Short Communications

Automated Cation-Exchange Chromatography of Peptides at a High Sensitivity (0–0.1 Absorbance Range)

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A method is described where leaching of the column resin by pyridine–acetate buffers is minimal and excessive rise of the base-line of the chromatogram is avoided. The method can be used to fractionate peptide mixtures at a concentration of 2–20nmol/component.

Pyridine–acetate buffers are used widely in cation-exchange chromatography of peptides because they are volatile and can easily be removed from the eluted fractions, so that the latter can be concentrated and used for further studies. The most commonly used systems are linear gradients from 0.2M, pH 3.1, or 0.1M, pH 3.5, to 2.0M, pH 5.0 or 5.1 (Schroeder *et al.*, 1962; Jones, 1970). These systems have given good results when there is sufficient material and the standard (0–5mV) range of a recorder can be used for the detection of the eluted fractions with ninhydrin

in automatic analysers. They have been found, however, quite unsuitable when a range of high sensitivity is required. The base-line rises gradually to high values as the concentration of pyridine increases. In addition, a wide peak of variable height (may reach the top of the chromatogram and beyond it) and width (eluted between 50 and 150ml of effluent) appears in the middle or second third of the chromatogram (Fig. 1, continuous lines). The pyridine developers seem to wash off from the column resin material that forms a precipitate when it is mixed with the ninhydrin

Fig. 1. Chromatographic profile of micromolecular fibrinogen derivatives eluted by a single pyridine gradient

Human fibrinogen was digested by human fibrinolysin (30–110 caseinolytic units/g) and the micromolecular derivatives were isolated by dialysis (against water) and gel filtration through Bio-Gel P-2. Chromatography was performed on a 23cm × 0.9cm column of the cation-exchanger Spinco PA-35 (Dowex 50W-X8) at 50°C with a Beckman amino acid analyser by increasing the pyridine concentration and the pH in the eluting buffer from 0.1M, pH 3.5, to 2.0M, pH 5.0. The flow rate of the buffer and the ninhydrin reagent was 50ml/h and the sample was applied at a volume of 0.25ml (0.7mg). The lower graphs show the buffer run (—) and the change in pyridine concentration (----). The upper graph shows the profile of the fibrinogen derivatives in a 6:1 machine-made reduction of the original chromatogram.

reagent. This precipitate gradually occludes the reaction coil in automatic analysers and is the cause of the large peak that appears on the chromatograms. Although it has been claimed that when the Spinco PA-35 resin (equivalent to Dowex 50W-X8) is used precipitate formation is not much of a problem (Jones, 1970), I found that occlusion of the coil occurs with this resin quite often also. By starting the elution with a very dilute pyridine buffer of pH 4.2 and by increasing the concentration of the pyridine first to 0.1M very slowly and then to 2.0M with another gradient, I have succeeded in establishing a procedure that does not raise the base-line much and it does not cause the appearance of the large peak on the chromatogram (Fig. 2, continuous lines). Occlusion of the reaction coil is less frequent. Judged from my experience with the micromolecular derivatives of fibrinogen, this method in addition gives a better separation of complex peptide mixtures than the standard procedure.

Materials and solutions

Pyridine (analysed reagent) and acetic acid (reagent grade) were purchased from J. T. Baker (Phillipsburg, N.J., U.S.A.). The pyridine was refluxed for 16–18h with solid ninhydrin (2–3g/l) and then redistilled to remove impurities. Ninhydrin (specially purified for use directly in automated analysis) was obtained from the Pierce Chemical Co. (Rockford, Ill., U.S.A.). Human fibrinogen, purified by the method of Blombäck & Blombäck (1957),

was purchased from Kabi Laboratories (Stockholm, Sweden). Glycerol-activated human fibrinolysin with a specific activity of 90–100 caseinolytic units/mg of protein (Sgouris *et al.*, 1960) was kindly supplied by Dr. J. T. Sgouris of the Michigan Department of Health. The spherical resin Spinco PA-35 (equivalent to Dowex 50W-X8) was obtained from Beckman Instruments Inc. (Palo Alto, Calif., U.S.A.). The resin was washed and packed in a 23cm × 0.9cm column as recommended by Beckman (1969). The polyacrylamide gel Bio-Gel P-2 was a product of Bio-Rad Laboratories (Richmond, Calif., U.S.A.).

The starting buffer was 0.01M-pyridine–acetate buffer, pH 4.2. The first gradient (linear) was made up as follows. A 300ml volume of starting buffer was placed in a reservoir, and 300ml of 0.15M-pyridine in another similar vessel connected with the reservoir at the bottom through a glass tubing with a stopcock and placed on the same level. The solution in the reservoir was stirred magnetically during the formation of the gradient, which was run for 7h until the concentration of the pyridine in the effluent became 0.1M. The second gradient (convex) was made up as follows. Pyridine (2M) was allowed to flow into a lower-placed air-tight vessel containing 120ml of 0.10M-pyridine, which was kept under continuous stirring with a magnetic bar. The ninhydrin reagent was prepared as recommended by Beckman (1969).

Digestion of fibrinogen was carried out as follows. The fibrinogen solutions (10mg/ml) in 100ml lots were dialysed against 20vol. of 0.2M-pyridine (final pH 7.8) or water five times, to remove the inorganic

Fig. 2. Chromatographic profile of micromolecular fibrinogen derivatives eluted successively with a dilute pyridine buffer and two gradients

The fibrinogen was digested as described in the legend to Fig. 1; the small fragments were isolated by dialysis and were concentrated by rotary evaporation. Chromatography was performed on the same column that was used in the experiments of Fig. 1. Elution was started with a straight 0.01M-pyridine–acetate buffer, pH 4.2, and the concentration of pyridine was increased first slowly to 0.1M by a linear gradient and then to 2.0M by a convex gradient as indicated by the broken line (----). The flow rates were the same as in Fig. 1. The lower graph shows the buffer run (—). The upper graph shows the profile of the small fibrinogen derivatives (1.2mg) in a 15:1 machine-made reduction of the original chromatogram.

salts and any pre-existing peptides. The fibrinolysin was dialysed against 500 vol. of water twice within 1 h to remove most of the glycerol and pre-existing peptides. The dialysed fibrinolysin was added to the fibrinogen suspension at 30–110 units/g in a water bath at 37°C. The clottability of fibrinogen was lost within 29–50 min; the digestion, however, was allowed to proceed for a period 10 times as long. The digest was dialysed against 3 vol. of water five times, and the combined diffusates were concentrated by rotary evaporation.

Chromatography

The chromatography was performed on a Beckman model 116 automatic amino acid analyser. The column was equilibrated with the starting 0.01 M-pyridine-acetate buffer, pH 4.2, and a sample of the diffusate containing 0.25–1.2 mg of protein was loaded at a volume of 0.25 ml. Elution was performed with the starting buffer, the first gradient and then the second gradient in the order mentioned for 2–4, 7 and 10–12 h respectively. Each of the eluents was applied until no more peaks were formed on the chromatogram of preliminary runs for at least half an hour. The flow rate of the equilibrating and eluting buffers and of the ninhydrin reagent was 50 ml/h, and the column pressure at the beginning of the run was 1.0–1.4 MPa (150–200 lbf/in²) and at the end 2.7 MPa (400 lbf/in²). The continuous line on the lower half of Fig. 2 represents a buffer run. Although the base-line is not completely straight throughout the 300–600 ml volume range, there is only one small peak that could interfere with the interpretation of the chromatogram. This peak appeared always at

the same position and is due to the absorption of ammonia by the buffers. The graph on the upper half of Fig. 2 is a machine-made 15:1 reduction of a representative chromatogram. Because of the reduction in graph size, components that gave weak reactions with ninhydrin do not show. About 80 ninhydrin-positive components were detected on the original, a far greater number than by any other single procedure (Triantaphyllopoulos & Triantaphyllopoulos, 1968a,b). Experiments with amino acid mixtures indicated that the sensitivity of the method is 2–20 nmol/constituent. Most of the chromatography can be performed during the night. If an automatic gradient-former is available, the increase in the pyridine concentration at the beginning of the second gradient can be programmed to occur at a lower rate, and a better separation of the components that are eluted between 600 and 800 ml of effluent can be obtained.

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