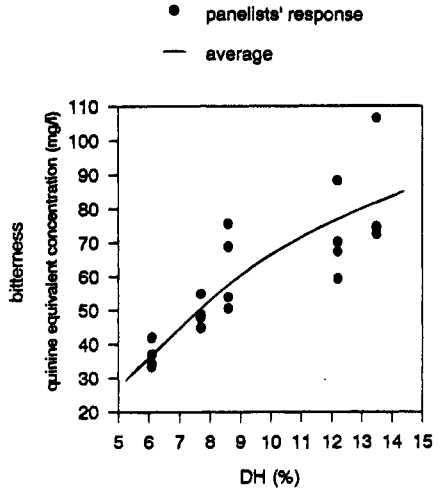

Figure 4. Evaluation of peptic hemoglobin hydrolysates bitterness. The degustation was performed with 200 μL of hydrolysate at 25 g/L, without retronasal olfaction. The hydrolysates were obtained by hydrolysis with 1% (E/S) pepsin. The bitterness intensity is expressed as isointensity quinine sulfate concentration (mg/L).

ness had been clearly proved, we wanted to elaborate separation methods to isolate bitter fractions. These methods would allow us to exhibit some bitter peptide characteristics and possibly to debitter hydrolysates.

Ultrafiltration Process. By ultrafiltration fractionating, four portions are obtained on the basis of molecular weight: MW >10 000, 10 000–5000, 5000–500, <500, although membrane cutoff is not very strict.

The YC05 membrane can be used for salt removal. Indeed, it was checked by conductivity measurement that most of the salt had run through that membrane, but only a few peptides were present in the permeate (checked by reversed phase chromatography and gel filtration).

Mass balance and fraction taste are shown in Table 1. Only the YM10 retentate has a brownish color, the other fractions presenting a yellow or light yellow color. There is little loss of matter (5%) during the process. The color and mass results have been verified by 226 and 410 nm absorbance. The fraction of molecular

Table 1. Successive Ultrafiltrations Process

	YM10 retentate	YM5 retentate	YC05 retentate	YC05 permeate
MW range	>10 000	<10 000 and >5000	<5000 and >500	<500
% (w/w)[a]	22	21	37	15
taste[b]	no or slight taste	slightly bitter	very bitter	salty

[a] Weight percentage was determined by dry matter weighing and expressed in relation to initial hydrolysate. [b] Taste was determined at a concentration equivalent to 25 g/L of initial hydrolysate.

Table 2. Organic Solvent Extraction

solvent	fraction type	% (w/w)[a]	taste[b]
2-butanol	organic phase 1	9	very bitter
	organic phase 2	9	bitter
	aqueous phase	80	slightly bitter
1-propanol	insolubles	26	no taste
	supernatant	72	bitter
2-propanol	insolubles	35	no taste
	supernatant	62	bitter
ethanol	insolubles	24	no taste
	supernatant	75	bitter

[a] Weight percentage was determined by dry matter weighing and expressed in relation to initial hydrolysate. [b] Taste was determined at a concentration equivalent to 25 g/L of initial hydrolysate.

weights between 500 and 5000, which represents 37% of initial hydrolysate, exhibits a strong bitter taste, while the fraction of highest molecular weight compounds has either not much taste or none at all. The salt taste of the YC05 permeate inhibits the detection of any other taste. The YM5 retentate (molecular weight between 5000 and 10 000) exhibits a slightly bitter taste, due to short overlapping with the next fraction.

Thus, the bitter peptides are concentrated in the permeate of 5000 cutoff membrane, which matches Ney's theory (Ney, 1979): bitter peptides have a molecular weight below 6000.

Organic Solvent Extraction. Organic solvent extraction is a fast separation step that could be easily scaled up. We expected to find a selective method and polarity and hydrophobicity features of bitter peptides by screening different solvents.

Hexane and toluene did not allow any extraction because an emulsion, which wholly solidified, appeared after mixing.

Methyl acetate and ethyl acetate took out only 3–4% but extracted no bitter compounds.

Extraction results with ethanol, 2-butanol, and 1- and 2-propanol are shown in Table 2. Supernatants obtained with ethanol and 1- and 2-propanol exhibit a strongly bitter taste and represent, respectively, 75%, 72%, and 62% (w/w) of initial hydrolysate. On the other hand, the two organic phases obtained after 2-butanol extraction contain only 18% of initial hydrolysate and the majority of the bitter taste. 1-Butanol gives the same results.

Extraction of peptic hemoglobin hydrolysate with secondary butyl alcohol seems to be an efficient method for removal of bitter compounds, as shown by Lalasidis and Sjöberg (1978). This method is selective, but complete debittering is not achieved with two successive extractions. The organic phase is less polar than the aqueous one, but is sufficiently polar to attract some peptides and amino acids, preferably hydrophobic ones.

Preparative Reversed Phase Chromatography. Separation by reversed phase chromatography is based on

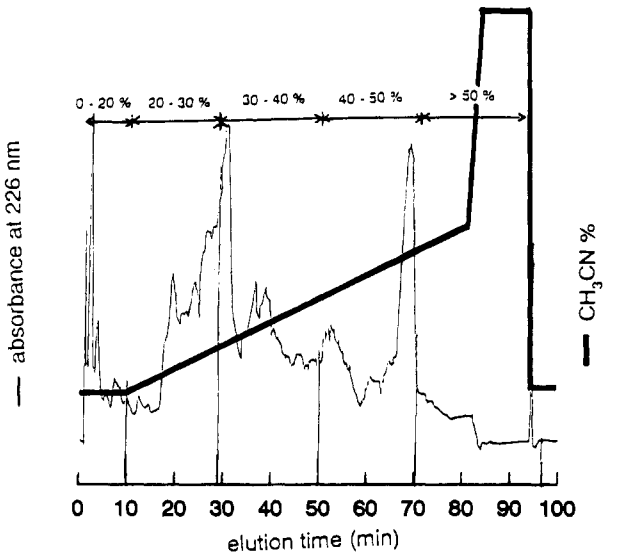

Figure 5. Preparative reversed phase chromatography of a peptic hemoglobin hydrolysate of DH = 13%. The chromatography was performed on a self-packed Lichroprep RP18 silica column (10 × 100 mm), eluted at 4 mL/min with gradients of H_2O–0.1% TFA as eluent A and CH_3CN–0.1% TFA as eluent B. After a 10 min step of 20% B, a linear gradient from 20% to 55% B in 70 min was applied. One hundred percent B for 10 min and then 10% B for 10 min cleaned and re-equilibrated the column after each injection. Absorbance was detected on line at 226 nm. Twenty milligrams of hydrolysate in 1 mL was injected. Fractions were collected according to the solvent rate by which they had been eluted: 0–20%, 20–30%, 30–40%....

the hydrophobic character of the molecules. As this technique cannot be suitably applied to great amounts of samples, we would use it only to demonstrate bitter peptide hydrophobicity. However, it would be used also as an analytical method for detecting bitter peptides.

Reversed phase chromatography has to be performed at semipreparative scale as tasting requires large samples, but results could be afterward extrapolated to an analytical column.

TFA is a nuisance as it gives a strong acid taste to the chromatographic fractions. Its use requires several (five) vacuum evaporations to remove it before tasting. However, its presence is necessary, as it allows the complete elution of all hydrolysate compounds, unlike methanol, 2-propanol, or acetonitrile used alone. Adsorption is stronger on the Lichroprep RP18 column with regard to the Pep RPC column; elution requires a further 5–10% CH_3CN.

Chromatographic profile and fractions are shown in Figure 5. The fraction corresponding to 40–50% CH_3CN contains heme and little degraded hemoglobin. Only the 30–40% CH_3CN fraction exhibits a bitter taste and represents 31% (w/w) of the first hydrolysate (Table 3). The 20–30% CH_3CN fraction exhibits a slight bitter taste, certainly because of fractions overlapping, which has been confirmed by analytical chromatography (Pep RPC). The amino acid composition analysis of the bitter fraction (30–40% CH_3CN) showed a composition unchanged with respect to the total hydrolysate.

Preparative reversed phase chromatography allows bitterness to be concentrated in a fraction eluted with 30–40% CH_3CN and representing 31% of the hydrolysate. Nevertheless, it does not appear to be the best analytical method for identifying bitter peptides in the whole hydrolysate. Indeed, bitter peptides are not sufficiently distinctly separated from the other compounds of the hydrolysate.

Comparison of the Fractionation Methods. The three methods, successive ultrafiltration process, 2-butanol extraction, and preparative reversed phase chromatography, exhibit only one very bitter fraction. Extraction and ultrafiltrations are easier and could be carried out more easily on an enlarged scale than chromatography. Extraction is more selective, as it extracts 18% (w/w), than reversed phase chromatography and the ultrafiltration process, whose bitter fractions represent, respectively, 31% and 37% of the hydrolysate.

Table 3. Preparative Reversed Phase Chromatography

	% CH_3CN				
	0–20%	20–30%	30–40%	40–50%	>50%
% (w/w)	19	39	31	9	1
taste	slightly salty	slightly bitter	**bitter**	no taste	no taste

[a] Weight percentage was determined by freeze-dried powder weighing and expressed in relation to initial hydrolysate. [b] Taste was determined at a concentration equivalent to 25 g/L of initial hydrolysate.

Figure 6, which presents chromatograms on Superose 12 of the different fractions obtained by separation methods (ultrafiltration, 2-butanol extraction, and reversed phase chromatography), shows that all bitter fractions are characterized by a high relative intensity of peak 10. This peak corresponds to compounds that interact specifically with the gel. Usually, these compounds are hydrophobic and aromatic, which confirms bitter peptide features.

The fraction of compounds whose molecular weight is 500–5000 exhibits high peak 10 (Figure 6a), just as the fraction eluted out of the preparative reversed phase column with 30–40% CH_3CN (Figure 6c). These two fractions are very bitter. The fraction that overlaps with the 30–40% fraction (20–30% fraction) and which is slighty bitter contains little peak 10 (Figure 6c). The YC05 permeate presents also little peak 10 (Figure 6a), but its salt taste inhibits the detection of the slightly bitter taste.

Compounds represented by peak 10 are distributed among the three phases stemming from 2-butanol extraction (Figure 6b). We noticed that bitterness had not been totally eliminated from the aqueous fraction, and all phases exhibited bitter taste. However, peak 10 size increases with bitterness intensity: slightly bitter, bitter, very bitter (Table 2).

The Superose 12 column would allow us to pick out bitter peptic hemoglobin hydrolysates or fractions by selectively delaying and eluting last (after total column volume) bitter peptides.

Conclusion. This paper presents a complete study scheme of enzymatic protein hydrolysate including hydrolysis curve, degree of hydrolysis determination, chromatographic profile according to size and hydrophobicity, and rigorous and systematic tasting.

The peptic hemoglobin hydrolysates exhibit a strongly bitter taste, whose intensity increases from DH 6% to 13%.

A fractionation process by successive ultrafiltrations through different membranes clearly signals one bitter fraction corresponding to molecular weight below 5000. Bitter-tasting compounds are eluted with 30–40% CH_3CN from a preparative reversed phase chromatographic column. 2-Butanol extracts an organic phase which represents 18% (w/w) of the initial hydrolysate and exhibits bitterness. Bitter peptides are small (PM < 5000) and rather hydrophobic as they are extracted by 2-butanol and adsorbed on C_{18} bonded silica.

These three methods show that a component category which is represented by only one peak on an analytical

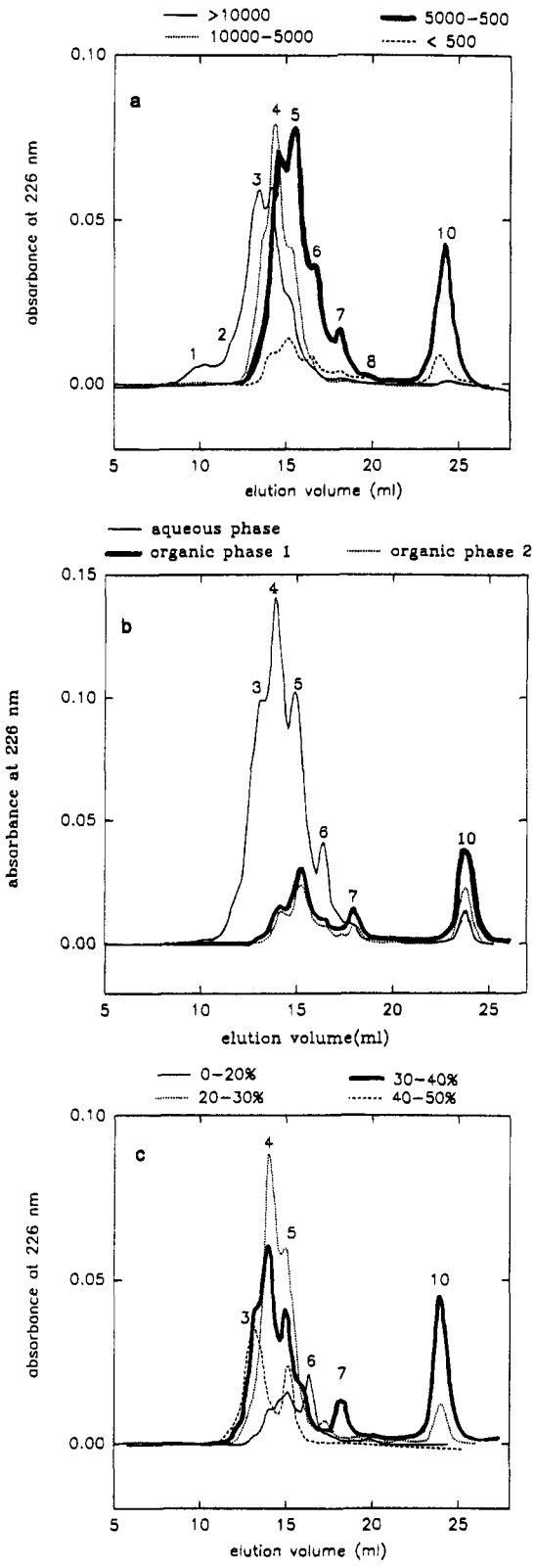

Figure 6. Superose 12 gel filtration profile of different obtained fractions. Experimental conditions were as indicated in Figure 2. Samples were injected at concentration equivalent to 25 g/L of initial hydrolysate (50 μL). The fractions came from a peptic hemoglobin hydrolysate (DH = 13%), which had undergone different separation types. The most bitter tasting fractions are plotted as a thick line. (a) The fractions obtained by successive ultrafiltration process, corresponded to different molecular weight ranges: >10 000, 10 000–5000, 5000–500, <500. (b) The fractions obtained by 2-butanol extraction corresponded to the two organic and the aqueous phases. (c) The fractions obtained by preparative reversed phase chromatography on a Lichroprep RP18 silica column were collected according to the acetonitrile rate by which they had been eluted: 0–20%, 20–30%....

Superose 12 column is obviously responsible for the majority of bitterness. This (these) compound(s) interact-(s) with Superose 12 gel and is (are) eluted later after the total packed bed volume. As it divides molecules by size and could interact specifically with hydrophobic peptides, chromatography on a Superose 12 column seems to be a good analytical method for following hydrolysate evolution with reaction time and particularly for detecting bitter peptide presence.

The exhibited peak has been isolated and its composition determined (Aubes-Dufau et al., 1995). A following study, made with other hydrolysates, would validate the different methods of analysis and fractionation elaborated in this paper. Moreover, different hydrolysis conditions or the use of other proteolytic enzymes could avoid the appearance of bitterness during hemoglobin hydrolysis.

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