Bitter peptide from hemoglobin hydrolysate: isolation and characterization

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Abstract Two separation methods, ultrafiltration and 2-butanol extraction, have shown that a peptide is the major agent responsible for bitterness in peptic hemoglobin hydrolysates. It was easily purified from these complex mixtures by specific hydrophobic adsorption on Superose 12, a gel-filtration column, which could constitute an original and interesting method for bitterness detection. The bitter peptide which corresponded to VV-hemorphin 7, the fragment 32–40 of the β chain of bovine hemoglobin, is first generated during proteolysis, then hydrolysed by pepsin. It exhibited a strong bitterness at 0.25 mM equivalent to 0.073 mM quinine sulfate or 21 mM caffeine.

Key words: Bitterness; Bitter peptide; Hemoglobin; Peptic hydrolysate; Gel-filtration

1. Introduction

It has long been known that the formation of bitter peptides is responsible for undesirable taste in foodstuffs. Their bitterness has been related to the presence of a high content of hydrophobic amino acids [1]. In 1971, Ney established the Q rule [2] which occupies a central position in the field of peptide bitterness studies. The Q value of one peptide, calculated from the hydrophobicity of the side chain of the involved amino acids, predicts its possible bitterness. Only peptides with molecular weights up to approximately 6000 Da and a Q value higher than 1400 cal/mol are bitter according to this rule [2]. Guigoz and Solms [3] have studied about 200 peptides and practically all complied with the so-called Q rule except many glycine-containing peptides. Nevertheless, the accuracy of the Q rule is sometimes thought to be limited because steric parameters and spatial structure, which are not reflected in the average hydrophobicity, are important for the intensity of the bitter taste.

Numerous bitter peptides have been isolated and their structures determined, from enzymatic hydrolysates, especially of casein because cheese ripening has been particularly investigated, and their structures determined. On the other hand, synthetic studies on the structure–taste relationship of bitter peptides have been chiefly carried out by Japanese workers with di- or tripeptides [4]. They concluded that bitterness is greatly enhanced when the hydrophobic residues are located at the C-terminal or in the C-terminal moiety.

A flavour compound must, presumably, interact with a receptor site, in order to bring about its physiological effect, which in general requires two major steps: (i) transport of the molecule to the receptor site, (ii) interaction with the receptor site. The first one is affected by factors such as volatility and lipid solubility, and the second involves the presence and correct stereo-alignment of interacting groups in the molecule [5].

Accepting a receptor model does not, of course, conclude anything with regard to the structure of real receptors, but the model allows a formal, uniform description of the molecular structure of bitter compounds [6]. Several models have been proposed in the literature [7]. Among others, Ishibashi et al. [8] have suggested two determinant sites, 4.1 Å apart: the 'binding unit' (BU) (bulky hydrophobic group, imino ring in proline) and the 'stimulating unit' (SU) (hydrophobic group or bulky basic group as an α-amino or a guanidino group). Tamura et al. [9] have completed this model with a hydrophobicity recognition zone located on the wall of a 15 Å pocket. In that way, the receptor recognizes the difference between very bitter and slightly bitter compounds.

The present paper deals with hemoglobin hydrolysis by pepsin in relation to its bitterness. The presence of a bitter peptide, the major cause of the whole hydrolysate bitterness, will be demonstrated. This peptide will be isolated and its structure determined. Finally, sensory analysis of the peptide and its kinetic study will complete this work.

2. Materials and methods

2.1. Materials

Bovine hemoglobin was obtained from Serva Feinbiochemica. Pepsin (2500 FIP-U/g) was purchased from Merck. Enzyme concentration is expressed by mass unit of enzyme commercial powder. Quinine sulfate was purchased from Prolabo. All reagents were of analytical grade.

The synthetic peptide used in this study was synthetised by Dr. H. Mazarguil (LPTF (CNRS), Toulouse, France).

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).

2.2. Hydrolysate preparation

The hydrolysis of hemoglobin (50 g/l) was carried out at pH 3 and 37°C and controlled by a pH-stat (Metrohm). Pepsin (50 g/l) was added in order to obtain an enzyme–substrate ratio (E/S) of 1% (w/w). The reaction was stopped by adding NaOH to pH 7. The hydrolysates were freeze-dried. The hydrolysate solutions were centrifuged before analysis or treatment. The degree of hydrolysis (DH) was determined with the TNBS (trinitrobenzene sulfonic acid) method described by Adler-Nissen [10].

2.3. Gel-filtration

2.3.1. Analytical. 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. 50 μl of hydrolysate powder sample (25 g/l) were applied to the column. The peptide concentration can be determined with an integrator (HP3396 A; Hewlett Packard).

2.3.2. Preparative. A glass column (XK 16/70; Pharmacia LKB)

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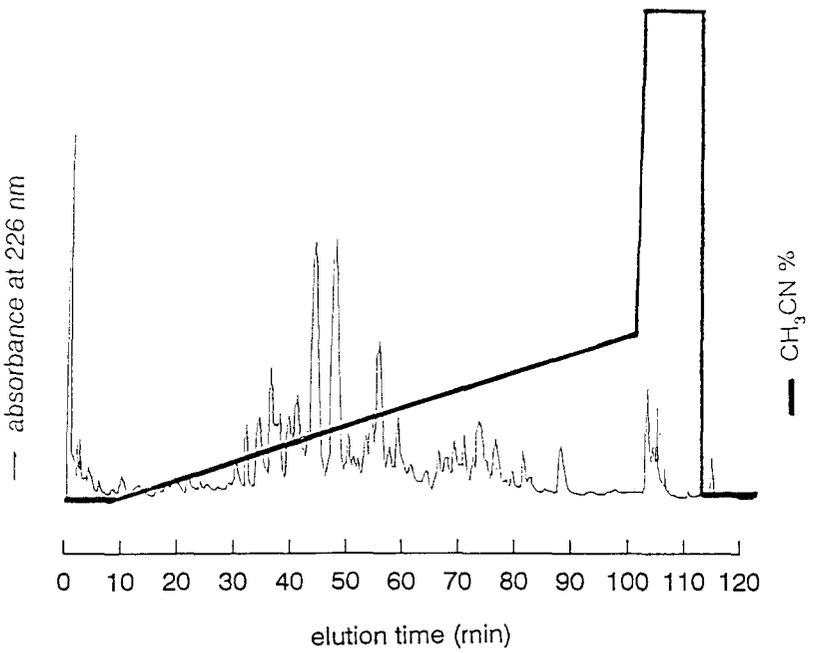

Fig. 1. Analytical reverse-phase chromatographic profile of a peptic hemoglobin hydrolysate (DH 13%) on a Pep RPC column.

packed with Superose 12 Prep Grade (Pharmacia LKB), was eluted at 1 ml/min with 20 mM acetate ammonium.

1 ml samples at a concentration equivalent to 100 g/l to initial hydrolysate were applied to the column.

2.4. Reverse phase chromatography

The separation was performed on a Pep RPC column (HR 5/5; Pharmacia LKB) eluted at 0.7 ml/min with a gradient of H_2O/0.1% TFA (trifluoroacetic acid) 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.

2.5. 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 two organic fractions were mixed.

The evaluation of the process was made by sampling and dry matter weighing, and expressed by weight percentage of the initial hydrolysate.

2.6. Ultrafiltration process

The hydrolysate (20 g/l in water) was fractionated into three parts by successive treatments with ultrafiltration membranes YM5, YC05 (Amicon GRACE), of which the cut-offs were respectively 5000 and 500 Da (g/mol). Ultrafiltration with YC05 membrane allowed salt elimination.

The evaluation of the process was made as indicated for 2-butanol extraction (section 2.5).

2.7. Amino acid sequence analysis

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

2.8. Sensory analysis

The bitterness of the samples was estimated in aqueous solution.

Only the taste character of the whole hydrolysate and its fractions was determined at a concentration of 25 g/l or equivalent to 25 g/l of initial hydrolysate for the different fractions. The bitterness intensity of the synthetic peptide was determined at a concentration of 0.3 g/l (0.25 mM) with 1 ml tasted volume. It was scored by comparison with a quinine sulfate concentration scale (dilution step of 1.2). A stimulus (peptide solution) and a standard solution (quinine solution with known concentration) were compared and the concentration of the standard solution was reduced or increased according to the subject's response, until it reached a balance. This sensory test was performed with forced choice and pair comparison: 7–8 pairs had to be tasted in order to determine one concentration. The equivalent quinine concentration or isointensity quinine concentration was calculated with the 'Up-and-Down' method of Dixon [11]. The concentrations given in this paper were the mean of at least four values for each taster [7]. The reliability of the subject throughout the experiment was controlled with a quinine solution of known concentration as stimulus.

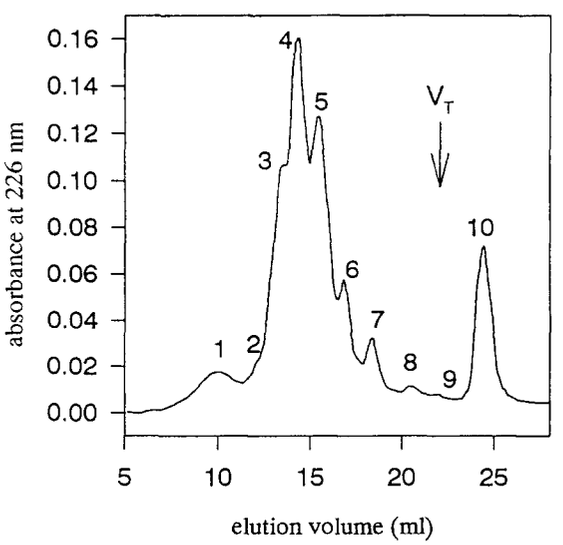

Fig. 2. Superose 12 gel-filtration chromatogram of peptic hemoglobin hydrolysate (DH 13%).

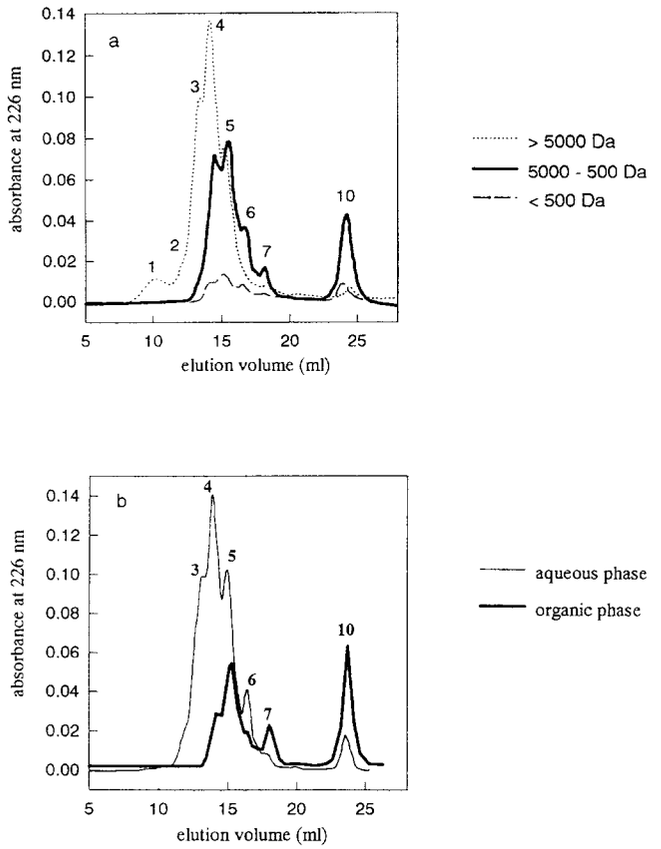

Fig. 3. Superose 12 gel-filtration profile of different fractions obtained from peptic hemoglobin hydrolysate (DH 13%). The most bitter fractions are plotted as a thick line. (a) Fractions obtained by successive ultrafiltration (molecular weight > 5000, 5000–500, < 500 Da). (b) Fractions obtained by 2-butanol extraction.

All the tasted solutions had to be previously neutralized and desalted, so that sour and salt tastes did not interfere with bitterness.

3. Results and discussion

Peptic hemoglobin hydrolysate exhibits a distinct and strong bitter taste. Its composition complexity is revealed by analytical reverse-phase chromatography performed on a Pep RPC column (Fig. 1) which shows numerous peptidic fragments.

On the other hand, the Superose 12 gel-filtration column offers a simple pattern of hydrolysate (Fig. 2). A high peak (no. 10), among the peaks numbered 1 to 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, but allows their distribution evolution to be monitored.

Two separation methods based on different characteristics, a successive ultrafiltration process for the size and a 2-butanol extraction for the polarity, allowed us to isolate strongly bitter fractions. The bitterness is concentrated in the fraction of molecular weights between 500 and 5000 Da, which represents 37% of the initial hydrolysate; that matches with Ney's theory [2] that bitter peptides have a molecular weight below 6000 Da. The organic phase obtained after 2-butanol extraction only contains 18% of initial hydrolysate and the majority of the bitter taste. This extraction seems to be an efficient method for removal of bitter compounds, as shown by Lalasidis and Sjöberg [12], but complete debittering is not achieved with two successive extractions: the aqueous fraction exhibits a slight bitter taste.

Fig. 3, which presents chromatograms on Superose 12 of the different fractions obtained by the two previous separation methods (a, ultrafiltrations; b, 2-butanol extraction) shows that only one peak (no. 10) is present in all the bitter fractions. This peak corresponds to compounds which are obviously responsible for the majority of bitterness.

In order to elucidate whether one or several peptides produce bitterness in peptic hemoglobin hydrolysate, the exhibited peak 10 should be isolated. A preparative method has to be used in order to obtain a large enough amount for analysing and, especially, tasting. Extraction with 2-butanol is a good first step separation which is selective and easily carried out on an enlarged scale. The mixture of the two organic fractions is injected on the Superose 12 Prep Grade preparative column. Its capacity can be exceeded and resolution can decrease without preventing good separation in this case. Indeed, the interesting peak is not separated for its size since it interacts with the matrix, and it is distinctly isolated from the other compounds of the hydrolysate on the Superose 12 analytical column. Ammonium acetate as volatile buffer was employed in order to get an easy recovery of peptides for further applications. The fraction corresponding to the targeted peak 10 is very bitter compared with the other fractions. The chromatographic control of this fraction on Pep RPC reverse-phase chromatography column, shows a large peak eluted at 23% of acetonitrile (Fig. 4). Thus, only one peptide would be the main agent responsible for the intense bitterness of peptic hemoglobin hydrolysate.

The bitter peptide amino acid sequence obtained with a protein sequencer is Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe. Its molecular weight is 1195 g/mol. The primary structure is identical to fragment 32–40 of the β chain of bovine hemoglobin.

This peptide has already been characterized by Piot et al. in a peptic bovine hemoglobin hydrolysate, for its opioid activity [13]. Another opioid peptide, β casomorphin 7, has also been described as tasting bitter [14]. The peptide was named

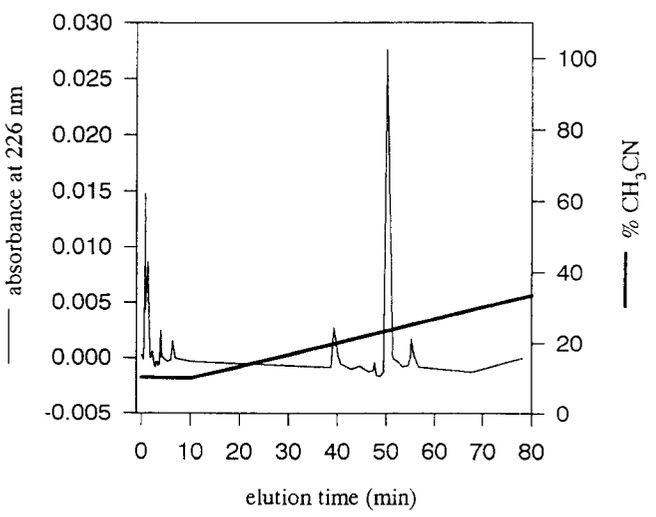

Fig. 4. Analytical reverse-phase chromatographic profile of Superose 12 peak 10 on a Pep RPC column.

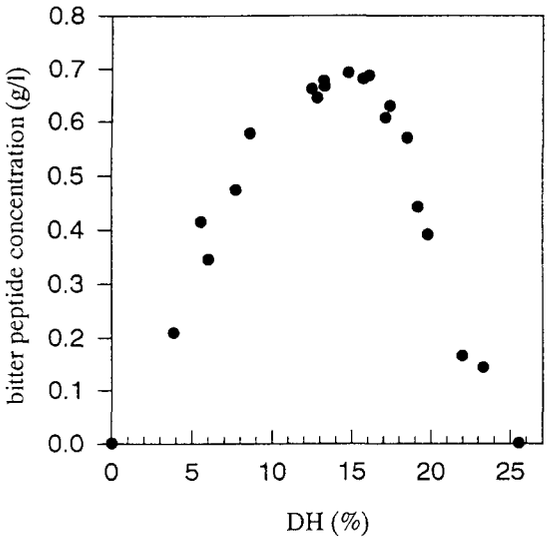

Fig. 5. Variation of bitter peptide concentration with hydrolysis degree (DH) in peptic hemoglobin hydrolysates.

VV-hemorphin 7 in view of its origin and activity. It was isolated by gel-permeation HPLC performed on a TSK G2000 SWG column and reverse-phase HPLC. The TSK G2000 SWG column is a semi-preparative HPLC column with a high efficiency and resolution but with a prohibitive cost [15]. The peptide was eluted normally from the gel-permeation column among several other peaks. Contrary to our case, there was not a specific adsorption on the matrix or consequently an easy separation from the other peptides. The separation on a Superose 12 column appears to be an original method as it utilises a major drawback of gel-permeation (i.e. hydrophobic interaction) and gives an unusually high resolution for peptide purification using this kind of chromatography. It would be of interest to determine if our simple method is specific for bitter peptides and could be employed for other bitter hydrolysates.

The Q value of the isolated bitter peptide, calculated following Ney's method [2] is 1730 cal/mol. Thus the Q rule can be applied to VV-hemorphin 7.

The isolated peptide has all the requirements for tasting bitter. It contains a high number of hydrophobic residues (6), which could act as the 'binding unit' (BU) and/or 'stimulating unit' (SU), and Phe, an hydrophobic amino acid, is located at the C-terminus. An arginine residue could play the role of SU. However, proline could bring them nearer in order to match with the receptor model. Indeed, the role of the proline residue in peptide taste is distinct from other hydrophobic amino acids. Its hydrophobic feature is certainly important, but by folding the peptide skeleton with its imino ring, the proline molecule could also form a suitable steric conformation for the bitter taste receptor [16].

Isointense concentration to a quinine concentration has been determined instead of detection or recognition thresholds which are difficult to obtain. Seven tasters, who determined a known quinine concentration with less than 5% average error, were chosen. On average, 0.3 g/l of peptide exhibits the same strong bitterness as 57 mg/l of quinine sulfate. In molar concentrations, 0.25 mM peptide corresponds to 0.073 mM quinine sulfate and 21 mM caffeine, which is much more than threshold. Thus, VV-hemorphin 7 is a strong potent bitter peptide. Its high content of hydrophobic residues and its spatial structure allow it to be very much more bitter than, for example, Val-Tyr-Pro with a 3 mM threshold [16]. This tripeptide corresponds to the amino acid sequence at the beginning of VV-hemorphin 7.

It is easy to measure the concentration of the bitter peptide by area peak integration, because it is available in a pure state and represented by a distinct peak with good resolution. In that way, it is possible to follow the kinetics of the apparition of VV-hemorphin 7 during the hemoglobin hydrolysis by pepsin. Fig. 5 shows the bitter peptide concentration evolution with the degree of hydrolysis (DH). In a first stage, the peptide is generated, then the pepsin hydrolyses it. The disparition happens after DH 16% and is complete at DH 25%. However, the 25% DH hydrolysate is still slightly bitter. The isolated bitter peptide seems to be degraded into other, less bitter peptides. We note by analogy with the opioid activity of VV-hemorphin 7, that several other hemorphins are known, like for example, hemorphin 4 (Tyr-Pro-Trp-Thr), hemorphin 6 (Tyr-Pro-Trp-Thr-Gln-Arg)... [17], which could be bitter and derived from VV-hemorphin 7.

The isolated bitter peptide was formed at an early stage in the peptic hydrolysis: it is present from 4% DH. The maximum concentration is 0.7 g/l, which corresponds to 2.8% of the whole hydrolysate (25 g/l), that is close to the rough percentage of 3% calculated after purification. 0.7 g/l corresponds to 0.58 mM VV-hemorphin 7, which represents 75% of the potential peptide molar concentration. The highest concentrations were reached in standard hydrolysates with 8–16% DH. These are the most interesting hydrolysates, because smaller DH hydrolysates have no special properties and higher DH hydrolysates are almost impossible to obtain due to the necessity of the very great amount of enzyme and the over-long proteolysis time.

Enzymatic protein hydrolysates could be used as feedstuff as they have functional and nutritional properties. Proteolysis could besides lead to valorization of hemoglobin, usually a waste product, by improving its properties and especially discolouring it. However, it is not possible to produce peptic hemoglobin hydrolysates without bitterness, even by making DH fluctuate. Thus, hemoglobin or blood proteolysis by pepsin is not a good process for feed purposes, because bitter peptide formation makes the hydrolysates unpalatable.

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