MICROSEQUENCE ANALYSIS: I. PEPTIDE ISOLATION USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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1. Introduction

Peptide mixtures, arising from such diverse sources as tissue extracts, urine or proteolysis, are usually fractionated and characterized by a combination of ion-exchange chromatography, gel-exclusion chromatography and/or electrophoresis. Appropriate combinations of these methods will normally yield a homogeneous product, but their costs, detection limits, analyses times and yields vary. The recently introduced technique of high-performance liquid chromatography is now being adapted to peptide–protein fractionation by numerous laboratories. The great versatility of this technique rests in the various column materials available for normal-phase adsorption (silica), reverse-phase adsorption (hydrocarbon bonded to silica) or ion exchange (ionic groups bonded to silica or polystyrene).

To date proteins and large peptide fragments have been separated using both ion exchange (CM-glycophase/CPG [1] and DEAE-glycophase/CPG [2]) and molecular or steric exclusion supports (glycophase G/CPG [1,2]). Smaller peptides resulting from enzymatic hydrolyses [1,3–7], solid-phase syntheses [8] and tissue extractions [5,6,9,10] have been chromatographed and/or isolated using reverse phase C18 columns and either isocratic or gradient elution systems. The detection systems, in most cases absorbance, limit both the buffer systems which can be employed and also the sensitivity. These restrictions can largely be overcome by diverting a portion of the eluant to a 'peptide' analyzer and reacting the primary amines with ninhydrin [11], OPA [12] or fluorescamine [13].

This article reports the use of HPLC for peptide isolation utilizing reverse phase columns. The flexibility and reliability of the system derives from the use of microprocessor-controlled pumps for gradient formation and a peptide-detection system based on fluorescamine or OPA. Protein digests of <100 nmol, where 100 pmol is required for detection, yield fragments which can then be readily sequenced by microsequence analysis on either solid- [14] or liquid-phase [15] instruments.

Abbreviations: DABITC, 4-*N*,*N*-dimethylaminoazobenzene 4'-isothiocyanate; HPLC, high-performance liquid chromatography; OPA, *O*-phthaldialdehyde

2. Materials and methods

The apparatus, for the analytical or preparative separation of peptides, consists of an HPLC system connected via a sampling valve to a fluorescence detection system. Two Altex pumps (model 110), controlled by an Altex microprocessor (model 420), delivered the gradient for column elution. Sample injection was via a Rheodyne injector (model 70 with a 500 μl sample loop). The sampling and detection systems have been detailed in [13]. Basically, the sampling system consists of a valve which, via an electronically controlled pneumatic system and a transfer solution (water; flowrate 14 ml/h), removes a predeterminable aliquot of the eluant (0–100%). This aliquot is then mixed with a buffer and a fluorogenic reagent (detailed below), the resulting fluorescence measured (Gilson Spectra/G10 Fluorometer

equipped with a standard flowthrough 65 μl cell, and fluorescamine excitation–emission filters) and recorded (W + W Recorder, model 312). The solution compositions and their flowrates used in this investigation, were for fluorescamine detection, borate buffer (0.6 M borate titrated to pH 9.6 with saturated LiOH and back to pH 9.4 with acetic anhydride, 2.5 ml; flowrate 28 ml/h) and the fluorescamine (Roche) solution (20 mg/100 ml in acetone; flowrate 14 ml/h). The single mixture necessary for OPA detection was a 0.6 M borate solution titrated to pH 10.3 with saturated LiOH prior to the addition of 2 ml acetic anhydride, 1 ml β-mercaptoethanol, 2 ml 30% Brij 35 (Pierce) and 3 ml methanol containing 200 mg OPA (Sigma); flowrate 28 ml/h.

Goldfish and human hemoglobins were prepared from freshly collected blood. The erythrocytes were washed 3 times in ice-cold saline, then lysed hypotonically. Globin was obtained by the normal acetone/HCl procedure. Chain separation of goldfish globin was achieved by chromatography on Sephacryl S200 (30% acetic acid) following denaturation (8 M urea, pH 8.6) and β-chain disulfide polymer formation (oxidized in presence of dehydroascorbic acid) [16]; for human globin chains by standard procedures [17]. Aminoethylation [18], cyanogen bromide fragmentation [19], citraconylation [20] and maleylation [21] were performed as indicated. Tryptic digestions (Sigma type XI, TPCK-treated enzyme) were done in 0.2 M *N*-ethylmorpholine acetate (pH 8.0) using a 2% (w/w) protease ratio at 37°C and terminated by diluting into the starting buffer for the chromatographic separation.

All glassware which came in contact with any of the solutions, for either the buffer or detection systems or the buffer–sample eluant following chromatography, was thoroughly washed in RBS-35 detergent (Fluka), rinsed in distilled water and oven-dried. Solutions were maintained in dark glass bottles and either kept tightly closed or covered with aluminium foil. Acetic anhydride (0.2%) was included in all solutions; no smoking was allowed in the laboratory. The chemicals used, unless otherwise noted, were analytical grade from Fluka or Merck. Acetic acid, formic acid, 1-propanol and pyridine were each distilled over ninhydrin just prior to use. Amino acid analyses were performed on either a Durrum D-500 or a Liquimat III analyzer following 6 N HCl hydrolysis.

3. Results and discussion

In primary structure analyses the isolation of peptides resulting from chemical or enzymatic cleavage has generally been a time consuming and tedious task at the micro-level (<50 nmol). Numerous authors [1–11] have reported the use of HPLC in peptide–protein isolation. However, a major limitation of this technique in microsequence analysis has been the lack of an adequate detection system. The advantages or disadvantages of using fluorogenic reagents over ninhydrin, e.g., higher sensitivity [22], or either fluorescamine or OPA [23] for monitoring column eluants have also been the topics of various reports. However, conditions employed in such studies were often not optimal for the respective fluorogenic reagent, e.g., buffer system, presence of reducing agent and its concentration, presence of a detergent and its concentration, and temperature. Figure 1 illustrates the presently achieved chromatographic separation of the tryptic peptides from the α-chain of goldfish hemoglobin and compares their detection with either fluorescamine (fig.1A,B) or OPA (fig.1C). As noted [24] peaks detectable with fluorescamine are not necessarily detectable with OPA; similarly, their relative fluorescence yields are not necessarily comparable.

Analyses of peptides obtained from a preparative isolation (fig.1A) at the 100 nmol level have shown, under conditions used for OPA detection, that peptides must contain the amino-group of lysine, consequently only fluorescamine has been utilized as the fluorogenic reagent. The reproducibility of the separation is shown in fig.1A,B. Chromatograms of 1 and 100 nmol of tryptic peptides are identical, thus optimal conditions worked out with <1 nmol of digested polypeptide can be used with confidence for the separation of preparative amounts of material (10–100 nmol). Overall yields as judged by amino acid analyses of the isolated peptides were between 30–70%. In this case, however, no attempt was made to determine the extent of the trypsin digestion.

Sequence analyses of >30 residues are readily obtained by the use of the modified Edman reagent DABITC in both the solid- and liquid-phase sequencers at the 5 and 20 nmol levels, respectively [14,15]. It is therefore desirable to isolate large peptides, and examples of chromatograms of peptides obtained

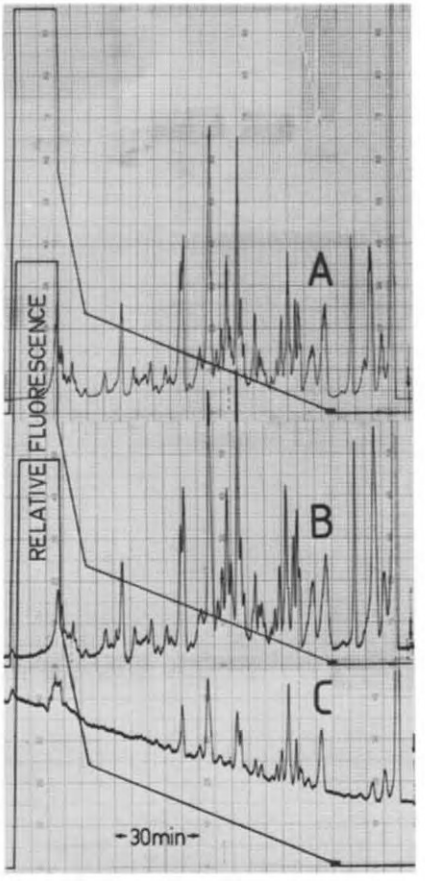

Fig.1. HPLC separations of the tryptic peptides from α-chain of goldfish hemoglobin: (A) 100 nmol material, 2% analyzed with fluorescamine; (B) 1 nmol material, 10% analyzed with fluorescamine; (C) 1 nmol material, 10% analyzed with OPA. Chromatography was on a LiChrosorb RP 18 (10 μm particle size) column (4.6 × 250 mm) developed with a gradient (dark line) at 0.7 ml/min using as buffers (*A*) 0.5 M pyridine formate (pH 3.0) and (*B*) 1.0 M pyridine acetate (pH 5.5), 60% (v/v) 1-propanol. See section 2 for description of the detection system.

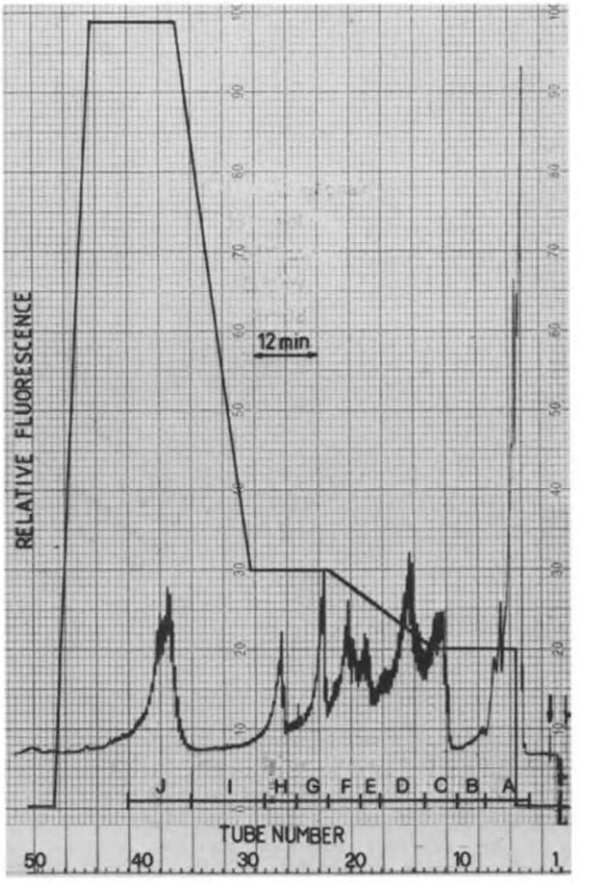

Fig.2. HPLC separation of the tryptic peptides from amino-ethylated and maleylated β-chain of goldfish hemoglobin. Chromatography was on a LiChrosorb RP 8 (10 μm particle size) column (4.6 × 250 mm) developed with a gradient (dark line) at 0.7 ml/min with the buffers (*A*) 1.0 M pyridine acetate (pH 5.5) and (*B*) 1.0 M pyridine acetate (pH 5.5), 60% (v/v) 1-propanol. 120 nmol digest applied and 1.7% of the eluant removed for detection with fluorescamine (see section 2).

from limited tryptic digest, or cyanogen bromide cleavage, of both goldfish β- and human α-chains, are shown in fig.2–4.

The separation of maleylated peptides from limited tryptic digestion of goldfish β-globin was performed with an equilibrium eluant at pH 5.5. At this pH all peptides were soluble (100 nmol/500 μl). Overall yields were 70% based on arginine content, and 3 peptides could, on the basis of amino acid analyses, be identified by comparison with the reported sequence of β-globin from carp [16]. The amino-terminal peptide, having all amino groups blocked by maleic anhydride produced no fluorescence, however from amino acid analysis of fraction B the peptide was obtained in 85% yield. The separation of modified peptides is often a useful technique when problems of solubility are encountered. However in view of the small quantity of peptide needed for sequence analysis

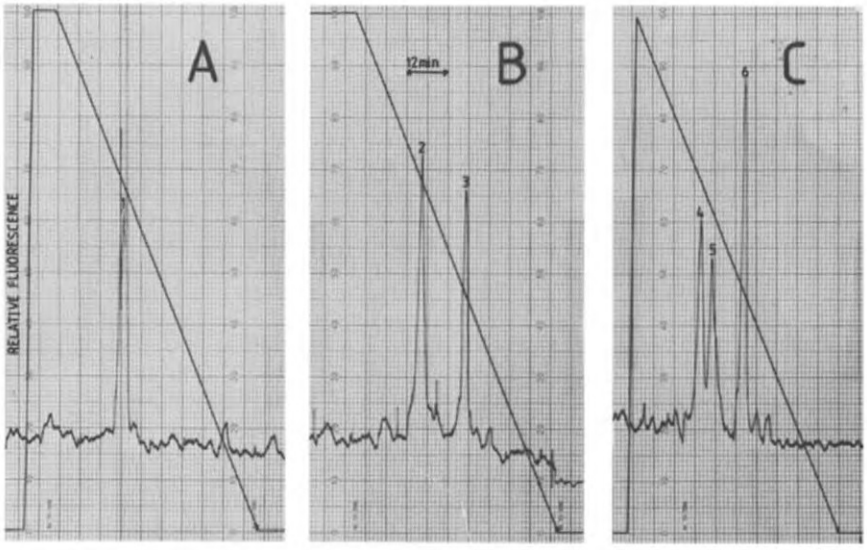

Fig.3. Timed tryptic digestions of the citraconylated and aminoethylated α-chain of human hemoglobin. The conditions for chromatography on a LiChrosorb RP 18 column were as in fig.1. Trypsin (1% by wt) was added to 106 nmol α-chain derivative and the digestion performed at room temperature. At 0 min (A), 2 min (B) and 25 min (C), 1% (1.06 nmol material) was chromatographed and 10% of the eluant removed for detection with fluorescamine (see section 2).

(<20 nmol) and the relative large volumes for injection (0.5–1.0 ml) insolubility in this procedure is less likely to be encountered than when working with material at higher concentration.

The differential rates of polypeptide bond cleavage by either enzymatic or chemical agents can be utilized to generate fragments not normally obtained by extended times of reaction. An example of the optimization of conditions for limited tryptic digestion, resulting in large fragments, is shown in fig.3 for citraconylated, aminoethylated human α-globin.

The speed and sensitivity of this system, in combination with micro-sequencing using DABITC, is illustrated by the separation (fig.4) and subsequent sequence determinations on peptides obtained from the cyanogen bromide cleavage of 53 nmol of human α-globin. The yield and composition of each fragment are shown in table 1 and the sequence determinations

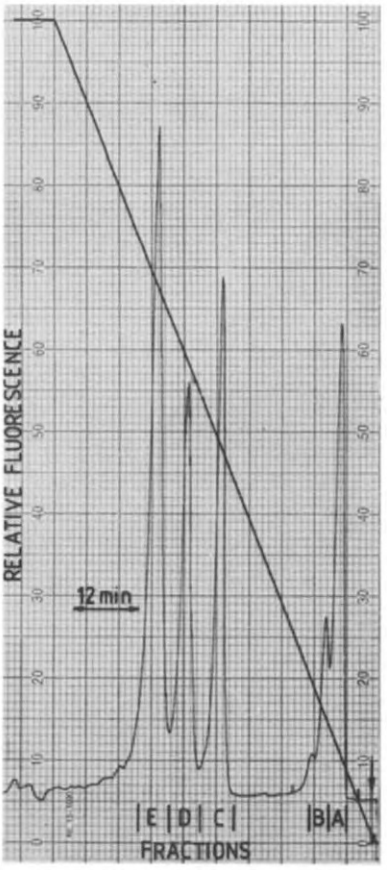

Fig.4. Separation of the CNBr peptides from aminoethylated α-chain of human hemoglobin. The material (53 nmol) was chromatographed on a LiChrosorb RP18 column as in fig.2 and 5% of the eluant removed for detection with fluorescamine (see section 2).

Table 1
Amino acid compositions and yields of the CNBr fragments from human α-globin[a]

	Pool C	Pool D	Pool E	α-Globin
Lysine	2.7 (3)	3.6 (4)	3.0 (4)	10.5 (11)
Histidine	0.9 (1)	3.2 (4)	4.3 (5)	10.1 (10)
Arginine	0.6 (1)	0 (0)	1.4 (2)	2.5 (3)
Aspartic acid	2.1 (2)	4.5 (5)	5.0 (5)	12.6 (12)
Threonine[b]	1.0 (1)	3.3 (4)	4.0 (4)	9.9 (9)
Serine[b]	1.1 (1)	2.4 (3)	5.0 (7)	10.7 (11)
Glutamic acid	2.9 (3)	1.4 (1)	1.1 (1)	6.0 (5)
Proline	0.9 (1)	1.7 (2)	3.2 (4)	7.6 (7)
Glycine	3.8 (4)	3.5 (3)	1.1 (1)	7.8 (7)
Alanine	6.3 (7)	5.3 (5)	8.4 (9)	22.1 (21)
Cysteine[c]	0 (0)	0 (0)	1.0 (1)	0.9 (1)
Valine	2.5 (3)	3.6 (4)	5.8 (6)	12.6 (13)
Methionine[d]	1.0 (1)	0.9 (1)	0 (0)	1.9 (2)
Isoleucine	0 (0)	0 (0)	0 (0)	0 (0)
Leucine	1.9 (2)	2.9 (3)	10.9 (13)	17.1 (18)
Tyrosine	1.1 (1)	0.9 (1)	1.0 (1)	2.7 (3)
Phenylalanine	0 (0)	3.0 (4)	3.0 (3)	6.4 (7)
Tryptophan[e]	1.0 (1)	0 (0)	0 (0)	0.9 (1)
% recovery	58.7	69.8	102.4	–

[a] Pools C, D and E represent those from fig.4
[b] Corrected for 9% (Thr) and 7% (Ser) destruction during hydrolysis
[c] Detected as aminoethylcysteine; calculated in pool C using the tyrosine value and in α-globin as 33% of arginine
[d] Detected as homoserine and homoserine lactone; calculated in pools A and B using the tyrosine value and in α-globin as 66% of arginine
[e] Calculated in pool A using the tyrosine value and in α-globin as 33% of arginine

of fractions C and D by liquid- [15] and solid-phase sequencers [14] are illustrated in the accompanying communications [14,15]. We wish to emphasize that peptide separation was obtained in <1 h and the resulting fractions (<4 ml) were dried in <1 h by vortex evaporation. The techniques and instruments described here therefore provide rapid and sensitive methods for the isolation of and subsequent sequence analyses of peptides and/or proteins available in limited quantities.

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