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PEPTIDE SEPARATIONS USING FLUORESCENCE DETECTION

ERNEST H. CREASER and GRAHAM J. HUGHES
Protein Biochemistry Unit, Research School of Biological Sciences, Australian National University, Canberra (Australia)
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SUMMARY

The formation of fluorophores by the action of *o*-phthalaldehyde with amino acids and peptides has provided a highly sensitive assay for these compounds. A relatively simple system for the analysis and separation of peptides, in the range 5 nmole to 10 μmole, normally derived from enzymic digestion of proteins, is described.

The system comprises a gradient-generating device feeding volatile pyridine buffers via a pump to a column of cation-exchange resin. Eluate from the column is fed through a proportioning pump to a fluorocolorimeter, output from which is displayed on a recorder. For analytical runs the eluate is mixed with *o*-phthalaldehyde in borate buffer containing Brij 35 and 2-mercaptoethanol prior to its passage into the detector. For preparative work the eluate stream is split, one reacting with *o*-phthalaldehyde, the other for collection.

Results on the analysis and preparation of tryptic peptides derived from cytochrome *c* and Salmonella histidinol dehydrogenase are discussed.

INTRODUCTION

Despite recent advances in liquid and solid phase automatic sequencing there is no methodology available to completely sequence entire proteins of molecular weight greater than 10,000. It is still necessary, therefore, to break the molecule into peptides of convenient sizes for complete analysis. The separation and detection of such peptides, derived from digestion with trypsin, is described in this paper.

In recent years two fluorogenic agents have been used for the detection of peptides, which are simpler in use and allow for a greater sensitivity than the previously used ninhydrin method. Such procedures enable sequencing work to be feasible on increasingly smaller quantities of proteins and open the way to structural studies on proteins whose restricted availability has hitherto precluded their sequence determination. These reagents are fluorescamine (4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione)[1] and OPA (*o*-phthalaldehyde)[2]. Fluorescamine has been used as a spray reagent for peptide detection on thin-layer chromatograms[3], but OPA seems to be the reagent of choice for peptide detection in eluates from ion-exchange chromatography[4]. The reagent was combined with high-pressure microbore ion-exchange chromatography

to yield tryptic peptide maps from microgram quantities of proteins[4]. We describe a relatively simple apparatus using OPA detection. A small-scale separation is performed initially as either an analytical peptide map at the 5–10-nmole level or a scale model to optimize elution conditions for larger-scale preparative peptide separation of up to 10 μmoles, using variable split stream eluate detection and collection. Volatile pyridine–acetic acid buffers are used throughout. We have developed the methodology for our studies on the evolution of cytochromes *c* of Australian animals and on bacterial dehydrogenases.

EXPERIMENTAL

All chemicals used were of analytical-reagent grade.

Buffered OPA reagent

Boric acid (247 g) plus KOH (200 g) were dissolved in distilled water and made up to 4 l. This solution was 1.0 *M* in borate and the pH was 10.62. OPA was obtained from Durrum Chemicals (Palo Alto, Calif., U.S.A.) or Sigma (St. Louis, Mo., U.S.A.) and was normaliy used at 1 g/l of buffer. The required amount of OPA was weighed out and dissolved in 10 ml methanol; then this solution was added to the borate buffer. Before use 1 ml/l of 2-mercaptoethanol and 2 ml/l of a 30% Brij 35 solution (Pierce, Rockford, Ill., U.S.A.) were added and thoroughly mixed.

Elution buffers

Pyridine–acetic acid was used. Limit buffers were 0.2 *M* pyridine adjusted to pH 3.1 with glacial acetic acid and 2.0 *M* pyridine adjusted to pH 5.0 with glacial acetic acid. The composition of the pH 3.1 buffer was: 16.1 ml pyridine, 278.5 ml glacial acetic acid, distilled water to 1000 ml; that of the pH 5.0 buffer: 161 ml pyridine, 143 ml glacial acetic acid, water to 1000 ml. Buffers of intermediate concentration were made up by mixing appropriate quantities of each limit buffer.

Proteins

Trypsin was TPKC tfeated Worthington (Freehold, N. J., U.S.A.) Batch 35K940. For digests a 1/50 weight ratio of trypsin to protein, which was in 0.25% ammonium hydrogen carbonate at a concentration of 10 mg/ml, was incubated for 24 h at 37°. Cytochrome *c* was Sigma Horse Heart Type III. Crystalline histidinol dehydrogenase was prepared by a published method[5] and was reduced and carboxymethylated by a reported procedure[6].

Apparatus

The apparatus employed consists of the following components:

(1) Gradient-forming device. We use a very simple gradient generator, two vessels of the same diameter in hydrostatic equilibrium. The concentrated solution flows into the mixing vessel from whence it is pumped via PTFE tubing. To date we have found that linear gradients are quite satisfactory.

(2) Buffer pump. A Beckman Accu-Flo positive displacement pump was available but any similar pump would suffice, the maximum pressure generated in the apparatus was *ca.* 600 p.s.i. and the minimum flow-rate was 6.0 ml/h.

(3) Chromatography columns. We used a range of columns depending on the amount and complexity of the peptide mixture to be fractionated: two microbore columns (Altex Scientific, Berkeley, Calif., U.S.A.), a 100 × 3 mm and a 200 × 3 mm; and two 9-mm diameter columns, 570 mm and 200 mm long (Beckman, Fullerton, Calif., U.S.A.). All columns employed could be operated up to 500 p.s.i. and were thermostated at 50°. Beckman PA35 cation-exchange resin was employed in this work.

(4) A split stream device was a simple T piece, which split the column eluate into a part for detection and a part that was collected in a fraction collector.

(5) A Technicon proportioning pump was used to pump the fraction of the split stream that was to be assayed and the fluorophore mixture. These two streams were united by a simple glass T piece and the combined column eluate and fluorophore buffer mixture passed to the fluorescence detector. A coil of PTFE tubing provides a 1-min delay to allow mixing. After detection the mixture was pumped to waste by the Technicon pump.

(6) Fluorescence detector. We used an Aminco fluorocolorimeter (4-7439). The excitation filter was Corning 7-51 and the emission filter was a Wratten 2A. The stream was passed through a de-bubbler detection cell (Aminco P/N 4-7393) and then went back to the proportioning pump. The electronic output from the detector was displayed on a suitable recorder; the detector output can be varied to match any recorder up to 100 mV full scale deflection.

RESULTS AND DISCUSSION

In early experiments we used fluorescamine as the fluorophore. We discontinued the use of this compound a sit is soluble in acetone but not in aqueous buffers. Fluorescamine, therefore, required the use of a third stream in the flow system, and it was also essential to use silicone tubing, which had a very short life, to pump acetone solutions. In addition, fluorescamine gave less yield of fluorescence and was more expensive[7].

The sensitivity of the OPA reaction and its detection by the fluorocolorimeter is very much greater than is necessary. Accordingly we used the fluorocolorimeter at its least sensitive setting (× 100) and fed the data to a 5-mV recorder. Under these conditions we observed a 7Q% f.s.d. with 2 nmoles norleucine and we could obtain a satisfactory peptide map with 5–10 nmoles (130 μg) of cytochrome *c*. No special precautions were taken to remove either ammonia- or OPA-reacting materials from the distilled water. Solvents for the eluting buffers were used as purchased from the manufacturers. We have not found the need to go to higher sensitivities than mentioned above.

Normally, we ran initially a 5–10-nmole sample of peptide mixture on the smallest column (100 mm × 3 mm) using a linear gradient of 30 ml pH 3.1 pyridine–acetic acid plus 30 ml pH 5.0 buffer. The column was eluted at a flow-rate of 6 ml/h (*i.e.* a 10-h run) and the tube in the proportioning pump was selected to sample at this rate (orange/green tube). In practice the flow-rate of the column pump was adjusted to be just slightly greater than the sample taken by the proportioning pump. The fluorophore buffer mixture was pumped at 101.4 ml/h in (green/green) Solvaflex tubing. This ratio of fluorophore to sample of *ca.* 17 was essential to bring the pH of

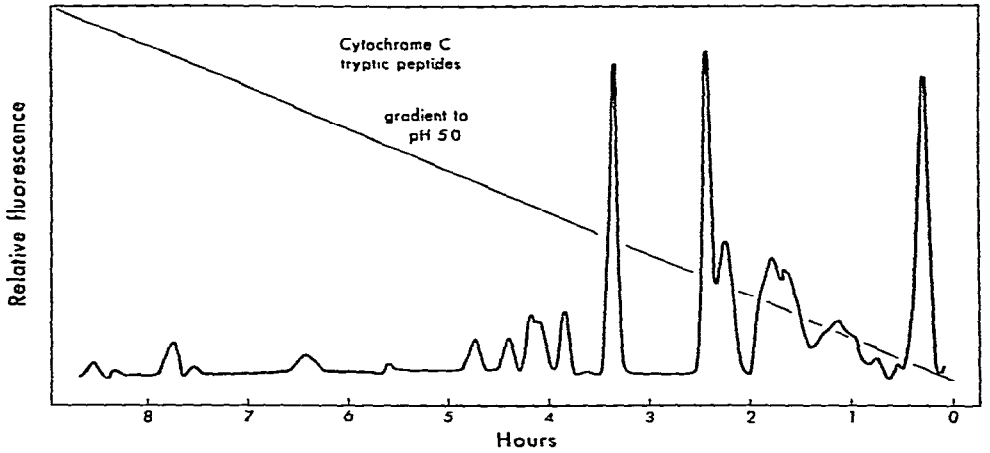

Fig. 1. Chromatography of a tryptic digest of cytochrome *c*. Five nanomoles of digest were applied to the column (100 × 3 mm) and eluted with a linear gradient, comprising of 30 ml of 0.2 *M* pyridine–acetic acid buffer, pH 3.1, and 30 ml of 2 *M* pyridine–acetic acid pH 5.0, as indicated in the elution diagram. The flow-rate was 6 ml/h and the entire column eluate was utilized for detection.

the mixture up to the pH range of fluorescence. Any significant reduction of this ratio resulted in insufficient pH elevation to permit detectable fluorescence. When using split stream operation and taking less samples for assay, the fluorophore-to-mixture ratio can be proportionately reduced. A chromatogram using 5 nmoles of tryptic peptides from cytochrome *c* is shown in Fig. 1. We used replicas of this chromatogram to establish three points. First, that the concentration of OPA was saturating; secondly, that if mercaptoethanol was omitted, there was very little reaction of OPA with the peptides; and thirdly, that when Brij 35 was omitted, several of the peptide peaks were much reduced in size. These latter observations are in agreement with

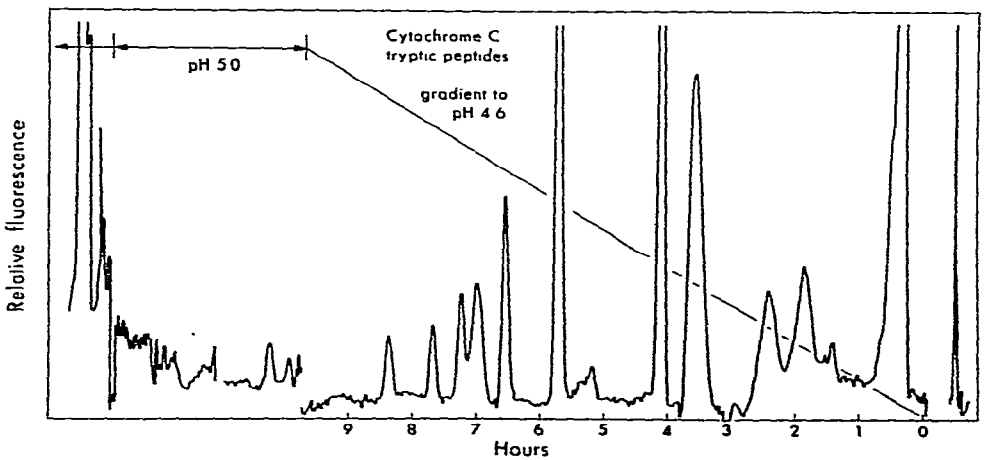

Fig. 2. Elution diagram of a tryptic digest of cytochrome *c* (50 nmoles) applied to a 200 × 3 mm column and eluted with a linear gradient comprising 30 ml each of pH 3.1 and pH 4.6 pyridine–acetic acid buffers. As indicated in the diagram, elution with 2.0 *M* buffer was commenced after 9 h. The flow-rate was 6 ml/h and the entire column eluate was passed through the detection system.

previous work[8] that the fluorescence yield of lysine peptides is enhanced by Brij 35. Presumably, the peaks whose intensities were not affected by the presence of Brij were due to peptides with arginine as C terminal.

The most valuable result obtained from operation of the small column was to gain information to optimize the elution conditions to allow separation of as many peptides as possible. This is shown in the next two sections.

The optimal separation of tryptic peptides from cytochrome *c* proved quite simple. From Fig. 1 it can be deduced that the gradient was far longer than necessary, and so it was reduced in slope by a factor of two for the next run; also as early peaks were a little crowded a longer column was used. In Fig. 2 the results of these two changes are shown. Nearly all of the possible 17 peptides were completely separated. Quantities of 50 nmoles were used to detect minor components. Preparative separations were performed by scaling up the Fig. 2 experiment by a factor of about 10. A 200 × 9 mm column was used, at a flow-rate of 60 ml/h. The amount of eluate taken for detection corresponded to *ca.* 50 nmoles. We have separated up to 125 mg of horse heart cytochrome *c* (10 μmoles) observing a peptide elution pattern that was identical with that seen in Fig. 2.

The histidinol dehydrogenase separation was more difficult. From the amino acid composition one would expect 33 soluble peptides in a tryptic digest of the reduced carboxymethylated protein[9]. The initial chromatographic separation of 10 nmoles was performed on a 200 mm column; however, this resulted in poor separation, as shown in the early part of Fig. 3. Elution with pH 3.1 buffer for 2 h followed by a gradient reduced to one half the volume (see Fig. 4) resulted in a greater separa-

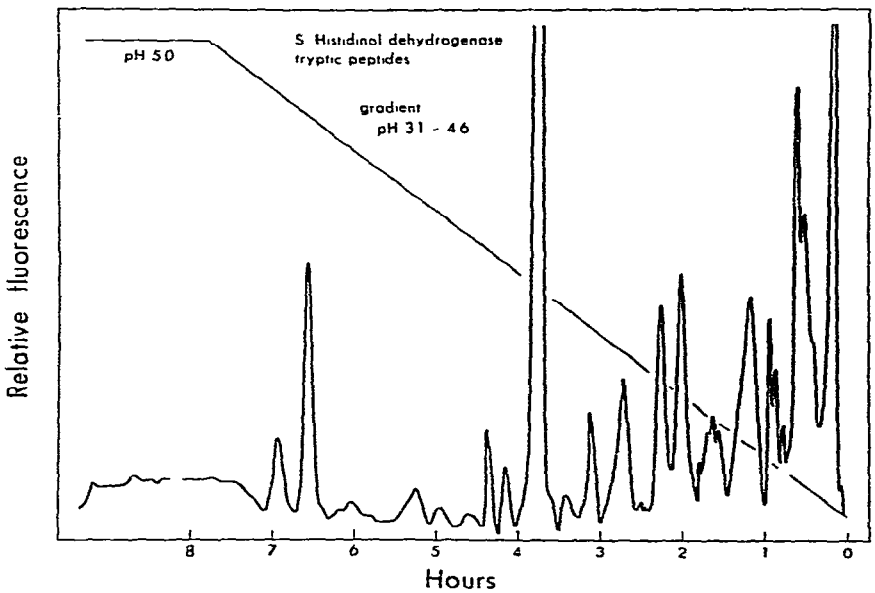

Fig. 3. Analysis of tryptic peptides from Salmonella reduced carboxymethylated histidinol dehydrogenase. Ten nanomoles of digest were applied to the column (200 × 3 mm) and eluted, at a flow-rate of 7 ml/h with a linear gradient comprising of 30 ml each of pH 3.1 and pH 4.6 buffers. After 7.5 h the gradient was replaced by 2 *M* pyridine–acetic acid pH 5.0.

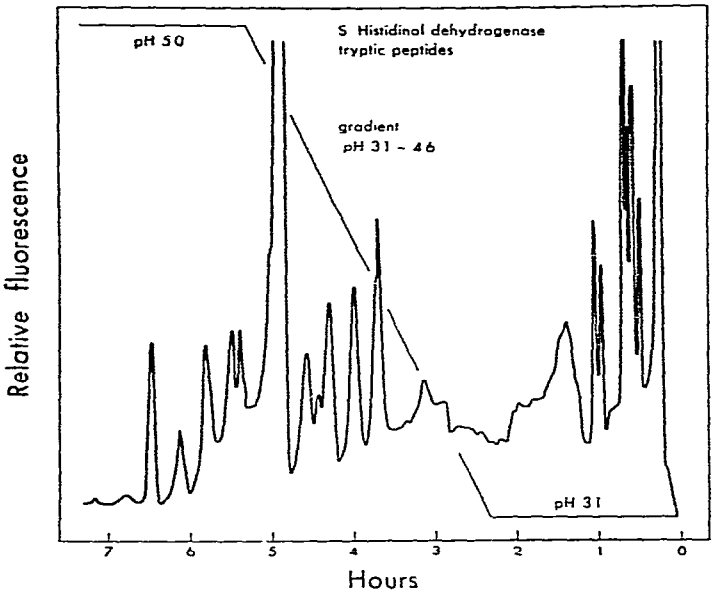

Fig. 4. Analysis of tryptic peptides from Salmonella reduced carboxymethylated histidinol dehydrogenase using a modified elution from the chromatogram shown in Fig. 3. The column was first eluted for 2 h with pH 3.1 buffer; then with a linear gradient comprising 15 ml each of pH 3.1 and pH 4.6 buffers; and finally with pH 5.0 buffer, as indicated in the diagram.

tion. Thus, for the preparative separation of 670 nmoles (27 mg) the column was eluted with pH 3.1 buffer for the first 90 min. From Fig. 4 it was calculated that a gradient of from pH 3.8 to pH 4.6 would effect a good separation. This was achieved in practice and the final peptides were eluted with pH 5.0 2.0 *M* pyridine–acetate buffer. The final result is shown in Fig. 5 and we consider it quite suitable for separating and collecting as many of the possible peptides as is feasible.

This strategy of scale model peptide maps with gradient manipulation would

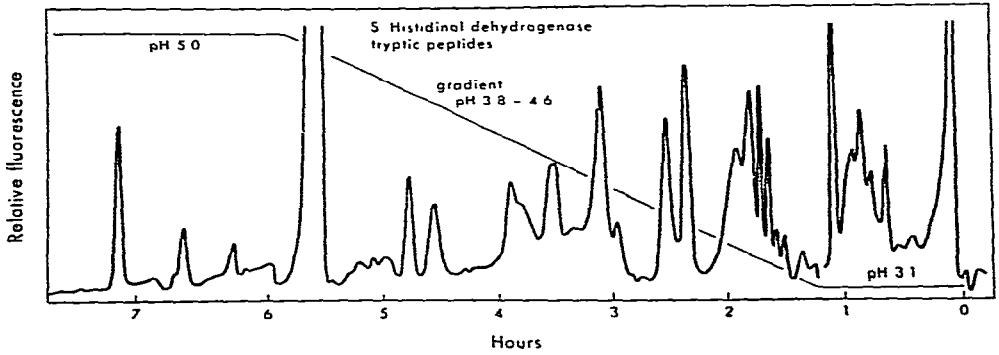

Fig. 5. Preparation of tryptic peptides from Salmonella reduced carboxymethylated histidinol dehydrogenase. A 670-nmole digest was applied to the column (200 × 9 mm), and eluted, as indicated in the diagram, with pH 3.1 buffer for 1.5 h, a linear gradient comprising of 150 ml each of pH 3.8 and pH 4.6 buffers; and finally with pH 5.0 buffer. The flow-rate was 40 ml/h and 10% of the column eluate was utilized for detection.

be applicable to tryptic or any other type of digest. The use of OPA as a detection agent produces a very simple, sensitive and repeatable procedure for the production of peptide maps, and to monitor preparative peptide separations. In addition, we have used this method of detection in the chromatography of peptides on Sephadex G-25 and on anion-exchange resins.

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