CHROM. 4148

CONTINUOUS CHROMATOGRAPHY APPARATUS

III. APPLICATION

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SUMMARY

A description is given of the use of the continuous chromatography apparatus for the purification of cow heart myoglobin and the separation of skim milk proteins from lactose and salt.

INTRODUCTION

The details of construction[1] and operation[2] of the continuous chromatography apparatus have been given in the previous parts of this series. In this section we will present the results of the purification of myoglobin on Sephadex G-75* (for which use the device was originally built) and add as an illustration the separation of the components of skim milk on Sephadex G-25 gels. A commercial application of gel filtration to the latter separation has been described[3] for the production of protein-enriched milks. Since continuous systems are inherently more convenient to operate, we thought it would be of interest to apply the continuous chromatography apparatus to the separation.

EXPERIMENTAL

Myoglobin

The account of the separation and purification of myoglobin from heart extracts using Sephadex G-75 was first published in 1963 (ref. 4). Since that time modifications and improvements in the technique have been made as follows. To reduce decomposition of the globin, the pigment was extracted from beef hearts processed within 3 h after slaughter. The hearts were trimmed of nonmuscular tissue and cut in 1 cm cubes. The crude extracts for chromatography were prepared as described in the flow diagram of Fig. 1. The continuous chromatography column was filled with Sephadex Superfine G-75 gel swelled in 0.02 M Tris, pH 8.1. The solution, which contains

* Mention of commercial names does not imply endorsement by the U.S. Department of Agriculture.

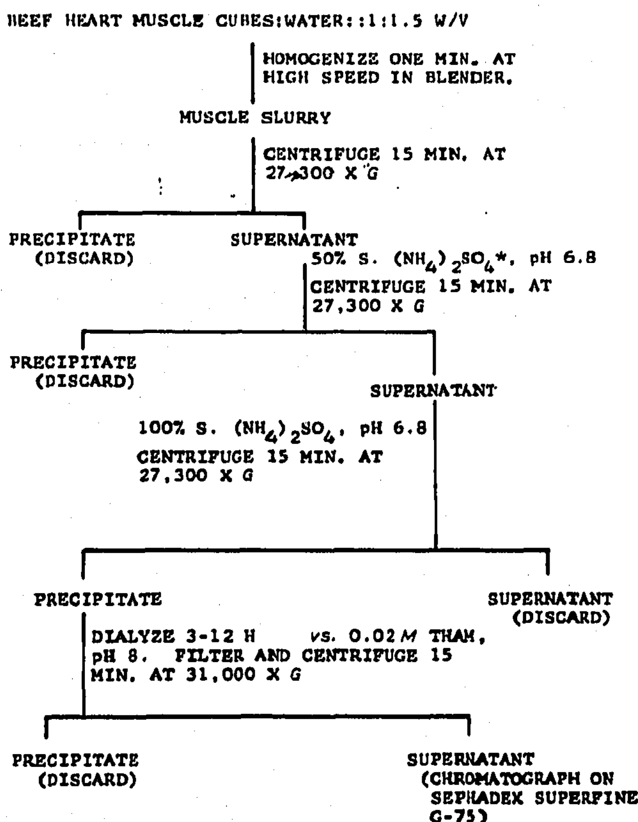
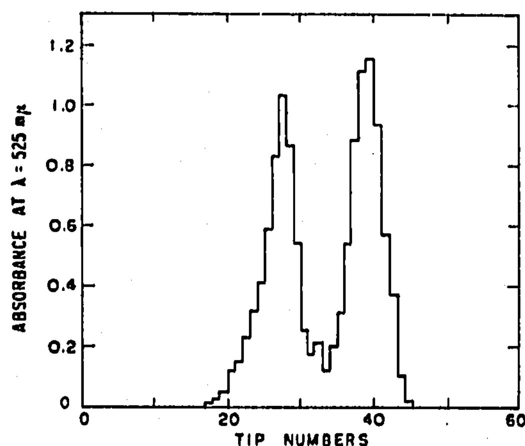


Fig. 1. Flow diagram for the preparation of heart muscle extracts for gel filtration. * Saturation calculated according to J. F. TAYLOR (1953)[6].

myoglobin, hemoglobin, and some extraneous proteins (total protein *ca.* 5%), was continuously fed into the column from a reservoir maintained at 5°. The eluting buffer was 0.02 *M* Tris–HCl, pH 8. This pH was chosen as being the lowest pH that would keep the proteins in solution from precipitating on the surface of the gel. After the chromatographic separation, the myoglobin fraction was concentrated in an Amicon Model 50 Ultrafiltration Pressure Cell, under 30 p.s.i., using a Diaflo UM-10 ultrafiltration membrane. The concentrated myoglobin solution (approx. 7 m*M* or 12.6% MetMb) was centrifuged 15 min at 31 000 × *g*. The myoglobin solution was made sterile by passing it from a syringe through a Millipore Swinnex-25 filter unit containing a sterile Millipore GS 0.22 μ filter membrane. From the filter unit the solution was filtered directly into a sterile, sealed serum vial. The vials were stored under refrigeration. Using this technique, we were able to prepare purified myoglobin in less than 24 h.

Skim milk

The use of G-50 for the separation of the proteins of skim milk from the lactose and salt was first reported in 1962 (ref. 5) and later the technique was developed to what may be maximum efficiency for commercial use[3], this time using Sephadex G-25. We used essentially the technique of the latter publication. The column was packed with Sephadex Superfine G-25 which had been swelled in water. The milk used was Walker-Gordon certified skim milk, containing 3.7% protein, 0.1% fat, and 4.9%



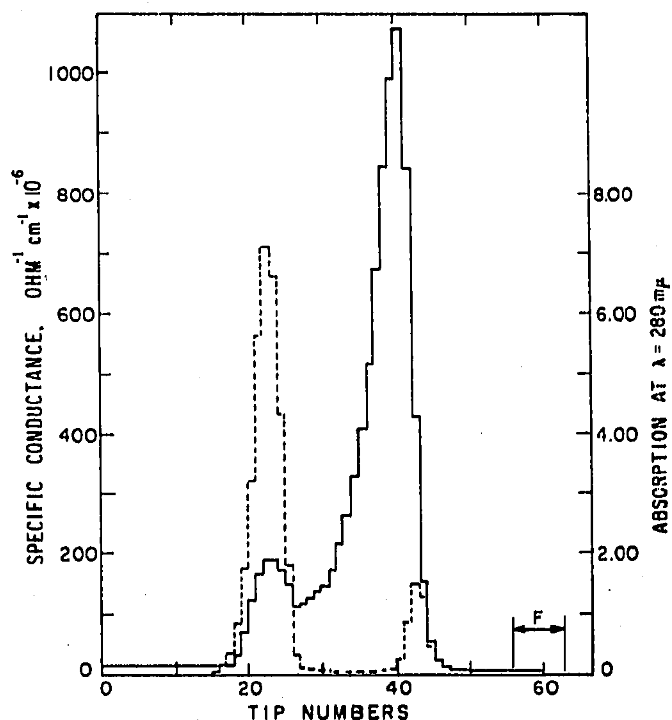


Fig. 2. The separation of myoglobin from hemoglobin in beef heart extracts on Sephadex Superfine G-75. Fractions 20–32, hemoglobin; fractions 34–43, myoglobin.

Fig. 3. The separation of the components of skim milk on Sephadex Superfine G-25. Fractions 19–26, protein; fractions 30–44, salts; fractions 40–44, lactose; band F, riboflavin. ———. Specific conductance; — — —, absorption, 280 mμ. F = fluorescent band.

lactose. Walker-Gordon is a milk from specially tested herds, flash-pasteurized, and of low bacterial count. The milk was fed into the column from a reservoir maintained at 5°, and eluted with distilled water. The input rate of the milk and the rotational speed of the column were adjusted so that all the components of the milk were eluted before one column revolution was completed.

Individual fractions were collected as described previously[2]. The heart extract fractions were analyzed for heme pigments by reading the optical absorption at 525 mμ. The skim milk fractions were analyzed for protein/lactose, salt, and riboflavin by UV absorption, conductivity and fluorescence, respectively.

RESULTS

The results of both chromatographic separations are shown in Figs. 2 and 3. Only the heme pigment fractions are shown in Fig. 2, but there is also a large non-heme protein band which precedes and overlaps the hemoglobin band. The myoglobin fraction comes off 97% pure, the remainder being a non-heme protein material. One curious observation was made. If fractions are collected across the myoglobin band and the absorbances are measured at 280 and 525 mμ (protein and heme, respectively) the ratio A_{280}/A_{525} drops from *ca.* 4.25 to *ca.* 3.90. The fractions were analyzed by disc electrophoresis, and gave identical patterns that showed four bands by Amido-Schwarz stain (protein) and three bands by benzidine–peroxide stain (heme), the odd

TABLE I

FLOW VOLUME AND DATA

	Myoglobin	Skim milk	
		Continuous	SAMUELSSON *et al.*[a]
Flow rates			
Solution	22 ml/h	22 ml/h	21.6 l/h
Solvent	920 ml/h	1460 ml/h	95.2 l/h
Bed volume	1200 ml	1200 ml	16 l
Elution position[b]			
Heart extract			
Hemoglobin	28		
Myoglobin	40		
Cytochrome *c*	60		
Skim milk			
Protein		23	
Salt		41	
Lactose		44	
Riboflavin		60	
Solvent volume to clear myoglobin	368 ml/h		
Milk protein		335.9 ml/h	7 l
All solutes	644 ml/h	876.0 ml/h	23.8 l
Column speed	128 min/rev	128 min/rev	15 min/cycle
Time on column			
Myoglobin	52 min		
Protein		29.4 min	5 min
Efficiency ratios			
Solution/solvent[c]	0.034	0.025	0.227
Solution/bed volume	0.031	0.031	1.35

[a] These are calculated values from the data of the table in the text, p. 124 of SAMUELSSON *et al.*[3]. Although our calculations may not be strictly correct, the figures form a basis for comparison, odious as it may be.
[b] Total number of tips between point of solution application and peak maximum.
[c] Total volume of solvent required to clear column.

protein band being $< 3\%$ of the total protein. If the myoglobin solutions were dialyzed, the ratio rose to *ca.* 4.25. Rechromatography would not remove the slight amount of non-heme protein. These results suggest a small amount of heme–globin dissociation, resulting in free globin and heme. The latter may be loosely bound to undissociated myoglobin molecules, and removed by dialysis. The latter process would result in an increase in the A_{280}/A_{525} ratio.

The separation and yield achieved was as good as by the usual column chromatographic method employed by us, but the time and effort required for the operation of the continuous column is a fraction of that of the discontinuous system.

The skim milk used for chromatography in the continuous column had the same protein, lipid and lactose content as that used by SAMUELSSON *et al.*[3], and the results are directly comparable in this respect. The separation achieved in the continuous system was about the same as that reported by SAMUELSSON *et al.*, but the efficiency of operation of the continuous system was considerably lower, as can

be seen by reference to Table I. The flow rate values for the continuous system are measured values, but those for the columns of SAMUELSSON *et al.* are calculated from the data in their paper. There are some uncertainties involved. For example, SAMUELSSON *et al.* report a volume of 23.8 l/column cycle, but it is not clear if the riboflavin had cleared the column. From the data of Fig. 4 in ref. 3 it would appear that the riboflavin from the preceding run came off with the protein of the succeeding run. Our total volume figure is that required to completely clear the column. In any case, the orders of magnitude would be the same and it can be seen that the continuous system is only 1/10 as efficient with respect to the amount of solvent required for a given volume of solution, and 1/44 as efficient with respect to volume of solution processed per hour for a given volume of gel. Our apology is that we were more interested in demonstrating the feasibility of the continuous chromatography system, rather than determining the efficiency of the operation. Our studies lead us to believe that there should be no difficulty in improving the efficiency of the continuous system, and even if the volume yield efficiencies cannot be made as good as those for the discontinuous system, the inherent ease of operation of the continuous system may offset the lower yields. We were, insofar as the myoglobin preparation was concerned, more interested in the purity of the pigment eluted from the column and therefore operated the column well below maximum capacity.

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