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Solvent effect on kinetics of appearance of haemorphins in the course of peptic hydrolysis of bovine haemoglobin

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The effect of the composition of the solvent on the peptic hydrolysis of bovine haemoglobin was studied to improve the preparation of two opioid peptides. Peptic hydrolysis was performed for 24 h at 23 °C in 0.1 M sodium acetate buffer, pH 4.5. A HPLC method was reported for isolating LVV-haemorphin-7 and VV-haemorphin-7 in one step. The kinetics of appearance of haemorphins was investigated in the presence of 20% (v/v) ethanol, a stabilizing solvent of haemoglobin or urea, a denaturant agent. Ethanol improved the yield of VV-haemorphin-7, whereas urea improved the yield of the two haemorphins. Because the amounts of haemorphins observed in urea were greater than in ethanol, the denatured state of haemoglobin is more favourable to obtaining LVV-haemorphin-7 and W-haemorphin-7. The peptide bonds that give rise to haemorphins would be more accessible to the pepsin.

Introduction

The enzymic hydrolysis of proteins is widely used in food industries to improve the proteins' functional properties such as taste, solubility, emulsification and gelation, or to prepare extensively hydrolysed proteins for hypoallergenic infant diets [1] and nutritional therapy [2]. More recently the pharmaceutical and food industries have shown increasing interest in peptides derived from protein hydrolysates as components of functional foods [3]. Much research has focused on the isolation of peptides derived from various protein sources. Numerous biologically active peptides have already been identified in, for example, casein [4], soya proteins [5], gluten [6], tuna myoglobin [7], mitochondrial cytochrome *b* [8]. These peptides exhibit immunomodulating [9], opioid [10–12], antithrombotic [13] or antimicrobial activities [14].

Blood from slaughterhouses is an unconventional but attractive source of proteins. Haemoglobin represents up to approx. 90% of the total cell-fraction proteins and is often a waste product. Therefore investigations have been reported to transform this agricultural by-product by enzymic hydrolysis. In a previous paper [15] we reported the preparation at the pilot-plant scale of a decolorized peptic hydrolysate

from bovine haemoglobin that was used for clinical enteric nutrition [16] and in cell-culture media [17]. Preparation by ultrafiltration of haem iron-enriched haemoglobin hydrolysates for treatments of iron deficiency has also been described [18]. It has recently been shown that bovine haemoglobin contains fragments or gives, after hydrolysis by pepsin or trypsin, peptides presenting different activities in bradykinin potentiation or as an opioid, coronar-constrictory or analgesic [19].

We have previously reported the isolation of two opioid peptides from a peptic hydrolysate of bovine haemoglobin prepared at a pilot-plant scale in a ultrafiltration reactor at pH 2 [20]. These two opioid peptides, LVV-haemorphin-7 (Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe) and VV-haemorphin-7 (Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe), correspond respectively to the sequences 31–40 and 32–40 of the β chain of bovine haemoglobin and are respectively identical with the sequences 32–41 and 33–41 of the β chain of human haemoglobin. However, these haemorphins are intermediate peptides and their concentrations in haemoglobin hydrolysates are often very low. Solvents have been used to improve the enzymic hydrolysis of proteins such as β -lactoglobulin [21], serum albumin [22], caseins [23] or gluten [24]. Several authors have observed a modification of the peptidic populations by alcohols such as ethanol and glycerol [21,24] and explained these changes in protease selectivity by a modification of the conformation of the native proteins and of the long intermediate peptides in relation with their hydrophobicities. In the present paper we report the appearance of two opioid peptides during the peptic hydrolysis of bovine haemoglobin at pH 4.5. These peptides had already been isolated from hydrolysates obtained at pH 2 for which the peptic activity is much higher than at pH 4.5. Therefore to improve the preparation of LVV-haemorphin-7 and VV-haemorphin-7 we have studied the kinetics of appearance of haemorphins during the peptic hydrolysis of bovine haemoglobin at pH 4.5 in the presence of 20% (v/v) ethanol, a stabilizing solvent of haemoglobin [25] or urea, a denaturing agent of proteins. We propose

Abbreviations used: DHc, corrected degree of hydrolysis; MALDI-MS, matrix-assisted laser desorption ionization MS.

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experimental conditions to increase haemorphin quantities in bovine haemoglobin peptic hydrolysates and to prepare these opioid peptides in one step by HPLC.

Materials and methods

Materials

All common chemicals and solvents were of analytical grade from commercial sources. Bovine haemoglobin and pig pepsin were purchased from Sigma Chemicals. Acetonitrile was of HPLC grade. Water was obtained from a Culligan system; the resistance was approx. 18 M Ω . All aqueous HPLC eluents were bubbled with helium.

Hydrolysate preparation

Native haemoglobin solution (100 ml) at 0.2% (w/v) was digested at 23 °C in 0.1 M sodium acetate buffer, pH 4.5, in the absence or the presence of 20% (v/v) ethanol, by pig pepsin (EC 3.4.23.1) (40 000 Anson units; Sigma Chemicals). Denatured haemoglobin was digested by pepsin under similar conditions but in the presence of urea (5.3 M). The enzymic hydrolyses were stopped by the addition of disodium tetraborate (0.32 M, pH 12.7) to yield a final pH of 10. The enzymic hydrolyses of haemoglobin were performed to different degrees of hydrolysis.

Determination of the degree of hydrolysis of haemoglobin

Degree of hydrolysis is defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds in haemoglobin. The cleavage of peptide bonds during hydrolysis was quantified by the trinitrobenzene sulphonate method [26]. The corrected degree of hydrolysis (DHc) is defined as the ratio of the number of cleaved peptide bonds (determined by the trinitrobenzene sulphonate method) to the total number of peptide bonds of haemoglobin effectively hydrolysed. The proportion of non-hydrolysed haemoglobin was deduced from the determination of its peak area under HPLC.

Amino acid analysis

Amino acids were analysed with a Waters Picotag Workstation. Peptide hydrolysis was achieved with constant-boiling (6 M) HCl containing 1% (w/v) phenol, for 24 h at 110 °C. The precolumn derivatization of amino acids with phenyl isothiocyanate and the HPLC separation of derivatized amino acids on a Waters reverse-phase Picotag column (150 mm \times 3.9 mm internal diam.) were performed by the method of Bidlingmeyer et al. [27]. The detection wavelength was 254 nm and the flow rate was 1 ml/min.

HPLC system

The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and analyse chromatographic data. All the chromatographic processes were performed with a Vydac C₄ column (250 mm \times 4.6 mm internal diam.). The mobile phase was water/trifluoroacetic acid (1000:1, v/v) as eluent A and acetonitrile/water/trifluoroacetic acid (600:400:1, by vol.) as eluent B. The flow rate was 1 ml/min. Samples were filtered through 0.22 μ m filters and then injected. The gradient applied was 0–67% (v/v) B over 30 min then 67–87% (v/v) B over 35 min. On-line instantaneous UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum every 1 s. The resolution was 1.2 nm. Chromatographic analyses were completed with Millennium software.

Matrix-assisted laser desorption ionization (MALDI)–MS

Matrix-assisted laser desorption ionization (MALDI)–MS was performed on a Finnigan Mat Vision 2000 time-of-flight mass spectrometer equipped with a viewing unit. All data were acquired at 500 MHz sampling frequency in reflector mode with external and/or internal calibration and also in linear mode. The nitrogen laser spot size was 70 μ m and the laser energy was adjusted close to threshold. The following matrix was used: a mixture of 2,5-dihydroxybenzoic acid (12 g/l) and 2-hydroxy-5-methoxybenzoic acid (12 g/l) (9:1, v/v) was prepared with acetonitrile/water (1:2, v/v) as solvent. A 1 μ l portion of the matrix solution was mixed with 0.5 μ l of sample solution on a stainless steel target, dried in a gentle stream of air and loaded into the mass spectrometer. Mass spectra were obtained preferably from the crystalline rim of the dried sample. Usually, 15–30 spectra were averaged in linear mode and 20–40 in reflectron mode. Linear configuration was used applying 30 kEv ion energy for reflectron time-of-flight analyser 5 kEv ion energy and 20 kEv post-acceleration to a discrete dynode secondary electron multiplier in positive-ion mode.

Results and discussion

Isolation of haemorphins from a haemoglobin peptic hydrolysate obtained at pH 4.5

The peptic hydrolysis of native bovine haemoglobin was performed for 24 h at 23 °C in 0.1 M sodium acetate buffer, pH 4.5. The resulting hydrolysate was analysed in one step by reverse-phase HPLC on a C₄ column as described in the

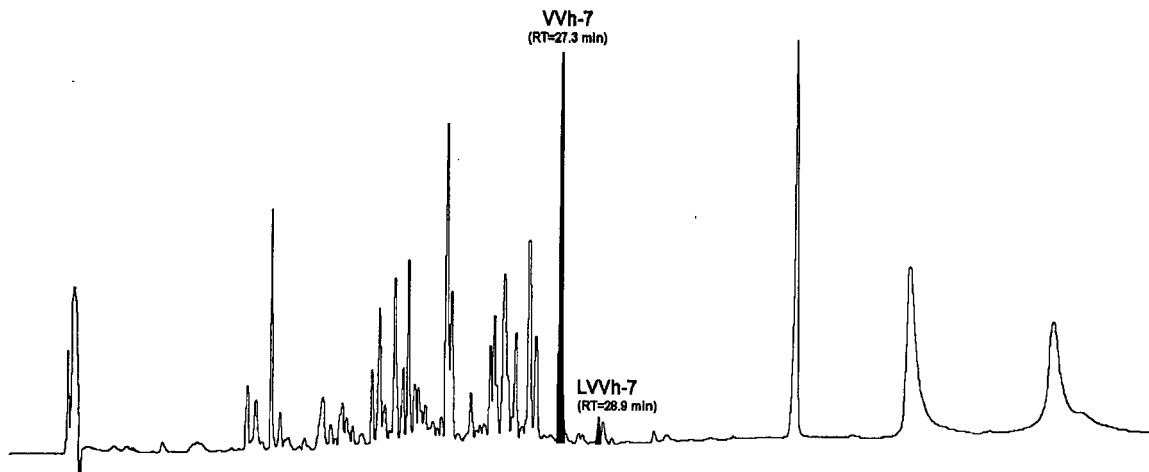


Figure 1 HPLC chromatogram of the haemoglobin peptic hydrolysate

The peptic hydrolysis was performed for 24 h at 23 °C in 0.1 M sodium acetate buffer, pH 4.5. The hydrolysate was analysed by reverse-phase HPLC on a C_4 column with the conditions described in the Materials and methods section.

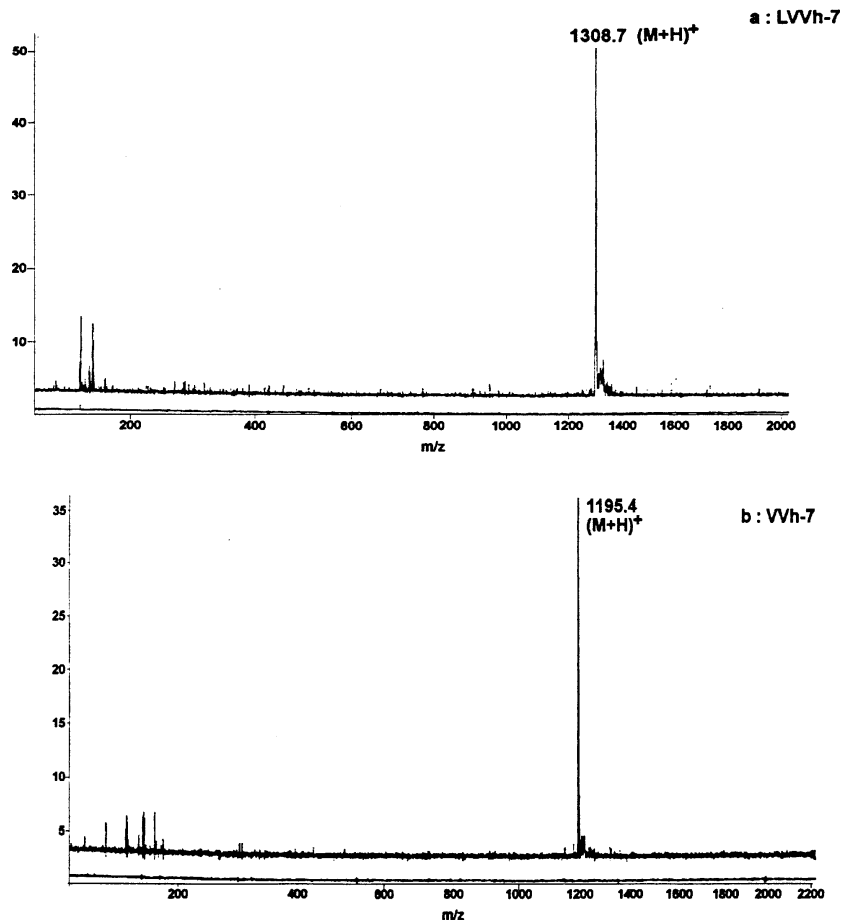


Figure 2 MALDI-MS of haemorphins isolated by HPLC

(a) LVV-haemorphin-7 (residues 31–40 of the β chain of haemoglobin); (b) VV-haemorphin-7 (residues 32–40 of the β chain of haemoglobin).

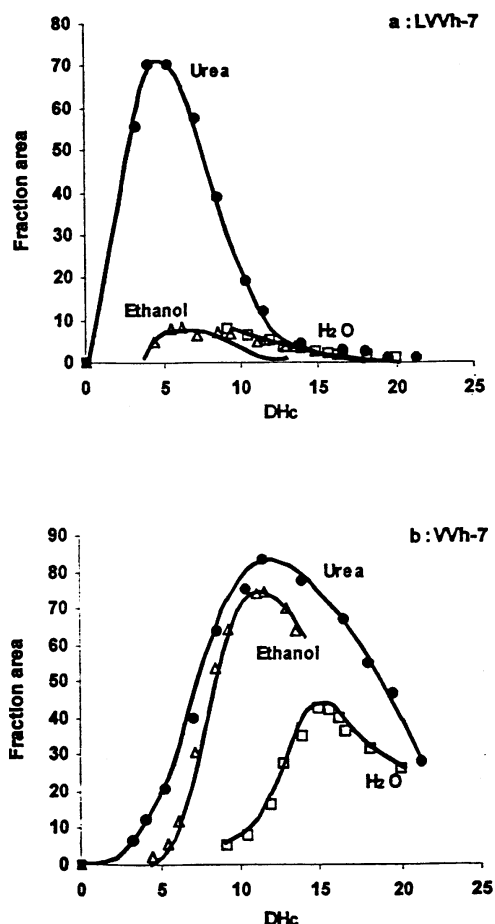


Figure 3 Kinetics of appearance of LVV-haemorphin-7 (a) and VV-haemorphin-7 (b) during haemoglobin hydrolysis in the presence of ethanol or urea

Peak areas corresponding to haemorphins were determined by reverse-phase HPLC coupled with detection with a photodiode array.

Materials and methods section; the chromatogram is shown in Figure 1. Peptides corresponding to each fraction were identified by amino acid analysis and MALDI-MS (results not shown). Two important fractions corresponding to pure peptides with retention times of 28.93 min (fraction 1) and 27.18 min (fraction 2) were recognized as haemorphins, which had already been isolated by successive size-exclusion chromatography and C_{18} reverse-phase HPLC from a haemoglobin peptic hydrolysate prepared at pH 2.

Figure 2 shows the positive MALDI spectra of these two peptides. The accurate relative molecular mass of the first peptide, deduced from the m/z value of $(M+H)^+$ by subtraction of one mass unit for the attached proton, is 1307 Da (fraction 1). On the basis of these results and taking into account the known amino acid sequence of α and β bovine haemoglobin chains, this peptide corresponds to the residues 31–40 of the β chain: Leu³¹-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe⁴⁰, i.e. LVV-haemorphin-7 [20]. The peptide

of the second fraction (27.18 min) presented a unit-mass resolution of 1194 Da, corresponding to the peptide originating at Val³² and extending to Phe⁴⁰ in the β chain: Val³²-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe⁴⁰, i.e. VV-haemorphin-7 [20]. These results show that the hydrolysis of bovine haemoglobin by pepsin at pH 4.5 also produces haemorphins. Moreover they can be isolated in one step by C_4 reverse-phase HPLC and the efficiency of the separation allows their easy on-line detection by using the UV-spectra comparison method published by other authors [28].

Effect of solvent on the yield of haemorphins

Two media were selected for studying the appearance of LVV-haemorphin-7 and VV-haemorphin-7: for peptic hydrolysis the solvent systems used were (1) a water/ethanol solvent containing 20% (v/v) ethanol, for which we had previously demonstrated a stabilizing effect on haemoglobin conformation [25], and (2) an aqueous solvent containing urea, which caused the denaturation of haemoglobin. The peptidic hydrolysates obtained in these solvents were compared with that obtained in the aqueous buffer alone at different degrees of hydrolysis of haemoglobin. DHc is more representative of the state of hydrolysis of haemoglobin than the simple degree of hydrolysis because it is independent of the mechanism of the hydrolysis, which is 'one by one' for native haemoglobin and 'zipper' for denatured haemoglobin. Figure 3 shows the appearance of each haemorphin in the water/ethanol solvent, in the presence of urea and in buffer alone as a function of DHc. Ethanol shifts the optimum concentration of haemorphins towards slight degrees of hydrolysis and increases the amount of VV-haemorphin-7. Ethanol has a small effect on the accumulation of LVV-haemorphin-7 which is observed in the form of traces as in the buffer alone. In contrast with the water/ethanol solvent, the denaturation of haemoglobin by urea markedly increases the amount of both LVV-haemorphin-7 and VV-haemorphin-7. As with ethanol, the optimum concentration was with urea observed at a lower DHc than in the buffer alone. The LVV-haemorphin-7 peak area was 7-fold that in the buffer alone, and the VV-haemorphin-7 peak area was 2.5-fold that in the buffer. Therefore urea-containing buffer is the best solvent for the preparation of haemorphins, probably because the peptidic bonds (Leu³⁰-Leu³¹, Phe⁴⁰-Phe⁴¹ and Leu³¹-Val³²) that give rise to the haemorphins are open to peptic digestion in denatured haemoglobin.

Solvent effect on the kinetics of appearance of LVV-haemorphin-7 and VV-haemorphin-7

We compared the kinetics of the appearance of haemorphins during the hydrolysis of bovine haemoglobin, as measured by DHc, in the water/ethanol solvent, in urea-containing buffer and in buffer alone. Their respective peak areas as a function

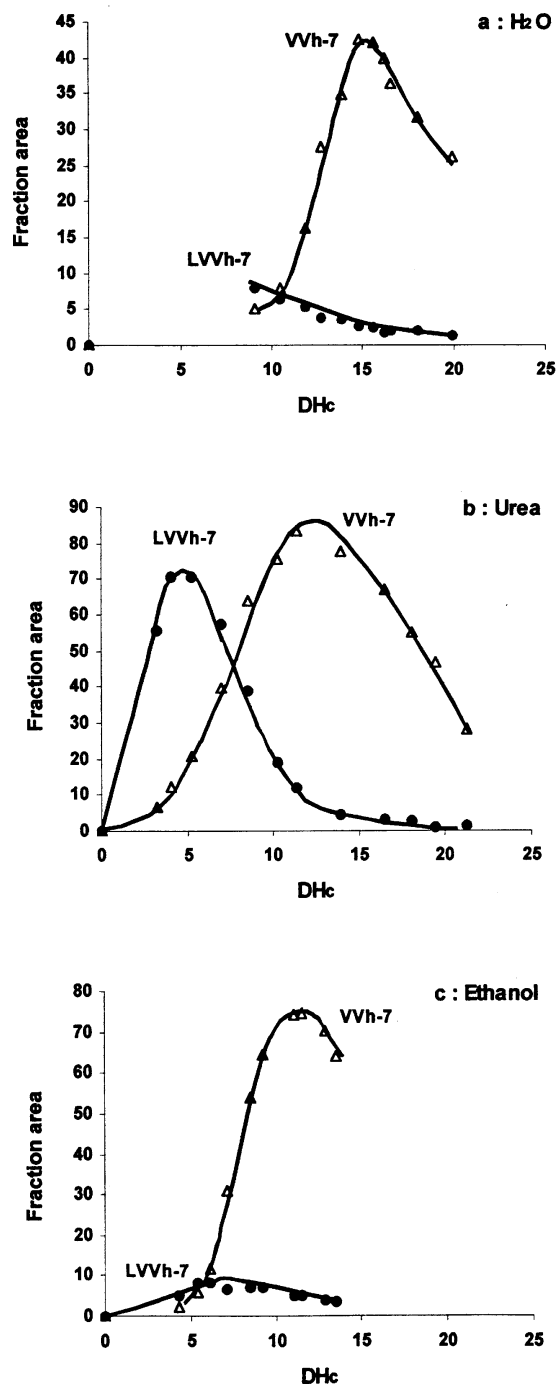


Figure 4 Comparison of kinetics of appearance of LVV-haemorphin-7 and VV-haemorphin-7 in buffer alone (a), in the presence of urea (b) or in the presence of ethanol (c)

Peak areas were determined as described in the legend to Figure 3.

of DHc of haemoglobin are shown in Figure 4. Ethanol or urea denaturation did not change the order of appearance of haemorphins, but in urea-containing buffer and water/

ethanol solvent haemorphins reached a higher concentration. In the urea-containing buffer, the only solvent allowing the observation of the two haemorphins in large proportions (Figure 4b), the concentration of LVV-haemorphin-7 was optimal at a DHc of 5%, whereas VV-haemorphin-7 attained its maximum concentration at a DHc of 12%. Therefore VV-haemorphin-7 was obtained only after the total disappearance of LVV-haemorphin-7. These results suggest that the two haemorphins were produced successively during the hydrolysis of the β chain of haemoglobin. Other authors [21,29] have also observed that, during the peptic hydrolysis of globin at pH 2, LVV-haemorphin-7 disappeared while the level of VV-haemorphin-7 increased, and they assumed that LVV-haemorphin-7 is a precursor of VV-haemorphin-7.

Conclusion

Our results show that LVV-haemorphin-7 and VV-haemorphin-7 are produced not only at pH 2, as reported by several authors during the peptic hydrolysis of bovine haemoglobin, but also at pH 4.5, at which pepsin is far less active. The peptic hydrolysis of bovine haemoglobin in the denatured state (in urea-containing buffer) or in the native state in the presence of 20% (v/v) ethanol permits a significant increase the production of these haemorphins. Therefore to prepare haemorphins we propose the hydrolysis of bovine haemoglobin at pH 4.5 in 5.3 M urea-containing acetate buffer up to DHc values of 5% and 11% respectively for the isolation of LVV-haemorphin-7 and VV-haemorphin-7. The isolation of haemorphins can be performed in one step by reverse-phase HPLC. This method of preparation of haemorphin at pH 4.5 in the presence of urea or ethanol could be interesting because, in contrast with pH 2, at which haem and haemoglobin partly precipitate, peptic hydrolysis is performed in homogeneous solutions.

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