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# Effect of Different Proteases on Bitterness of Hemoglobin Hydrolysates

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## ABSTRACT

Hemoglobin was hydrolyzed by several enzymes (Proctase, Alcalase, Neutrase, papain). Hydrolysates were analyzed (degree of hydrolysis, gel permeation on Superose 12 column, tasting) and fractionated by ultrafiltration and 2-butanol extraction. The bitter peptides were isolated and identified. The results were compared with those already obtained with peptic hemoglobin hydrolysates. All the findings were confirmed. Ultrafiltration concentrated bitter compounds in the fraction corresponding to 500–5000 Da, and these compounds were selectively extracted by 2-butanol. All the bitter peptides belonged to the same fragment of the β-chain of bovine hemoglobin. Finally, the use of a Superose 12 chromatographic column for easy detection of bitter hydrolysates without sensory analysis could be generalized for hemoglobin hydrolysates.

**Index Entries:** Hemoglobin; protease; hydrolysate; bitterness; bitter peptides.

## INTRODUCTION

Protein hydrolysates have been used as food ingredients for many years and, since the 1940s, enzymic hydrolysis of food proteins has been an established technology *(1)*. Protein digests can be produced for numerous purposes in animal or human foodstuffs, more particularly in dietetic foods and as flavoring agents *(2)*. Indeed, hydrolysis produces a decrease in peptide size and therefore modifies the functional characteristics of the proteins, thereby improving their quality. Moreover, the enzymic process preserves nutritional values, as it can be carried out under mild conditions.

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Nevertheless, problems related to taste have prevented the commercial success of food-protein hydrolysis, because it frequently leads to the production of a bitter taste. The formation of bitter peptides, well-known since 1952 *(3)*, is not the result of unwanted side reactions but is a natural consequence of proteolysis. Unfortunately, this bitterness compromises the quality of hydrolysates *(4)*.

At the beginning of the 1970s, interest focused on ways of making unconventional food proteins more attractive *(1)*. In this context, utilization of blood proteins—particularly hemoglobin, as it makes up about 90% of the total cell-fraction proteins—seems to be interesting *(5)*. Slaughterhouse blood represents a large amount of protein, but it is normally a waste product. However, hemoglobin could be valorized by proteolysis.

In a previous paper *(6)*, we studied hemoglobin hydrolysis by pepsin and we ascertained the bitterness of the hydrolysates produced. Different separation processes were developed to isolate and to characterize the bitter fractions *(6)* and we demonstrated that a single peptide was the major agent responsible for bitterness in peptic hemoglobin hydrolysates *(7)*. This peptide was also identified *(7)*.

The purpose of the present study is to use other peptidases in order to produce hemoglobin digests and to determine their influence on bitterness formation. This gave the opportunity to retest the previously established procedures. With these aims in view, four industrial enzymes (Proctase, Alcalase, Neutrase, papain) were used and hydrolysis was studied in accordance with the scheme elaborated with peptic hemoglobin hydrolysates *(6)*: hydrolysate production and analysis (degree of hydrolysis, gel permeation on Superose 12 column, tasting), and separation of bitter fractions (ultrafiltrations, 2-butanol extraction).

## MATERIALS AND METHODS

### Materials

Bovine hemoglobin was obtained from Serva Feinbiochemica (Heidelberg, Germany) (ref. 24510). Pepsin (ref. 7189–2500 FIP.U/g), Proctase and papain (ref. P3375) were purchased in powder form, from Merck (Darmstadt, Germany), Meiji Seika Kaisha Ltd (Japan), and Sigma Chemicals (St. Louis, MO), respectively. Alcalase 0.6 L and Neutrase 0.5 L, in liquid form, were a generous gift from Novo Nordisk (Copenhagen, Denmark). Quinine sulfate came from Prolabo (Paris, France). All reagents were of analytical grade.

### Proteolytic Activity Determination

The reaction was carried out at 37°C with stirring. Hemoglobin solution (20 g/L) was used as substrate. At time $t = 0$, 50 µL of enzyme were added to

5 mL of substrate. Samples of 200 µL were withdrawn at 1, 3, 5, 7, and 10 min and the reaction was stopped by adding 400 µL of trichloroacetic acid (TCA) (5% w/v). After 10 min of cooling, the samples were centrifuged for 15 min at 13,500*g*, and then the absorbance of the four time-diluted samples was read at 280 nm.

The initial-reaction rate was determined by plotting 280 nm absorbance vs reaction time. The specific activity corresponds to the slope of the curve between the initial rate and enzyme concentration. Its unit is (AU/min)/(g/L) of enzyme commercial preparation.

The protein concentrations were determined as equivalent of g bovine serum albumin (BSA)/g commercial preparation, by using the Lowry method *(8)*.

## Hydrolysate Preparation

Hemoglobin (50 g/L) was dispersed in 60 m*M* HCl or $H_2O$, and the pH was set to the desired value depending on peptidases: pH 3.0 for pepsin and Proctase, pH 8.0 for Alcalase, pH 7.0 for Neutrase and papain. Hydrolysis was carried out at 37°C and controlled by a pH-stat (Metrohm, Herisau, Switzerland). The absence of protease autolysis was verified. The reaction was stopped by increasing or decreasing pH according to the proteases employed to irreversible inactivation: pH 7.0 for pepsin and Proctase, pH 3.0 for Alcalase and Neutrase, pH 1.0 for papain. The hydrolysates were freeze-dried.

Finally, the hydrolysate solutions were centrifuged (5 min at 12,000*g*) before analysis or treatment. The degree of hydrolysis (DH) was determined with the trinitrobenzene sulfonic acid (TNBS) method described by Adler-Nissen *(9)*.

## Gel Filtration

All chromatographic methods were performed on a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia LKB, Uppsala, Sweden). Absorbance was detected on line at 226 nm (Uvicord SII LKB).

### Analytical

A prepacked column of Superose 12 (HR 10/30) (Pharmacia LKB) was eluted with 20 m*M* ammonium acetate buffer at pH 7.0 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 ($V_t$) (determined with acetone) of the column were respectively 6 and 18 mL; 50 µL of hydrolysate powder sample (25 g/L) were applied to the column.

### Preparative

A glass column (XK16/70) (Pharmacia LKB) packed with Superose 12 Prep Grade (Pharmacia LKB), was eluted at 1 mL/min with 20 m*M*

acetate ammonium. A 1 mL sample at a concentration equivalent to 100 g/L to initial hydrolysate was applied to the column.

## 2-Butanol Extraction

Two successive extractions were made by vigorously mixing, in equal volume, hydrolysate aqueous solution (50 g/L) and solvent, for 10 min, and by centrifuging to separate the two phases. The solvent was eliminated by vacuum evaporation and the fractions were freeze-dried. The evaluation of the process was made by sampling and dry-matter weighing, and expressed by weight percentage of the initial hydrolysate.

## Ultrafiltration Process

The hydrolysate (20 g/L in water) was fractionated into four parts by successive treatments with ultrafiltration membranes YM10, YM5, and YC05 (Amicon Grace; Witten, Germany), of which the nominal cuts-off were 10,000, 5000, and 500 Da, respectively. Ultrafiltration with YC05 membrane allowed salt elimination. The detailed ultrafiltration operation has been previously described *(6)* and the process was evaluated as indicated for 2-butanol extraction.

## Amino-Acid Sequence Analysis

Amino-acid sequence analysis was performed on a protein sequencer (Applied Biosystems 470A; Warrington, UK) with on line a phenylthiohydantoin amino-acid analyzer (Applied Biosystems 120A) (J. Capdevielle, Sanofi Recherche, Labège, France).

## Sensory Analysis

The bitterness of the hydrolysates and the different fractions obtained was estimated in aqueous solution. The hydrolysates exhibited an extremely unpleasant odor, which could mask the bitter taste. Thus, air was blown into the nose during the degustation, to suppress retronasal olfaction. The degustation was performed with 500 µL of sample put with a syringe onto the base of the tongue.

The bitterness intensity was scored by comparison with a quinine sulfate-concentration scale and expressed as an isointensity quinine concentration. The development of the test has been previously presented *(6)*. The concentrations given in this chapter were the mean of at least four values for each taster. The reliability of the subject throughout the experiment was controlled with a quinine solution of known concentration as stimulus. All the tasted solutions had to be neutralized and desalted beforehand, so that sour and salt tastes did not interfere with bitterness.

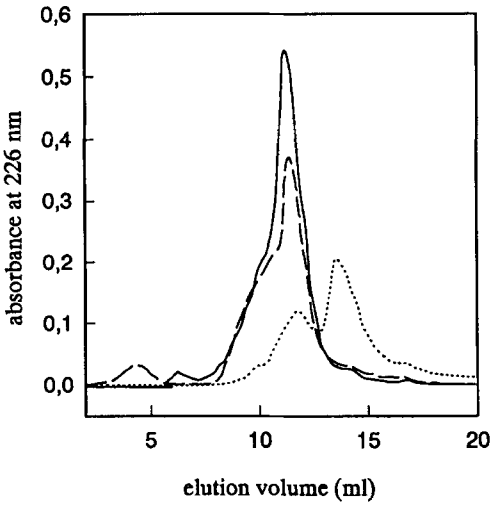


Fig. 1. Superose 12 gel filtration chromatograms of hemoglobin. Superose 12 (HR 10 × 30) column (Pharmacia LKB) was eluted with 20 m*M* ammonium acetate buffer at pH 7.0, containing 0.15 *M* NaCl, at a flow rate of 0.5 mL/min. Absorbance was detected on line at 226 nm. Symbols: —— hemoglobin (pH 8.0); – – – hemoglobin (pH 3.0 then 8.0); ····· hemoglobin (pH 3.0).

## RESULTS

In order to monitor the evolution of molecular-weight distribution during the hydrolysis, we used the gel permeation on Superose 12. The gel-permeation profile of hemoglobin (Fig. 1) was the reference (DH = 0). It exhibits several peaks that correspond to several hemoglobin forms: tetra-, di-, and monomeric forms, with regard to the molecular-weight column calibration. At pH 3.0, hemoglobin is essentially di- and monomeric, so dissociated. This denaturation is partially reversible, because when pH increases from 3.0 to 8.0, the majority of hemoglobin (75%) precipitates, but the soluble form is tetra- and dimeric. As thermal denaturation leads to precipitation, in order to obtain dissociated hemoglobin at neutral pH, urea (5 *M*) has to be used.

The activity of the studied proteases (pepsin, Proctase, Alcalase, Neutrase, papain) was determined with hemoglobin as substrate. The activities (Table 1) could be expressed with both commercial preparation masses (activity 1) and protein concentration (BSA equivalent) of commercial preparation (activity 2). However, for a practical application, activities 1 are more useful.

In order to compare the acid proteases (pepsin, Proctase) and the others (Alcalase, Neutrase, papain), activity was determined not only with denatured hemoglobin (diluted in urea 5 *M*), but also with native hemoglobin (diluted in $H_2O$). Indeed, comparison is more rigorous under the same conditions (dissociated hemoglobin), but 5 *M* urea is not suitable for

Table 1
Activity of the Used Proteases

| | Activity (denatured hemoglobin) (AU/min)/(g/L) | | Activity (native hemoglobin) (AU/min)/(g/L) | |
|---|---|---|---|---|
| | 1 | 2 | 1 | 2 |
| Pepsin | $9.2 \times 10^{-3}$ | $15.9 \times 10^{-3}$ | — | — |
| Proctase | $8.7 \times 10^{-3}$ | $20.8 \times 10^{-3}$ | — | — |
| Alcalase | $0.7 \times 10^{-3}$ | $14.3 \times 10^{-3}$ | $0.3 \times 10^{-3}$ | $6.1 \times 10^{-3}$ |
| Neutrase | $0.5 \times 10^{-3}$ | $11.6 \times 10^{-3}$ | $0.2 \times 10^{-3}$ | $4.6 \times 10^{-3}$ |
| Papain | $3.4 \times 10^{-3}$ | $9.4 \times 10^{-3}$ | $0.3 \times 10^{-3}$ | $0.8 \times 10^{-3}$ |

[a] Hydrolysis was carried out at 37°C. Native hemoglobin was diluted in $H_2O$. Denatured hemoglobin was diluted in 60 m*M* HCl (pH 3.0) or 5 *M* urea.

[b] Activity 1 was expressed with commercial preparation concentration. Activity 2 was expressed with protein (bovine serum albumin equivalent) concentration.

degustation. The activities (activities 1) of Alcalase, Neutrase, and papain determined with urea-diluted hemoglobin are greater than with native hemoglobin. However, in both cases, these activities are lower than those of pepsin and Proctase (activities 1). According to these results and for a practical use, the enzyme-substrate ratios (E/S) have been chosen to produce hemoglobin hydrolysates: E/S = 1% for Proctase as in the case of pepsin *(6)*, E/S = 6% for Alcalase, Neutrase, and papain. E/S was expressed with mass unit of enzyme commercial preparation.

The variations of DH with reaction time for the different endopeptidases are presented in Fig. 2. Hemoglobin proteolysis by Neutrase is weak and 6% DH is only reached after 14 h with E/S = 6%. However, 12% DH could be obtained in the same time, but with 12% Neutrase (data not shown). Papain weakly hydrolyses hemoglobin too, and a papain/hemoglobin ratio of 6% and 14 h reaction were necessary for a DH of 12%. Although Proctase and pepsin have quite similar activities, the DH reached with Proctase is higher. Thus, DH greater than 20% could be obtained easily with the use of Proctase (1%) and Alcalase (6%), whereas pepsin has to be added at a ratio of 12% for the same result.

The different kinds of hydrolysates were tasted. Hemoglobin hydrolysates produced with papain and Neutrase are tasteless at a concentration of 50 g/L, but all the other hydrolysates are bitter at a lower concentration (25 g/L). Their bitterness intensities are presented in Table 2. Peptic hydrolysates are about twice as bitter as the others. Bitter taste of hemoglobin hydrolysates produced with Proctase and Alcalase are in the same range and do not increase with a DH between 13 and 20%.

Because chromatography on a Superose 12 column seemed to be a worthwhile analytical method in our previous works *(6)*, the hemoglobin

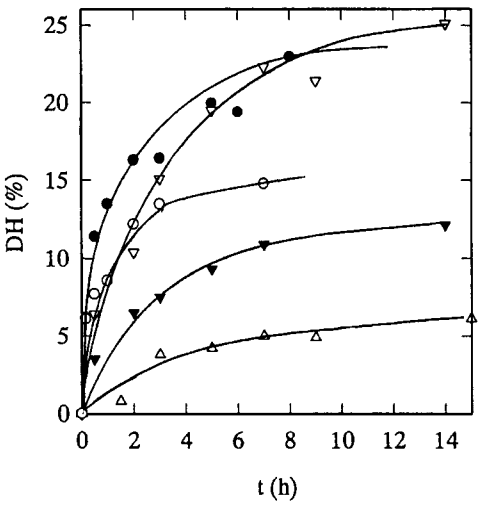


Fig. 2. Variation of the hydrolysis degree with time, for different proteases. Hydrolysis was carried out at 37°C. Enzyme-hemoglobin ratios are expressed with commercial-preparation masses. Symbols: ∇, Alcalase 6%; Δ, Neutrase 6%; ▼, papain 6%; ○, pepsin 1%; ●, Proctase 1%.

Table 2
Bitterness Intensity of Hemoglobin Hydrolysates

| Enzyme | Hemoglobin hydrolysate DH (%) | Isointensity quinine concentration (mg/L) |
|---|---|---|
| Pepsin | 13 | 220 |
| Proctase | 13 | 130 |
| Proctase | 20 | 140 |
| Alcalase | 15 | 80 |
| Alcalase | 20 | 100 |

[a] The degustation was performed with 500 µL of sample at 25 g/L, without retronasal olfaction. Bitterness intensity was expressed as equivalent quinine concentration (mg/L).

hydrolysates produced in this study were also analyzed with this technique (Fig. 3). A high-intensity peak appears after the mobile-phase volume ($V_t$ = 18 mL) in all the bitter hydrolysates (Fig. 3A, B, C). Nonbitter hydrolysates do not present such a peak (Fig. 3D). This peak is eluted late and so represents compounds that interact with Superose 12 matrix.

In order to separate the bitter part, hydrolysates were fractionated by ultrafiltration process using several different membranes. Mass balance and fraction taste are shown in Table 3. Papain hydrolysate YC05 permeate represents a great percentage because of salt generated during inactivation of papain (pH 1.0, in place of pH 3.0 for the other proteases) and neutralization.

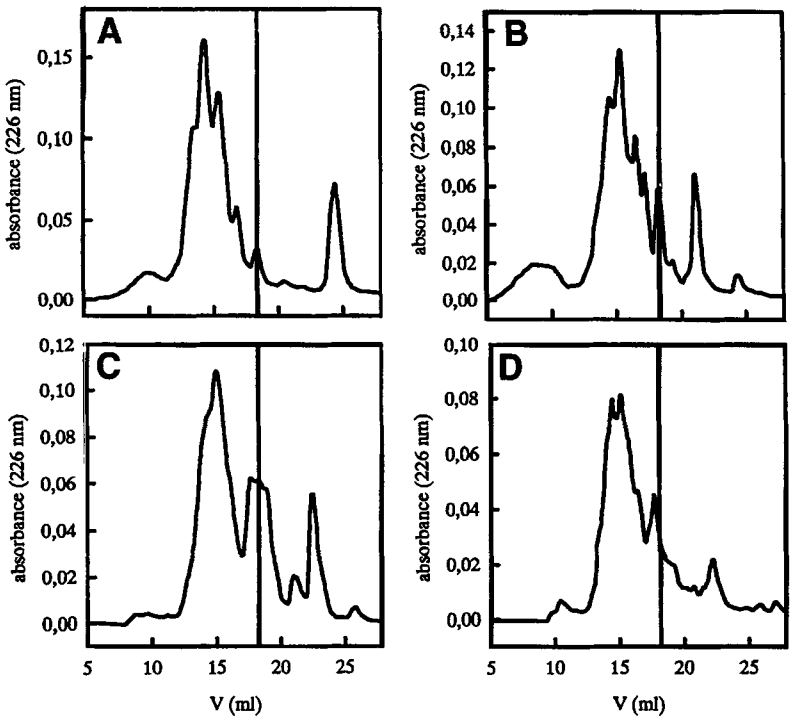


Fig. 3. Superose 12 gel filtration profiles of hemoglobin hydrolysates. Experimental conditions were as indicated in Fig. 1. The vertical line represents the mobile-phase volume (Vt = 18 mL). Hydrolysate samples produced with pepsin (DH 13%) (**A**), Proctase (DH 17%) (**B**), Alcalase (DH 22%) (**C**), papain (DH 12%) (**D**) were injected at 25 g/L (50 µL).

Table 3
Ultrafiltration of Hemoglobin Hydrolysates: Taste and Mass Balance of Obtained Fractions

| Hemoglobin hydrolysate (used enzyme, DH) | YM10 retentate (MW > 10000) (% w/w) | YM5 retentate (MW < 10000 and > 5000) (% w/w) | YC05 retentate (MW < 5000 and > 500) (% w/w) | YC05 permeate (MW < 500) (% w/w) |
|---|---|---|---|---|
| Pepsin | 22, | 21, | 37, | 15, |
| (DH 13%) | no taste | slightly bitter | very bitter | salty |
| Proctase | 20, | 15, | 43, | 16, |
| (DH 14%) | no taste | very slightly bitter | bitter | salty |
| Alcalase | 17, | 5, | 54, | 19, |
| (DH 15%) | no taste | no taste | bitter | salty |
| Neutrase | 25, | 12, | 33, | 27, |
| (DH 12%) | no taste | no taste | slightly salty | salty |
| Papain | 12, | 7, | 41, | 37, |
| (DH 12%) | no taste | no taste | slightly salty | very salty |

[a] Weight percentage was determined by dry matter weighing and expressed in relation to initial hydrolysate. Taste was determined at a concentration equivalent to 25 g/L (pepsin), 50 g/L (Proctase, Alcalase), 75 g/L (Neutrase, papain) of initial hydrolysate.

Table 4
2-butanol Extraction of Hemoglobin Hydrolysate:
Taste and Mass Balance of Obtained Phases

| Hydrolysate used enzyme, DH) | Organic phase 1 (% w/w) | Organic phase 2 (% w/w) | Aqueous phase (% w/w) |
|---|---|---|---|
| Pepsin | 9, | 9, | 80, |
| (DH 13%) | very bitter | bitter | slightly bitter |
| Alcalase | 12, | 14, | 72, |
| (DH 22%) | very bitter | slightly bitter | very slightly bitter |
| Proctase | 10, | 12, | 78, |
| (DH 20%) | bitter | bitter | bitter |
| Neutrase | 7, | 11, | 82, |
| (DH 12%) | no taste | no taste | no taste |
| Papain | 8, | 10, | 82, |
| (DH 12%) | no taste | no taste | no taste |

[a] Weight percentage was determined by dry-matter weighing and expressed in relation to initial hydrolysate. Taste was determined at a concentration equivalent to 25 g/L (pepsin) or 50 g/L (other proteases) of initial hydrolysate.

Although papain and Neutrase hydrolysates are not bitter, they could contain bitter compounds that have been masked in whole hydrolysate. But no fraction obtained from these hydrolysates had a bitter taste. For other hydrolysates, bitterness is concentrated in the fraction corresponding to the molecular weight range of 500–5000 Da. The YC05 retentate represents 37 to 54% of initial hydrolysate and so ultrafiltration is more or less selective in isolating bitter compounds, depending on hydrolysate. Chromatography on Superose 12 column of the different ultrafiltration fractions showed that only the bitter fractions exhibited peaks eluted after the mobile phase volume (results not shown).

In a previous study *(6)*, we demonstrated that 2-butanol was the more efficient organic solvent for bitter-peptide extraction. Thus, we used it again and the results obtained are shown in Table 4. No fraction from papain and Neutrase hydrolysates was bitter. This result confirms the ultrafiltration result: papain and Neutrase hydrolysates do not contain bitter peptides, or not enough to exhibit a detectable taste. This fact has to be compared with the absence of the retained peak on Superose 12 column or with its small area. In the case of the other hydrolysates (pepsin, Alcalase, Proctase), the majority of the bitter taste is concentrated in the two organic phases, which represent 18 to 26% of initial hydrolysate, according to the protease used. Thus, although this extraction method is rather selective, the aqueous phase remains bitter. In the case of pepsin and Alcalase hydrolysates, this phase is only slightly bitter. Nevertheless, 2-butanol extraction is not as efficient for Proctase hydrolysates, whose aqueous fraction is bitter, but less so than the initial mixture.

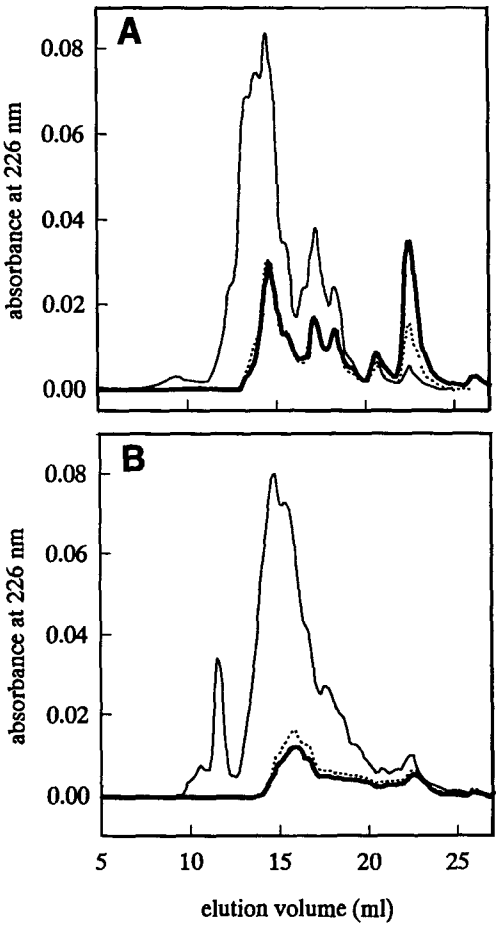


Fig. 4. Superose 12 profiles of 2-butanol extraction fractions of hemoglobin hydrolysates. (**A**) Example of bitter hydrolysate produced with Alcalase; (**B**) example of nonbitter hydrolysate produced with papain. Experimental conditions were as indicated in Fig. 3. Samples were injected at concentration equivalent to 25 g/L of initial hydrolysate (50 µL). Symbols: —— aqueous phase; – – – organic phase 1; ····· organic phase 2.

The different 2-butanol extraction phases were analyzed by chromatography on Superose 12 column and it appeared that 2-butanol selectively extracted molecules that adsorbed on Superose 12 stationary phase. Indeed, peaks eluted after mobile-phase volume ($V_t$) are chiefly present in bitter organic phases. Figure 4A presents an example of bitter hydrolysate (produced by Alcalase). However, no peak is selectively extracted by 2-butanol in nonbitter-tasting hydrolysates, in particular papain hydrolysate (Fig. 4B).

There is an interesting analogy between all these findings and the results already obtained with peptic hydrolysates *(6)*. Bitter hydrolysates produced by pepsin, Alcalase, and Proctase present peaks retained on Superose 12 column, and these peaks are found in all bitter fractions (ultrafiltration, 2-

butanol extraction). Thus, we isolated these peaks with the separation process previously described *(7)*. In each hydrolysate (produced by Alcalase and Proctase), a single peptide was isolated. These peptides had a bitter taste and their amino-acid sequences were determined with a protein sequencer:

peptide isolated from Proctase hemoglobin hydrolysate:
Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg
peptide isolated from Alcalase hemoglobin hydrolysate:
Val-Val-Tyr-Pro-Trp.

These peptides belong to fragment 32–40 of the β-chain of bovine hemoglobin and are the major cause of bitterness, as previously demonstrated in the case of VV hemorphin 7, the bitter peptide isolated from peptic hemoglobin hydrolysates *(7,10)*.

## DISCUSSION

Although the high Q value of hemoglobin would predict bitter hydrolysates *(11)*, all the hydrolysates produced in this study with several proteases of different characteristics are not bitter: papain and Neutrase do not produce bitterness. Thus, peptidases have a great influence on the hydrolysate taste. The chosen enzymes have broad specificity, especially for aromatic or hydrophobic amino acids. Nevertheless, they did not all produce bitterness, and the bitter peptides are different. It is rather difficult to consider specificity of protease, because it was generally determined with the oxidized B-chain of insulin or synthetic peptides. For entire proteins, the secondary specificity of the proteases, the fold structure of the proteins and the peptide-bond weakness may be as important as the primary specificity *(12)*. Thus it seems rather difficult to obtain a relationship between protease characteristics and bitterness formation.

From a practical point of view, in order to use hemoglobin hydrolysates for feed purposes, preferably papain or Neutrase should be employed, but DH would be lower than 12%. Hydrolysates produced with Proctase or Alcalase could also be integrated into food formulations, although masking compounds *(13)* should be added. On the other hand, the use of pepsin is excluded.

The fractionation methods developed in our previous work *(6)* with peptic hemoglobin hydrolysates were also effective for other hemoglobin hydrolysates. Bitter compounds are always concentrated by ultrafiltration in fraction corresponding to molecular range between 500–5000 Da, in agreement with Ney's theory *(11)* that the molecular weights of bitter peptides is below 6000 Da. Also they were selectively extracted by 2-butanol, which demonstrates rather hydrophobic properties.

The peptides chiefly responsible for bitterness of hemoglobin hydrolysates were isolated and identified. They all stemmed from fragment 32–40 of the B-chain of bovine hemoglobin.

Finally, the bitter peptides of hemoglobin hydrolysates interacted specifically with the matrix of Superose 12 column. This kind of interaction is usually observed with hydrophobic and particularly aromatic compounds. Thus, in Superose 12 chromatogram, the bitter peptides were represented by peaks eluted after the mobile-phase volume, which refutes gel-permeation theory. Thus, because of this property, bitter hemoglobin hydrolysates could be easily detected by chromatography on Superose 12 column. Indeed, hydrolysates with a high-intensity peak eluted after the mobile-phase volume of the column (elution volume of the smallest molecules) are bitter. Sensory analysis, a detailed and complex methodology, could thus be avoided. This interesting detection method has been validated for all hemoglobin hydrolysates, bitter and nonbitter, but other kind of hydrolysates need to be studied in order to generalize it.

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