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IDENTIFICATION OF PEPTIDES, FROM A PEPTIC HAEMOGLOBIN HYDROLYSATE PRODUCED AT PILOT-PLANT SCALE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

JEAN-MARIE PIOT*, DIDIER GUILLOCHON and QUIYU ZHAO
Laboratoire de Technologie des Substances Naturelles, IUT A-Lille I, B.P. 179, 59653 Villeneuve d'Ascq Cedex (France)
GUY RICART
Laboratoire de Spectrométrie de Masse, Lille I, 59655 Villeneuve d'Ascq Cedex (France)
BERNARD FOURNET
Laboratoire de Chimie Biologique, Lille I, U.A. 217 du C.N.R.S., 59655 Villeneuve d'Ascq Cedex (France)
and
DANIEL THOMAS
Laboratoire de Technologie Enzymatique, E.R.A. 423 du C.N.R.S., Université de Technologie de Compiègne, B.P. 649, Compiègne Cedex (France)
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SUMMARY

Gel-permeation high-performance liquid chromatography (HPLC) and reversed-phase HPLC were used to separate a mixture of peptides, produced at pilot-plant scale by peptic hydrolysis of bovine haemoglobin. Volatile buffers were employed in both HPLC techniques in order to get an easy recovery of peptides for further applications. The method is more rapid than low-pressure gel filtration. Amino acid analysis and fast atom bombardment mass spectrometry confirmed the purity, and allowed accurate molecular weights to be determined, for isolated peptides. These data demonstrate that such efficient techniques, usually used to resolve hydrolysates obtained in batch with pure substrates and highly specific enzymes, can be employed to resolve complex enzymatic hydrolysates of crude protein.

INTRODUCTION

Protein hydrolysates have been used as food ingredients for many years[1]. Additional applications have been investigated within the past few years[2], such as nutritional therapy[3,4], research on biological activity[5,6], stimulating effect on fermentation[7]. Such applications require very well defined and reproducible hydrolysates, especially when they are produced on a large scale. In order to gain a better understanding of the peptide population of such complex hydrolysates, precise analytical methods must be employed.

Since the initial uses of high-performance liquid chromatography (HPLC) for

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protein digest mapping[8,9], this method has been largely applied as a means of separating small to medium sizes peptides (less than 100 amino acids)[10–13]. Currently reversed-phase HPLC is most frequently used for peptide fractionation[14–16], because excellent resolutions are obtained. Gel-permeation HPLC is seldom employed, because it appears not to have so high a resolution for peptides separated from complex hydrolysates[17] as for pure standard peptides[18]. The major problem connected with gel-permeation HPLC is the unpredictable behaviour of peptides because complex mechanisms including size exclusion, hydrophobic adsorption and electrostatic interactions operate[13].

As for the determination of accurate peptide molecular weights, a new approach is offered by fast atom bombardment mass spectrometry (FAB-MS)[19–21]. This technique, therefore, allows a direct comparison of the molecular weights of the peptides, expected from the proposed structure after specific enzymatic or chemical cleavage, with those weights experimentally determined by FAB-MS[20].

We report here work on the analysis of a peptic haemoglobin digest, obtained at pilot-plant scale by an ultrafiltration process[22]. Studies on the applications of this hydrolysate are now being undertaken in many areas such as nutritional therapy and the isolation of biologically active peptides. Preliminary studies were successfully confirmed with microbiological culture experiments[23]. The aim of this work is accurately to determine the composition and molecular weight of any peptide in this complex haemoglobin hydrolysate. Peptide fractionation and purification are performed by using reserved-phase HPLC to supplement gel-permeation HPLC separation. The molecular weight of each peptide estimated from the amino acid composition is compared with the value determined by FAB-MS. The results demonstrate that it is possible to know precisely any peptide from a very complex peptic hydrolysate, produced at pilot-plant scale. The efficiency of the procedure is discussed and compared with previous values.

EXPERIMENTAL

Materials

All common reagents were of analytical reagent grade from Merck (Darmstadt, F.R.G.). Acetonitrile was of HPLC grade. Water was obtained from a Waters (Saint-Quentin, France) Milli-Q water system. Standard peptides were insulin A, bacitracin, bradykinin, actinomycin and PZ-Pro-Leu-Gly-Pro-Arg (where PZ is 4-phenylazobenzyloxycarbonyl), from Serva (Heidelberg, F.R.G.). The amino acids standard kit H was from Pierce (Rockford, IL, U.S.A.). All aqueous HPLC eluents were filtered prior to use on Sartorius (Palaiseau, France) 0.45-μm filters, and degassed with helium (Air Liquide, Bois d'Arcy, France) during analysis.

High-performance liquid chromatography

The HPLC apparatus was a Waters 600 E pump-system controller with a Waters Model U6K injector and a Waters Model 445 detector set at 215 nm and connected with a Waters Model 745 integrator.

Gel-permeation HPLC. The elutions were performed on a TSK (LKB, Bromma, Sweden) G2000SW column (600 mm × 7.6 mm I.D.) with 10 m*M* ammonium acetate buffer adjusted to pH 6.0 with acetic acid. Hydrolysate powder samples of 8.5

mg were dissolved in 85 μl of the same buffer before being applied to the column. The flow-rate was 0.75 ml/min. Fractions were collected, and freeze dried. The gel was calibrated with standard peptides under the same conditions.

Reversed-phase HPLC. Analyses of the peptidic fractions eluted from the TSK G2000SW column were performed by reversed-phase HPLC on an LKB ODS-120T-C_{18} column (300 mm × 7.8 mm I.D.). Elutions were accomplished with eluent A, 10 m*M* ammonium acetate pH 6.0, and eluent B, 50% (v/v) acetonitrile in eluent A. The gradient applied was 0–80% B in 80 min and 15 min reequilibration at 0% B between each analysis. The flow-rate was 1.5 ml/min. Samples were dissolved in eluent A (10 mg/ml) and 200 μl were injected. Each peak eluted from the column was collected manually.

Amino acid analysis

Amino acids were analysed using a Waters "Picotag Work Station". Peptides (0.1–10 μg) were hydrolysed by constant boiling HCl containing 1% phenol, for 24 h at 110°C. Precolumn derivatization of amino acids with phenyl isothiocyanate and HPLC separation of derivatized amino acids on a Waters RP-Picotag column (150 mm × 3.9 mm I.D.) were performed according to Bidlingmeyer *et al.*[24]. The wavelength was 254 nm and the flow-rate 1.0 ml/min.

Fast atom bombardment mass spectrometry

A Kratos MS 50 RF high resolution mass spectrometer equipped with a DS 90 (DGDG/30) data system was used. The mass spectrometer was operated at an 8-keV accelerating potential. An Ion Tech Model B 11 NF saddle field fast atom source energized with the B 50 current-regulated power supply was used with xenon as the bombarding atom (operating conditions: 7.3 kV, 1.2 mA). Peptides were dissolved in water (250 μg in 50 μl) and 1 μl of the solution was loaded on the copper tip with thioglycerol as a matrix. In this case the source housing was not heated. The mass range was scanned at 10 s/decade with a mass resolution of 3000. Caesium ionide was the standard for mass calibration.

Peptic haemoglobin hydrolysis

Decolorized bovine haemoglobin hydrolysate was obtained at pilot-plant scale by peptic proteolysis in an ultrafiltration reactor followed by decolorization with magnesia, desalting and atomization as described in ref. 22. The nitrogen content determined by the kjeldahl method allowed us to evaluate amounts of peptides in the hydrolysate greater than 90% (N × 6.25).

RESULTS AND DISCUSSION

Gel permeation HPLC separations

Fig. 1 shows the elution positions of the peptide standards for the TSK G2000SW column. The plot of the logarithms of their molecular weights as a function of the elution volumes is linear in the range from 800 to 2500 according to Richter *et al.*[18]. The gel-permeation HPLC analysis of 8.5 mg of the peptic digest of haemoglobin is shown in Fig. 2. Twenty elutions were performed and gave the same elution patterns. Peaks corresponding to fractions I–VII were collected and pooled. The

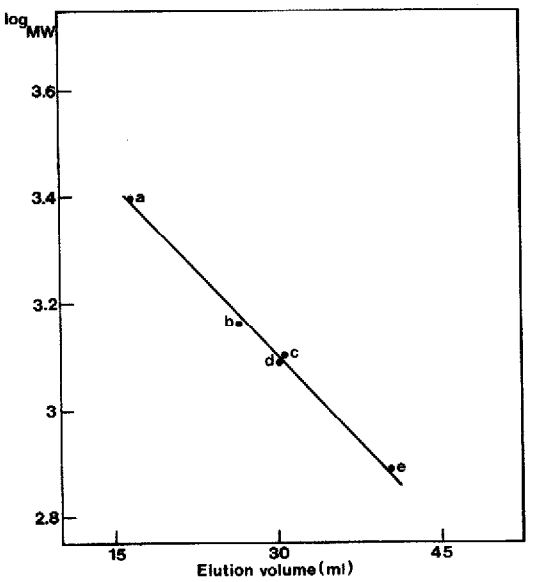

Fig. 1. Calibration plots of peptides using the TSK G2000SW column: (a) insulin A; (b) bacitracin; (c) actionomycin; (d) bradykinin; (e) Pz-Pro-Leu-Gly-Pro-Arg. Eluent: 10 m*M* ammonium acetate buffer (pH 6.0); flow-rate 0.75 ml/min. Detection: UV (215 nm).

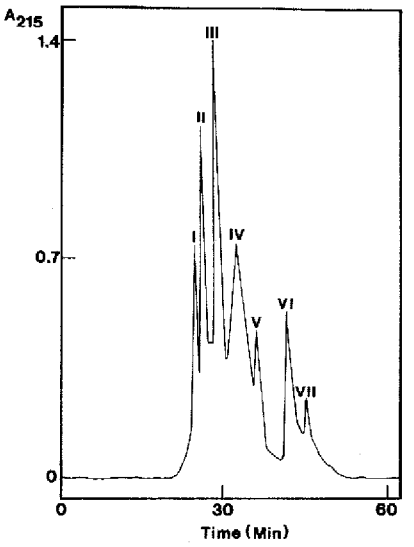

Fig. 2. Elution profile of total decolorized peptic hydrolysate, from bovine haemoglobin, using the TSK G2000SW column. Conditions as in Fig. 1.

TABLE I

APPARENT MOLECULAR WEIGHT DISTRIBUTION OF PEPTIDES SEPARATED BY TSK G2000SW AND QUANTITIES RECOVERED IN EACH FRACTION

	Fractions						
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*
TSK G2000 estimated MW	2200	2100	1950	1650	1430	1170	1050
Efficiency of recovery of peptides (%)	8.8	14.1	25.6	27.9	10.8	9.0	3.8

quantities of peptides recovered in each fraction and their estimated molecular weights, in regard to the calibration curve, are shown in Table I. The use of gel-permeation HPLC provides a good separation for this complex hydrolysate in comparison with a casein hydrolysate, for example[17]. Concerning the elution time, this technique is more effective than conventional low-pressure gel filtration often used for such hydrolysates analysis[25–27]. Therefore gel-permeation HPLC can be performed principally in order to obtain a good and rapid resolution of peptide fractions for further reversed-phase HPLC analysis, and for roughly assessing the reproducibility of the peptidic hydrolysate. Moreover studies have been undertaken in our laboratory in order to follow continuously, by gel-permeation HPLC, the evolution of the elution pattern of a protein hydrolysate, in an enzymatic ultrafiltration reactor at laboratory scale.

Reversed-phase HPLC

For the reversed-phase HPLC peptide mapping, a wide variety of buffers have been investigated in attempts to combine the desirable characteristics of UV transparency and volatility, and it appeared that ammonium acetate–acetonitrile gradient elution provides one of the most efficient systems due to its excellent volatility and the high solubility of many peptides in the buffer. The wavelength selected is 215 nm for both HPLC techniques because it corresponds to an isosbestic point at which the absorptions of random and helical peptide bonds are equal[28].

Whereas single reversed-phase HPLC of the total haemoglobin peptic hydrolysate is inadequate for a complete peptide separation (data not shown), the use of reversed-phase HPLC shows excellent separation of any fraction obtained by gel-permeation HPLC. Fig. 3a–g shows the elution profile of each of the seven fractions separated from the TSK G2000 column. Elutions with linear gradients give optimal separations of peptides having largely different hydrophobicities. With regard to tryptic peptides of haemoglobin[29], the peptic hydrolysate exhibits greater complexity. This is principally due to the lower selectivity of pepsin for peptide bond cleavage[30].

The objective of this publication is to demonstrate the use of FAB-MS to identify components separated by HPLC and gel filtration chromatography in the monitoring of an enzyme hydrolysis reaction. Because the protein is well known, it is not necessary completely to analyze all the components as separated by chromatogra-

phy, but merely to use the MS technique to verify the composition of selected chromatographic fractions to confirm that the hydrolysis is proceeding as expected. In this case, fractions II, III, V–VII have been arbitrarily selected for analysis of the amino amino acids and accurate molecular weight determination by FAB-MS as described in the text. One or two of the peptides have been collected at random in each fraction and named according to the process of isolation as follows: II_8 for fraction II, III_3 and III_{21} for fraction III, V_{16} for fraction V, VI_4 and VI_6 for fraction VI, VII_4 and VII_6 for fraction VII.

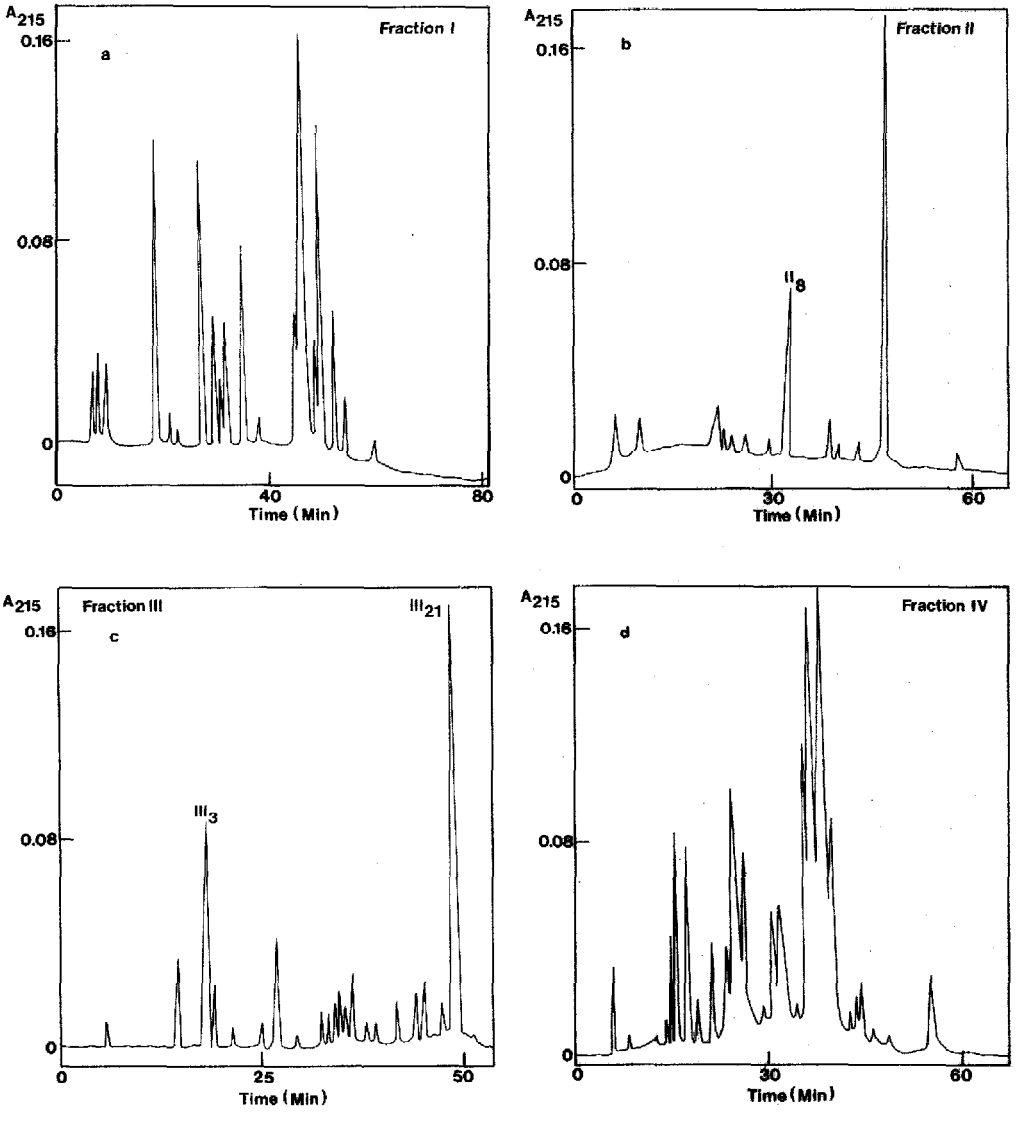

Fig. 3.

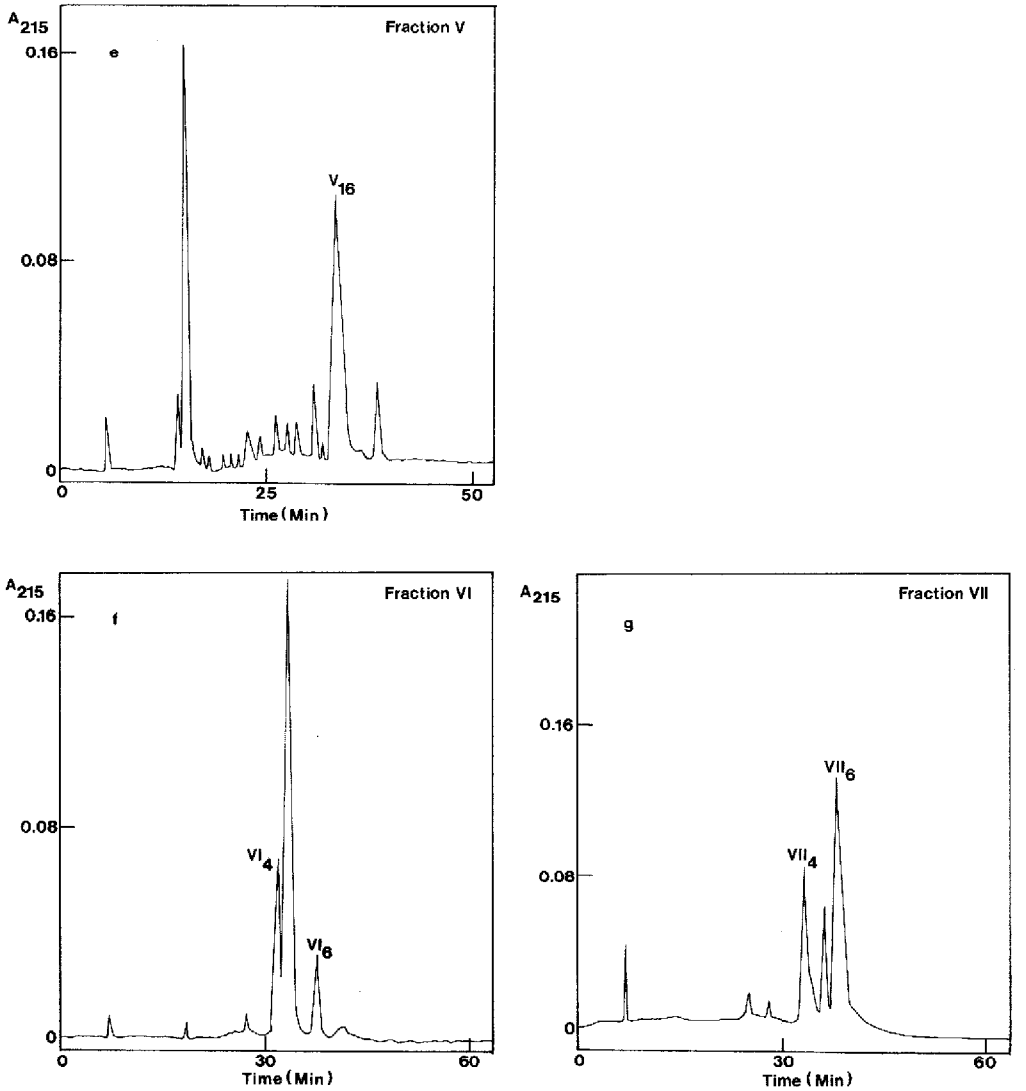

Fig. 3. (a)–(g) Elution profiles of each of the seven peptidic fractions separated from the TSK G2000SW column (Fig. 2). Conditions: column ODS-120 T-C 18; gradient, (A) 10 m*M* ammonium acetate (pH 6.0), (B) 50% (v/v) acetonitrile in eluent A, 0–80% B in 80 min; flow-rate 1.5 ml/min; detection, UV (215 nm).

Amino acid analysis and mass spectrometry

The amino acid compositions and deduced molecular weights of the eight peptides isolated are shown in Table II. Furthermore, owing to their amino acid composition, the peptides can be located in the well known globin sequence (Fig. 4). These data indicate a reasonable purity of these peptides. In Fig. 4 parentheses are used to enclose regions of the globin chain for which the sequence of amino acids has not been established but has been inferred from the amino acid composition by homology

TABLE II

MOLECULAR WEIGHTS OF THE EIGHT PEPTIDES ISOLATED DEDUCED FROM AMINO ACID COMPOSITION AND FROM MASS SPECTROMETRY

N.D. = Not detected.

Amino acid	II_8	III_3	III_{21}	V_{16}	V_4	VI_6	VII_4	VII_6
Asp		1.23(1)	1.33(1)	0.61(1)				
Glu	1.17(1)					1.17(1)		
Ser			2.74(3)					0.83(1)
Gly	1.04(1)			1.07(1)				
His			1.89(2)		0.97(1)		1.04(1)	1.07(1)
Arg						1.06(1)		
Thr			0.86(1)		2.82(3)	1.19(1)	2.60(3)	2.51(3)
Ala	1.76(2)		2.57(3)					
Pro			1.95(2)		2.04(2)	1.27(1)	1.96(2)	1.94(2)
Tyr	0.88(1)				1.03(1)	1.22(1)	0.96(1)	0.92(1)
Val			0.99(1)	1.00(1)		1.52(2)		
Met								
Cys								
Ile								
Leu	0.90(1)		1.83(2)	2.86(3)		0.93(1)		1.16(1)
Phe		0.91(1)	1.01(1)	0.93(1)	1.95(2)	1.24(1)	1.78(2)	2.72(3)
Lys		0.86(1)		1.42(1)	1.30(1)		1.21(1)	0.78(1)
Trp						N.D.(1)		
Deduced MW	622	408	1648	902	1237	1307	1237	1585
Mass spectrometry deduced MW	622	408	1648	902	1237	1307	1237	1585

with known sequences[31]. It seems that some peptides are broken down to others, *e.g.*, VII_6 into VII_4 (Fig. 4). Moreover, the same peptide can be eluted in different fractions during gel-permeation HPLC, *e.g.*, VII_4 to VI_4.

FAB-MS is particularly suitable for peptide analysis because it is capable of analyzing polar and ionic compounds without chemical derivatization. Moreover, it offers the additional advantage of being performed on liquid samples[32]. An highly viscous solvent (thioglycerol) is used in order to keep the sample in the liquid state during insertion into the high-vacuum source of the instrument and throughout the analysis.

As an example, Fig. 5 is the FAB-MS spectrum of a selected peptide (V_{16}). Accurate molecular weights of the eight peptides selected, determined by FAB-MS, are shown in Table II. The use of a FAB ion source in a mass spectrometer allows the m/z values of $(M+H)^+$ or $(M-H)^-$ ions of a peptide to be detected[33]. In our study only the $(M-H)^-$ ions are detected. The molecular weight of any peptide deduced from amino acid composition is rigorously the same as that determined mass spectrometrically. This indicates unambiguously the purity of the peptides isolated.

Important differences, between the accurate molecular weights of the peptides (Table II) determined by FAB-MS and those determined by gel-permeation HPLC (Table I), can be observed. So we can emphasize the failure of gel-permeation HPLC

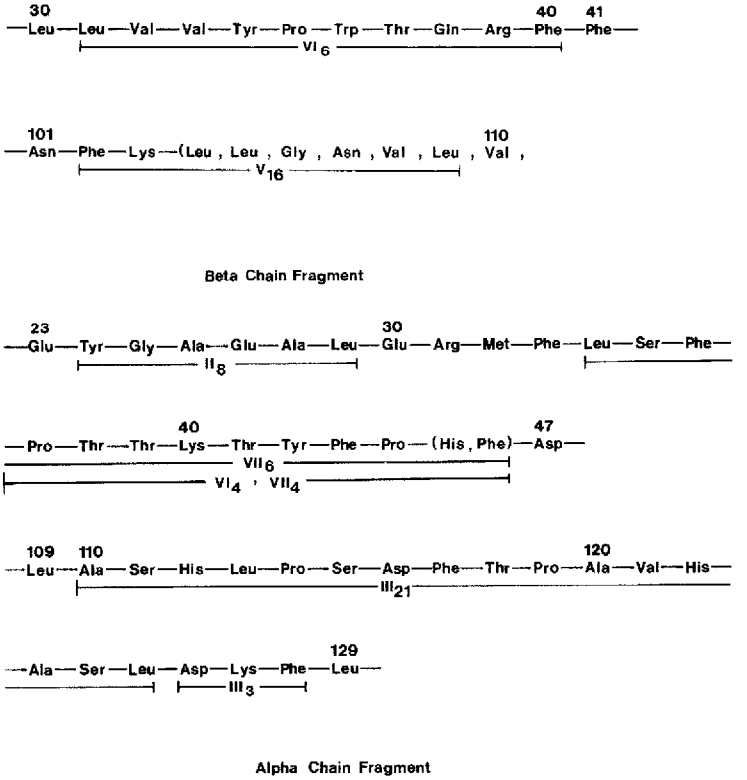

Fig. 4. Location of isolated peptides in the primary structure of bovine haemoglobin (α and β chain fragments).

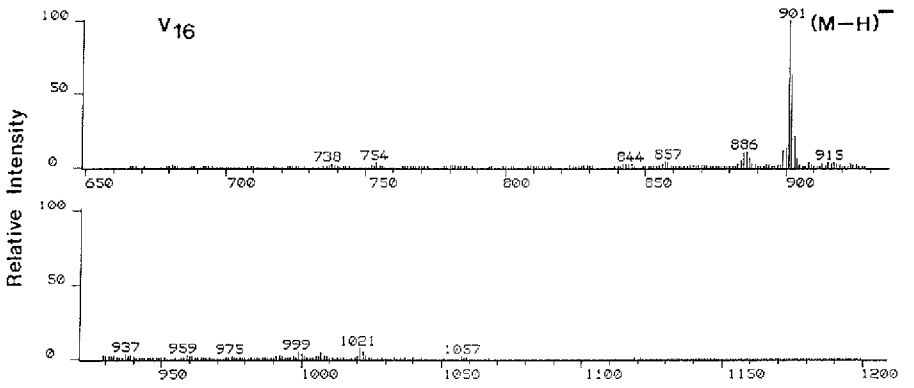

Fig. 5. Negative FAB-MS spectrum of peptide V_{16}. Experimental conditions are given in the text.

to give accurate molecular weights of peptides from such a complex enzymatic hydrolysate. However, in regard to this kind of complex peptidic population from a pilot-plant scale process, the combination of gel-permeation HPLC with reversed-phase HPLC has proven effective for a rapid fractionation and purification of peptides.

FAB-MS, coupled with amino acid analysis and those two simple HPLC methods, seems to be the best choice for determining the accurate composition of such a mixture. Thus, FAB-MS can be efficiently used for some peptides shown, by preliminary experiments, to be active for specific applications in areas such as cell culture or pharmacology. Tandem mass spectrometry (MS–MS) may be a powerful tool for sequencing these peptides[20]. Moreover, owing to the scale of hydrolysate production and its reproducibility[22], preparative gel-permeation HPLC and reversed-phase HPLC can be considered for such peptides.

Recently HPLC techniques have been combined with FAB-MS for the mass specific detection of mixtures of peptides produced by proteolytic hydrolysis of proteins in batch[32]. In the field of enzymatic ultrafiltration reactors, similar studies can be undertaken in order to get a better knowledge of the hydrolysis mechanism.

In conclusion, the results of this investigation show that HPLC and FAB-MS can be used to identify peptides from a complex haemoglobin hydrolysate produced at pilot-plant scale. FAB-MS can provide reliable molecular weight information by direct off-line analysis of HPLC fractions. This procedure should improve the technology of quality control for monitoring the enzymatic hydrolysis of proteins.

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