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:HROMATOGRAPHY OF LYSOSOMAL ENZYMES ON HYDROXYLAPATITE

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;UMMARY

Soluble proteins were extracted from Triton WR 1339-filled rat liver lysosomes .nd were chromatographed on hydroxylapatite by stepwise elution with increasing oncentrations of phosphate buffer (pH 6.8). The distribution of the activities of the :athepsins A, B, C, and D; β-galactosidase; α-mannosidase; β-glucuronidase; β-N-ıcetylglucosaminidase; acid phosphatase and arylsulfatase in the resulting fractions vas determined. Ammonium sulfate fractionation of lysosomal proteins was combined vith separation on hydroxylapatite in an attempt to improve the resolution of the :athepsins. Lowering the pH of the phosphate buffers to 5.8 greatly increased the .ffinity of lysosomal proteins for hydroxylapatite. By a combination of these manip-ılations fractions were obtained which contained cathepsin D without contamination)y other cathepsins.

NTRODUCTION

The lysosome is now known to contain about 40 enzymatic species with the :apacity to degrade all the major classes of biological macromolecules. These enzymes .ct in combinations determined by their specificity to digest individual complex nolecules within the lysosome. Precise definition of these digestive pathways in terms)f their individual enzymes depends, at least in part, on separation of the enzyme :omponents of the lysosome. This is particularly true of the pathway of protein :atabolism for which only limited knowledge exists concerning the contribution of :ach of the cathepsins to proteolysis. Availability of purified lysosome fractions allows ıpplication of conventional chromatographic methods to the separation of the en-zymes in the lysosome. The properties of lysosomal enzymes in several chromatographic ;ystems (DEAE-cellulose, Sephadex G-200, CM-cellulose) have been described else-vhere[1]. The usefulness of hydroxylapatite for fractionation of lysosomal enzymes was ;uggested by the work of HAYASE[2], who partially purified lysosomal lipase with ıydroxylapatite and demonstrated its capacity to resolve lysosomal protein into a ıumber of distinct zones. Descriptions of the properties of hydroxylapatite[3,4] also ;uggest its high resolving power and its adaptability to either analytical or preparative)perations. In view of the complexity of the mixture of proteins extractable from

lysosomes, hydroxylapatite seems particularly well suited to be a general method for separating lysosomal enzymes. This paper describes the fractionation of soluble lysosomal enzymes on hydroxylapatite columns with special emphasis on the behavior of the cathepsins. Factors that limit the capacity of hydroxylapatite to resolve complex mixtures of proteins are discussed.

EXPERIMENTAL

Materials

Hydroxylapatite (Bio-gel HT) in 1 m*M* sodium phosphate buffer, pH 6.8, was purchased from Bio-Rad Laboratories. Cytochrome *c*, type III, and hemoglobin, type I, were purchased from Sigma Chemical Co., N-CBZ-L-glutamyl-L-phenylalanine, benzoyl-L-arginine amide hydrochloride, and glycyl-L-tyrosinimide acetate were purchased from Mann Research. Materials used for the automated analyses are given by BECK AND TAPPEL[5].

Fractionation and concentration of lysosomal protein

Triton WR 1339-filled lysosomes were prepared from rat liver by the method of MAHADEVAN AND TAPPEL[6]. Soluble protein was extracted from these lysosome preparations by two methods. In the first method, protein was precipitated by bringing the lysosome fraction to 100% saturation with solid ammonium sulfate. Protein was collected by centrifugation at 13 000 × *g* for 20 min. Because of the high density of sucrose in these lysosome preparations, the precipitated protein floated to the surface during centrifugation. The subnatant liquid was removed by suction and the protein was resuspended in 1 m*M* sodium phosphate buffer, pH 6.8. Membranes were removed from the protein fraction by centrifugation at 95 000 × *g* for 45 min. The clear supernatant fraction was dialyzed overnight against 1 m*M* sodium phosphate buffer, pH 6.8. The dialyzed solution was concentrated 25-fold by vacuum filtration through dialysis tubing. In the second method, part of a batch of Triton-filled lysosomes was brought to 100% saturation with ammonium sulfate. From the remainder of the batch, three fractions of lysosomal protein were obtained by fractional precipitation between 0–30, 30–60, and 60–90% saturation of ammonium sulfate. Precipitated protein was collected by centrifugation and separated into membrane and soluble portions as described above. All fractions were dialyzed overnight against 1 m*M* sodium phosphate buffer, pH 6.8.

Column operation

Hydroxylapatite beds were poured either in hand-blown glass columns (0.9 × 15 cm) with glass wool bed supports or in a Pharmacia K 15/30 column. Protein was eluted from the columns by stepwise application of increasing concentrations of sodium and potassium phosphate buffers. Chromatographic separation of lysosomal proteins was carried out at 4°.

Enzyme and protein determinations

Protein concentration was determined by measuring the absorbance at 280 mμ and by the method of MILLER[7]. Both methods were standardized with bovine serum albumin. Acid phosphatase, β-N-acetylglucosaminidase, arylsulfatase, β-glucuronidase

and β-galactosidase activities were determined by the automated methods of BECK AND TAPPEL[5]. Measurements of α-mannosidase activity were made by automated analysis in the same manner as the above enzymes using a method adapted from CONCHIE AND FINDLAY[8].

Cathepsin A activity was measured by the method of IODICE *et al.*[9]. Cathepsin B was measured by the method of GREENBAUM AND FRUTON[10]; liberated ammonia was collected by the microdiffusion method of SELIGSON AND SELIGSON[11]. The procedure of METRIONE *et al.*[12] was used to measure cathepsin C activity. Cathepsin D determinations were performed according to GIANETTO AND DE DUVE[13].

RESULTS

The extraction of soluble protein from Triton-filled lysosomes was monitored by measurement of protein content at each step. Since the separation of the cathepsins was of especial interest, the activities of these enzymes were followed throughout this procedure. These measurements (Table I) indicated that the recovered soluble lysosomal proteins were enriched in cathepsins B, C, and D, while constituting only about 20% of the original protein. There was considerable loss of total cathepsin A activity in this procedure and, thereby, a decline in the specific activity of this enzyme. The process by which the proteins were concentrated resulted in some further loss of protein and a decline in the total and specific activities of the cathepsins. Only traces of catheptic activity were associated with the membrane fraction.

The pattern produced by the stepwise elution of lysosomal protein from hydroxylapatite consisted of twelve distinct zones (Fig. 1). In a trial operation of the column, Triton WR 1339 showed no tendency to bind to hydroxylapatite even at low phosphate concentrations and appeared in the void volume. The large initial peak obtained by the elution of soluble lysosomal proteins with 1 m*M* sodium phosphate buffer was due to the presence of Triton. The completeness of the recovery of protein from the column was difficult to determine due to the interference of Triton with the protein determinations. However, HAYASE[2] has shown that lysosomal protein was eluted by buffers up to 0.6 *M*, but not by buffers of higher concentration even with extended washing. Thus, it is likely that the lysosomal protein was completely eluted from this column.

Representative fractions from each of the protein peaks were assayed for the activities of lysosomal enzymes and the resulting values are presented as percent of

TABLE I

RECTION OF SOLUBLE PROTEIN FROM TRITON-FILLED LYSOSOMES

	Volume (ml)	*Protein*		*Cathepsin A*			*Cathepsin B*			*Cathepsin C*			*Cathepsin D*		
		mg	%	*S.A.*[a]	*T.A.*[b]	%	*S.A.*	*T.A.*	%	*S.A.*	*T.A.*	%	*S.A.*	*T.A.*	%
ial	340	221	100	168	1115	100	0.9	5.8	100	136	451	100	5	34	100
zed															
ible	125	59	27	160	281	25	2.5	4.4	76	231	204	45	37	66	197
ntrated	3.9	46	21	64	88	8	2	2.7	46	163	112	25	21	29	9

[a] Specific activity expressed as mμmoles of product/mg protein/min.
[b] Total activity expressed as μmoles of product/min/ml × ml.

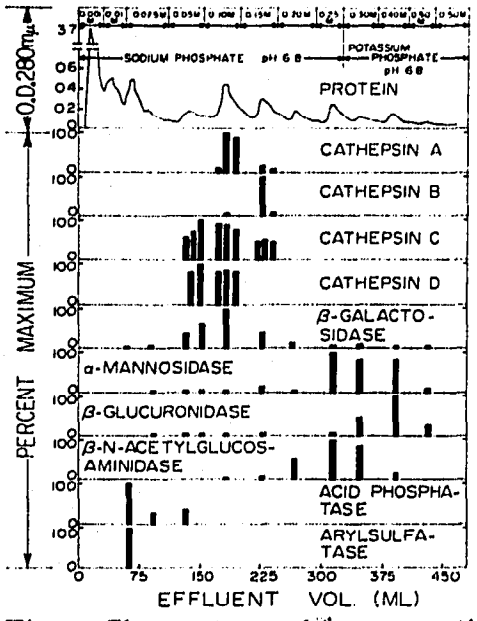

Fig. 1. Chromatographic separation of lysosomal enzymes. Concentrated lysosomal protein (35 mg) was applied to a 1.5 × 16 cm hydroxylapatite column. The flow rate was 35 ml/h and elution was by stepwise increases in phosphate buffer concentration. The fraction volume was 3 ml and protein was monitored by measuring absorbance at 280 mμ. Activities are presented as percent of maximum specific activity for each enzyme.

maximum specific activities (Fig. 1). The distribution of enzyme activities in these fractions reveals the capacity of hydroxylapatite to separate the components of complex protein mixtures. Acid phosphatase and arylsulfatase, for example, are maximally active in fractions which are virtually free of the other enzymes measured. Even in regions of the protein pattern that contain a number of enzymes, useful partial separations (*e.g.* of cathepsins A and B, of β-glucuronidase and β-N-acetylglucosaminidase) were achieved. Cathepsins B and D were completely separated. Several of the enzymes were partly concentrated in one or a few peaks and were partly separated from the other enzymes, although none of the enzymes was completely separated from all of the others.

The catheptic enzymes were all eluted within a narrow range of phosphate concentration and their distributions, especially those of cathepsins B, C, and D, overlap considerably. A series of experiments was undertaken to determine if the elution of the cathepsins from hydroxylapatite could be modified to improve the separation of these enzymes. Another portion of soluble lysosomal proteins, including those prepared by fractional precipitation with ammonium sulfate, were used for these experiments. The further concentration of protein by vacuum filtration after ammonium sulfate precipitation proved to be not only disadvantageous but unnecessary. The great affinity of hydroxylapatite for proteins in the presence of low phosphate concentrations makes it possible to apply proteins to these columns in very dilute solutions; protein is effectively concentrated during application because of its adsorption in a narrow zone of the column bed. The concentration step was, therefore, abandoned in the second method of preparation of lysosomal protein. The distribution of catheptic activities in these preparations indicated that a partial separation of the cathepsins had been achieved by ammonium sulfate treatment (Table II).

II

UM SULFATE FRACTIONATION OF TRITON-FILLED LYSOSOMES

	Volume (ml)	*Protein (mg)*	*Cathepsin A*			*Cathepsin B*			*Cathepsin C*			*Cathepsin D*		
			S.A.[a]	*T.A.*[b]	%	*S.A.*	*T.A.*	%	*S.A.*	*T.A.*	%	*S.A.*	*T.A.*	%
l	270	135	312	129		26	11		1380	285		295	122	
inal	176	90		86	100		7	100		190	100		81	100
, sat.	42	29	175	15	18	59	5	72	1223	54	28	565	49	61
inal	88	45		43	100		4	100		95	100		41	100
,% sat.	33	3.3	860	9	20	52	0.5	14	3420	17	18	414	4	10
,%sat.	11	4.5	13	0.2	0.4	0	0	0	653	4	5	531	7	18
,% sat.	11	2.8	19	0.2	0.4	15	0.1	4	308	1	1	181	1	4

[a] Specific activity expressed as mμmoles of product/mg protein/min.
[b] Total activity expressed as μmoles of product/min/ml × ml.

The protein solutions were equilibrated with the appropriate 1 m*M* phosphate buffer and applied to small (0.9 × 2.5 cm) columns. Elution was carried out by stepwise application of 3 or 5 ml of each of the buffers in a series. The eluent in each step was collected as a single fraction and analyzed for protein (280 mμ absorbance) and for the activity of the cathepsins.

Fig. 2 (A, B) indicates the effect of the pH of sodium phosphate buffer on the elution of lysosomal protein. At pH 6.8 the pattern of cathepsin elution is similar to that presented in Fig. 1. However, at pH 5.8 the cathepsins are more strongly adsorbed and are eluted only with the application of higher phosphate concentration. In similar

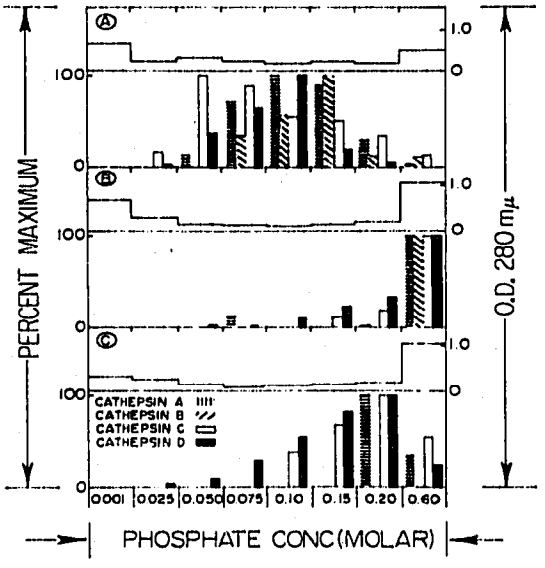

Fig. 2. Effect of pH on elution of cathepsins from hydroxylapatite. Protein was obtained by ammonium sulfate fractionation of Triton-filled lysosomes (see Table II). Samples containing 5 mg of lysosomal protein were applied to 0.9 × 2.5 cm hydroxylapatite columns and eluted by stepwise application of increasing concentration of phosphate buffer. Sodium phosphate was used at each step except the last, which was 0.6 *M* potassium phosphate. Activities are presented as percent of maximum specific activity for each enzyme. (A) Protein from 100% ammonium sulfate fraction eluted with pH 6.8 phosphate buffer. (B) Protein from 100% ammonium sulfate fraction eluted with pH 5.8 phosphate buffer. (C) Protein from 30–60% ammonium sulfate fraction eluted with pH 5.8 phosphate buffer.

experiments, sodium phosphate at pH 7.8 and potassium phosphate buffer at pH 6.8 produced elution patterns like that produced by sodium phosphate at pH 6.8.

Attempts were made to utilize this pH effect to enhance the separation of the cathepsins on hydroxylapatite. The protein precipitated between 30 and 60% saturation of ammonium sulfate was relatively richer in cathepsin D than were the other preparations and was free of cathepsin B. Elution of this protein from hydroxylapatite with sodium phosphate buffer at pH 5.8 yielded some fractions which contained only cathepsin D and which were free of other cathepsins (Fig. 2C).

DISCUSSION

The results reported here illustrate both the advantages and limitations inherent in the use of hydroxylapatite for the separation of complex protein mixtures. The separation of as many as ten enzymes into four distinct groups in a single step is clearly advantageous. Further, hydroxylapatite chromatography, which is not based on the ionic properties of the solutes, emerges as a useful supplement to ion-exchange methods. BECK *et al.*[1] have recently described the separation of rat liver lysosomal enzymes on DEAE-cellulose, Sephadex G-200, and CM-cellulose and the resolving power of hydroxylapatite compares quite favorably with that achieved by their methods. Several nonproteolytic lysosomal enzymes were partly or completely separated from other enzymes on hydroxylapatite. For example, acid phosphatase and arylsulfatase can be clearly separated from a number of other enzymes. BECK *et al.*[1] showed that arylsulfatase and acid phosphatase can be resolved on DEAE-cellulose. Thus, the combination of these chromatographic methods could purify and separate the two enzymes. Similarly, β-N-acetylglucosaminidase, β-glucuronidase, and arylsulfatase appear in the same region of the eluent from a DEAE-cellulose column. Yet these three enzymes are well separated from each other by either CM-cellulose or hydroxylapatite chromatography. Likewise, CM-cellulose will separate β-glucosidase from β-N-acetylglucosaminidase on one hand and from a combination of β-galactosidase and β-glucuronidase on the other. Separation of β-galactosidase and β-glucuronidase is readily achieved on hydroxylapatite. These examples suggest the usefulness of hydroxylapatite for the separation of the soluble lysosomal enzymes when combined with ion-exchange methods.

None of the above chromatographic systems, either separately or in combination, yielded good separations of the cathepsins. The combination of ammonium sulfate fractionation and "batch" type elution at pH 5.8 and pH 6.8 from small hydroxylapatite columns resulted in partial separations of the cathepsins (Fig. 2). Recent experiments in this laboratory (LIAO, unpubl. results) have indicated that it may be possible to combine DEAE-cellulose and Sephadex G-100 to separate partly the four cathepsins.

The exact mechanism by which proteins are adsorbed to hydroxylapatite and displaced from it by phosphate ions has not been defined. From studies of the effects of various anions and cations on the elution of proteins from hydroxylapatite[3,4,14], it appears that protein molecules, as well as phosphate ions, are bound to the calcium sites of the hydroxylapatite crystal. BERNARDI AND KAWASAKI[15] have recently proposed that the adsorption of proteins to hydroxylapatite is due to the interaction of their carboxyl groups with calcium sites on the hydroxylapatite crystal. Their

onclusion is based on the observation that only polypeptides containing free carboxyl roups are strongly adsorbed to hydroxylapatite. While their data establish the interction of carboxyl groups with hydroxylapatite they do not exclude interactions by ther groups. In fact, phosphoproteins have an unusually high affinity for hydroxylpatite[16]. Their hypothesis is also not necessarily consistent with their own observation hat denatured proteins are poorly adsorbed to hydroxylapatite or with the results eported here on the increased affinity of proteins for hydroxylapatite at lower pH alues.

Despite these uncertainties about the mechanism of hydroxylapatite chromatoraphy, it is clear that the protein–hydroxylapatite interaction is decreased by increased phosphate ion concentration. At low phosphate concentrations, proteins are trongly adsorbed to hydroxylapatite. As phosphate concentration increases, the nteraction between protein and hydroxylapatite is reduced and the rate of protein nigration begins to approach that of the solvent phase. If the shift from strong to veak interaction between a particular protein and hydroxylapatite is produced by a mall increase in phosphate concentration, the protein will be eluted within a narrow olvent zone. In practice, however, the behavior of proteins on hydroxylapatite is nore complex and the poor separation of the cathepsins described above exemplifies he principal limitation of this method. The cathepsins were eluted from the column vithin a narrow range of phosphate concentration and were difficult to resolve on ıydroxylapatite by small increases in phosphate concentration. This is due in part to he formation of multiple "false peaks" by the same protein. This phenomenon is vident in the pattern shown in Fig. 1.

The formation of false peaks is probably due to the tendency of proteins to be etarded or readsorbed by hydroxylapatite after their initial elution. Because of this ı given protein will not be completely eluted by a phosphate ion concentration high nough to cause its desorption. With further increase in phosphate concentration, nore of the retained protein is eluted in a second solvent zone to produce a false econdary peak of the same solute in the effluent. This results in mixing of proteins hat would otherwise be separable if critical elution concentration were the only perational factor.

It is difficult to explain this retention of a protein by lower regions of a column ıfter the protein has been initially eluted. HJERTEN[4] observed that a portion of the nigrating zone of the colored protein ceruloplasmin was readsorbed to the hydroxylapatite column and could not be eluted by extended washing with the same buffer. We have observed the same phenomenon with cytochrome *c*. The bulk of adsorbed cytochrome *c* is eluted from hydroxylapatite by 0.15 *M* sodium phosphate buffer (pH 6.8). However, some protein becomes distributed along the length of the column and is resistant to extended elution with 0.15 *M* buffer. Most, but not all of the retained cytochrome *c* can be eluted by 0.2 *M* buffer. This suggests that the retained protein may be even more tightly bound than it was prior to its initial elution.

The readsorption of protein could be explained if phosphate concentration in the lower portion of the column were to fall below the critical elution concentration. Since hydroxylapatite has an affinity for phosphate ions as well as for protein, it is possible that phosphate ions are removed from solution during the passage of buffer through the column. The effect of this would be a deterioration of the advancing front of increased phosphate concentration. The phosphate ion concentration would then

fall below the level at which the protein had been desorbed. At this point the protein would be readsorbed. TISELIUS *et al.*[3] reported that phosphate ions are retarded by hydroxylapatite and we have also found this to be the case; effluent phosphate buffers approach only about 80% of the phosphate concentrations of applied buffers. This suggests that the ionic environment within the hydroxylapatite column is complex and that the migration of a protein cannot be expected to follow the simple pattern that would be obtained if sharp interfaces between the steps of phosphate concentration could be maintained.

Even with the uncertainties and limitations of hydroxylapatite chromatography, its large capacity to resolve complex protein mixtures makes it an effective method for the analysis of the enzymatic complement of lysosomes. The combination of hydroxylapatite with ion-exchange methods offers many possibilities for the separation and purification of lysosomal enzymes.

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