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DISCONTINUOUS RECYCLING CHROMATOGRAPHY

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SUMMARY

A chromatography technique whereby the effluent is recycled in open one-column systems and which avoids the remixing of components of considerably different elution volumes is described.

INTRODUCTION

Chromatography using a recycling technique (recycling chromatography) was first introduced by PORATH AND BENNICH[1] in 1962, and represents a substantial improvement in those column chromatography systems in which the resolution is independent of the solute concentration, such as gel filtration. In this cyclic process, the effluent is pumped back to the influent end of the column in a closed circuit and the effective bed height can be increased several times. Since its introduction recycling chromatography has become a method of wide application[2].

If the process is carried out in an open system, as described in this paper, the effluent is collected in fractions, which after their evaluation, are reapplied to the column in the order of their emergence at an appropriately selected starting point in the elution profile. This discontinuous procedure, in which the column system is not connected in a closed circuit, permits the recycling, in one column, of mixtures of components with distribution coefficients ranging between 0 to 1 without their subsequent remixing. The number of cycles is not limited by the size of the column used. Recycling chromatography in an open system can be performed with conventional column systems without any additional equipment.

The procedure for discontinuous recycling chromatography is demonstrated in this paper by recycling on a Sephadex G-100 column of fragments from cyanogen bromide and trypsin hydrolysates of hog pepsin; the characterization of these components is the subject of further studies[3,4].

EXPERIMENTAL

Recycling of cyanogen bromide hydrolysate of hog pepsin

The discontinuous recycling procedure was carried out in a 260 × 10 cm bed of Sephadex G-100 equilibrated with a urea-containing eluant (0.3 *M* ammonium acetate, in 8 *M* urea, pH 5.0) which was also employed for the elution of the column.

The influent end (bottom) of the column was tapered to a thin tube, and was provided with a glass filter of medium pore size, 10 cm in diameter. The conical dead space below the filter was packed with glass beads, 2–3 mm in diameter. The effluent (top) part of the column was contracted to an internal diameter of 4 cm; it was closed off by another glass filter tapering to an outlet suitable for tubing of internal diameter approximately 1.2 mm (Fig. 1). This filter, which prevents the displacement of Sephadex particles from the column, was fitted in the bore of a rubber stopper fixed by a screw clamp into the neck of the column.

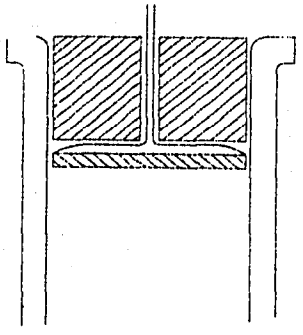

Fig. 1. Effluent part of the column. The chromatographic tube was shut off by a glass filter fitted into the bore of a rubber stopper.

The column was packed in the conventional manner, *i.e.* a slurry of the gel, prepared by allowing Sephadex G-100 (40–120 μ) to swell for 72 h in the urea-containing eluant described above, was allowed to sediment by gravity in the tube. The outlet end of the tubing connected to the bottom part of the column was adjusted so that the vertical distance between the level in the column and the outlet end of the tube did not exceed 1 m. After the packing had been almost completed, the column was closed by insertion of the rubber stopper with the outlet filter. The urea-containing eluant was then passed upward through the bed at a flow rate of 100 ml/h using a piston pump (Micropump MC 300, Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague). During this step the gel layer filled up the small dead space around the filter at the effluent end. The inlet tubing of the micropump, dipped into the urea-containing eluant in a reservoir, was provided with an inlet filter preventing the entry of impurities into the system.

The chromatography was effected in the upward flow arrangement. The inlet filter was immersed in the sample solution and transferred to the eluant reservoir after application of the sample to the column. The effluent emerging from the column passed through an ultraviolet absorptiometer (8300 Uvicord II, LKB Producter AB, Stockholm, Sweden), set at 280 nm, and then to a fraction collector. Fractions (100 ml) were collected at 1-h intervals and their absorbance at 280 nm was measured. The absorptiometer record was used to obtain instant information of the existent stage of separation in the column.

The preparation and purification of S-sulfo-pepsin from commercial twice crystallized hog pepsin (Worthington, Freehold, N.J., U.S.A.) as well as its cleavage with cyanogen bromide[5] were carried out as described elsewhere[6]. For the fractionation, 6.2 g of the cyanogen bromide hydrolysate was dissolved in 200 ml of the

urea-containing eluant (see above) whose pH had been adjusted to 8.5 by ammonium hydroxide. After the sample had dissolved completely, glacial acetic acid was added to decrease the pH of the sample to 5.0, and the sample solution was pumped into the column. The course of the fractionation is shown in Fig. 2. When fraction No. 170 was emerging from the column (as indicated by an arrow) reapplication of fractions Nos. 52–165 was begun; these fractions account for the entire effluent area corresponding to peaks CB1 – CB5. The inlet filter was placed at the bottom of a 100-ml measuring cylinder into which the first fraction (No. 52) to be recycled had been poured. After this fraction had been pumped almost completely into the column (avoiding the entry of air into the filter), fraction No. 53 was poured into the cylinder. The reapplication was continued in this manner up to fraction No. 165. The inlet filter was then transferred to the reservoir of eluant with which the development of the column was completed.

In the second cycle, during which the effective bed height amounted to 520 cm, the fractionation of the components present in the sample was substantially improved. Fractions corresponding to the individual peaks CB1 – CB5 were pooled, desalted on a Sephadex G-25 column, lyophilized, and investigated further[3].

Recycling of tryptic digest of an aminoethylated fragment CB2

The cyanogen bromide fragment of S-sulfo-pepsin was obtained as described above. It represents the N-terminal portion of the pepsin molecule and contains approximately 150 amino acid residues[4], among their number the only histidine of pepsin[7] and two half-cystine residues which form a disulfide loop in the molecule of the native enzyme ... –Cys–Ser–Ser–Leu–Ala–Cys–Ser–Asp–His–... (refs. 8, 9). This fragment does not contain arginine or lysine residues[3]. The conversion of fragment CB2 into its aminoethyl derivative[10], the digestion of the latter with trypsin, and the characterization of the individual tryptic fragments will be described in detail in a forthcoming paper[4].

The cleavage of the aminoethylated fragment CB2 at the carboxyl side of the two S-(β-aminoethyl)-cysteinyl (AEC) residues gave rise to a pentapeptide, Ser–

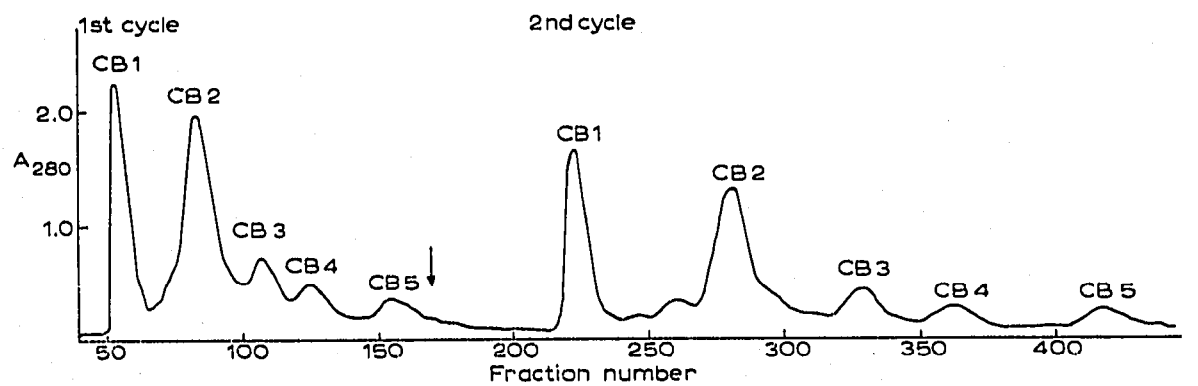

Fig. 2. Discontinuous recycling chromatography of the cyanogen bromide hydrolysate of S-sulfo-pepsin. The fractionation of 6.2 g of sample dissolved in 200 ml of 0.3 *M* ammonium acetate, in 8 *M* urea, pH 5.0, was effected on a Sephadex G-100 column (260 × 10 cm) equilibrated and eluted with the same solution. 100 ml fractions were collected at 1-h intervals. When fraction No. 170 was emerging (as marked by an arrow) reapplication of fractions No. 52–165 to the column was begun.

Ser–Leu–Ala–AEC, derived from the area between the AEC-residues, and to two larger fragments representing the rest of the molecule of fragment CB2. The pentapeptide was separated by gel filtration on a Sephadex G-25 column from the high molecular weight portion of the digest (designated CB2–RAE–T1). The latter was subjected to discontinuous recycling chromatography on the Sephadex G-100 column. The fractionation of this material is adduced here as another example of the applications of the fractionation technique described.

For the fractionation, 2.5 g of CB2–RAE–T1 was dissolved in 500 ml of 0.3 *M* ammonium acetate, in 8 *M* urea, pH 5.0. The fractionation was carried out with the same equipment and under conditions identical to those described above for the fractionation of the cyanogen bromide hydrolysate. As is apparent from Fig. 3, the recycling was only aimed, in this case, at the isolation of the two main components, designated by the abbreviated symbols –T11b and –T12. After four cycles the effective bed height amounted to 10.4 m.

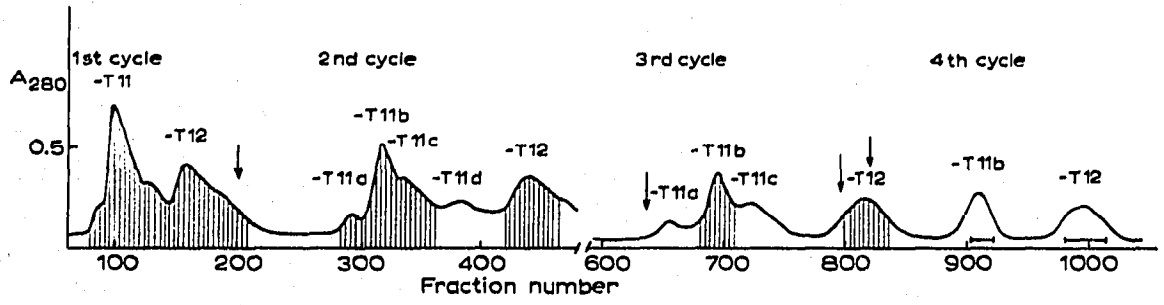

Fig. 3. Discontinuous recycling chromatography of tryptic digest of aminoethylated fragment of pepsin, CB2. The sample (2.5 g) was dissolved in 500 ml of 0.3 *M* ammonium acetate, in 8 *M* urea, pH 5.0. The column and the conditions of the fractionation were identical to those described in the legend to Fig. 2. The areas selected for recycling are hatched. The sites of reapplication of the fractions to be recycled are marked by arrows, save for the application of areas –T11a through –T11c in the third cycle, which is at fraction No. 560 lying in the interrupted part of the diagram. The arrow at fraction No. 637 designates the beginning of the reapplication of area –T12.

RESULTS AND DISCUSSION

Exceptionally long beds are required to achieve good resolution of certain components by gel filtration. The height of the columns used is limited by various factors, such as, *e.g.* the mechanical properties of the gels, the required quantity of the bed material, etc. Recycling chromatography, first introduced by PORATH AND BENNICH[1], permits the effective bed height of smaller columns to be increased by pumping the effluent back to the influent end of the column. The fractionation of the components present is thus continued during their subsequent passage through the column. These cycles can be repeated several times and the course of the separation can be monitored by a flow analyzer connected into the circuit. It is important that the dead space of the system, where remixing takes place, be kept at a minimum. A commercial apparatus designed for chromatographic separations by recycling is available (4900A ReCyChrom, LKB Produkter AB, Stockholm, Sweden).

The recycling chromatography arrangement described, however, is subject to certain limitations. In the case where the mixture to be separated contains components with considerably different distribution coefficients (K_D), then a component charac-

terized by a low K_D value, which emerges from the column in a small elution volume, can after reapplication and passage through the column get mixed with some other, "slower" component with a higher K_D value. The possibility of this remixing increases, especially in the subsequent cycles due to the broadening of the zones. This effect together with the column size are factors which determine the number of cycles possible. These drawbacks can be circumvented by limiting the recycling to components with K_D values distributed over a relatively narrow range. The number and the distribution of the components and the program of recycling are determined in an exploratory experiment. A selector valve, connected in the circuit, permits the reapplication of a narrower effluent range, followed by the opening of the closed circuit, and the "bleeding out" of the remaining peaks to a fraction collector. In an alternative procedure[11], the selected component is transferred to another column in which its recycling is effected.

In the discontinuous procedure, the circuit is open and the effluent is collected in fractions. After their evaluation, the fractions to be recycled are reapplied to the column at an appropriately chosen point of the elution process and the circuit is thus again closed.

There is an essential difference between mere rechromatography and discontinuous recycling chromatography. In the first case the fractions selected for rechromatography are pooled and all the components present are mixed in the reapplied sample and enter the column as a homogeneous mixture. In the second case the fractions to be recycled enter the column in sequence and the mutual shift of zones, arising from the preceding separation step, is thus retained. The interruption of the circuit offers the possibility of a more complete evaluation of the effluent between the cycles, including the determination of enzymatic activity.

If the recycled region includes all the peaks (Fig. 2), then the reapplication is usually started after the first cycle has been completed. If the procedure is merely aimed at the isolation of some of the components, only selected peaks are recycled (Fig. 3). The recycled peaks are applied to the column in the order of their increasing elution volumes, *i.e.* in order of their emergence from the column. For a better distribution of peaks in the effluent, a volume of eluant can be inserted between individual recycled regions. At the beginning of each successive cycle the sequence of the increasing elution volumes is broken, *i.e.* the last peak of the largest elution volume is followed again by the first, "fastest" component. Reapplication can be begun after the "slowest" component has emerged or, alternatively, the point of application has to be determined by a comparison of the elution volumes of both components as determined in the first cycle. Under these conditions, components with K_D values distributed over the entire range, from 0 to 1, can be recycled in one single column.

The broadening of peaks as a result of diffusion is relatively low. No irregularities in the elution profile due to the discontinuity in the applied samples were observed when the volume of the fractions collected was small. Thanks to the upward flow arrangement, the flow resistance of the 260 cm tall bed of Sephadex G-100 did not increase even after several months of operation. The discontinuous procedure has also been used in the downward flow arrangement and with considerably smaller columns[12]. A factor of crucial importance is the keeping of the dead space at a minimum.

A certain drawback of the discontinuous procedure represents the time-consuming application of samples, which can be controlled automatically, however, at the cost of the simplicity of the equipment.

The procedure described can be used in combination with the continuous method. By recycling in an open system a degree of resolution can be obtained which allows cuts to be made unambiguously. The latter can then be subjected to final separation by the continuous procedure.

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