A RAPID METHOD FOR REMOVAL OF ZINC FROM THE METALLO NEUTRAL PROTEASES

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SUMMARY

The use of long-term dialysis techniques to prepare apoenzyme from some of the microbial neutral proteases is limited by their instability to these conditions. Gel filtration over Sephadex G-25 equilibrated with pH 7.2 Hepes buffer (0.01 M) containing 2×10^{-3} M 1,10-phenanthroline and 0.01 M $CaCl_2$ has proved to be a rapid and efficient method for preparation of apoenzyme for thermolysin and the B. subtilis and B. cereus neutral proteases.

The removal of enzymatically essential metal ions has been achieved by a number of methods most employing dialysis techniques with and without the use of chelators (1-4). With most enzymes these methods are very efficient and dependable, particularly if the protein is stable to long-term exposures to buffer and dialysis. However, some enzymes such as the metallo neutral proteases are quite susceptible to denaturation and autolysis under these conditions and considerable losses of enzyme are obtained by these methods. In particular the B. subtilis neutral protease is quite sensitive to long exposure in solution with and without chelators (5). In the course of studies on the mechanism of inhibition of this enzyme and other neutral proteases by 1,10-phenanthroline, it was found that metal-free apoenzyme could be prepared rapidly by use of gel filtration techniques employing chelator equilibrated Sephadex gel columns. These studies are reported here.

MATERIALS AND METHODS

Reagent 1,10-phenanthroline was purchased from Fisher Scientific Company. Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) was purchased from Calbiochem. Only reagent grade salts and distilled water (conductivity 5×10^{-7} mho) were used throughout these studies. Particular care was exercised to extract glassware with dithizone/CCl_4 before use.

Sephadex G-25-300 was purchased from Sigma Chemical Company. The beads were equilibrated with pH 7.2 Hepes buffer (0.01 M) containing 0.01 M $CaCl_2$ both with and without 2×10^{-3} M 1,10-phenanthroline. For preparation of apoenzyme the 1,10-phenanthroline equilibrated Sephadex G-25 was packed into columns of various sizes depending upon the amount of enzyme to be used. Generally, for the preparation of apoenzyme from large amounts of enzyme (25 mg) a column size of 10x3 cm was used. The enzyme was put on the column in the pH 7.2 Hepes buffer (0.01 M) containing 2×10^{-3} M 1,10-phenanthroline and 0.01 M $CaCl_2$ and eluted with the same buffer. The apoenzyme came off at the void volume and was detected by assay in the presence of 10^{-5} M zinc.

The substrate 3-(2-furylacryloyl)-glycyl-L-leucine amide (FA-Gly-Leu-NH_2) (m.p. 175-176°) was synthesized as described (6). Thermolysin was purchased from Daiwa Kasei (Osaka, Japan) and recrystallized from calcium acetate solutions. The *B. subtilis* NRRLB341 neutral protease was isolated from filtration cultures as described (7). *B. cereus* neutral protease was isolated in highly purified form from culture filtrates (to be published).

The enzyme catalyzed hydrolysis of FA-Gly-Leu-NH_2 was monitored spectrophotometrically at 345 mμ using a Cary 14 PM recording spectrophotometer equipped with a thermostated cell compartment maintained at 25.0±0.1° as described (6,8). Zinc

content was determined by atomic absorption using a Beckman atomic absorption accessory (acetylene-air laminar flow burner) attached to a Beckman DU spectrophotometer and a ten-inch recorder.

RESULTS AND DISCUSSION

The stability of the bacterial neutral proteases is very sensitive to the calcium concentration of the preparations. This is true for both the thermostable thermolysin and the much less stable *B. subtilis* and *B. megaterium* enzymes. Methods designed to remove the catalytically important zinc, therefore, must not remove the calcium ions. The problems encountered in preparing the apo-enzyme of the *B. subtilis* neutral protease by use of EDTA are most probably due to the simultaneous removal of calcium. On the other hand, phenanthroline binds calcium very weakly (log K_1 = 0.7) while exhibiting a good binding for zinc (log K_1 = 6.36; log K_2 = 5.64; log K_3 = 5.20) (9) and such chelator calcium solutions would be useful for preferential removal of zinc. At a concentration of 2×10^{-3} M 1,10-phenanthroline and 10^{-2} M $CaCl_2$ less than one per cent of the calcium ions are complexed by the chelator leaving excess calcium for stabilization of the enzyme and excess chelator for zinc removal. Vallee and coworkers (4) successfully used dialysis against 1,10-phenanthroline containing calcium ions to prepare stable zinc-free thermolysin.

Figure 1 compares the chromatograms of the *B. subtilis* neutral protease obtained from a Sephadex G-25 column (21x1 cm) equilibrated and eluted with pH 7.2 Hepes buffer (0.01 M) (0.01 M $CaCl_2$) with and without 2×10^{-3} M 1,10-phenanthroline. In the presence of chelator greater than 95 per cent of the zinc was removed from the enzyme to yield an inactive protein peak which fully reactivated by addition of zinc (10^{-5} M) to the substrate enzyme solution. These columns ran quite rapidly to give the

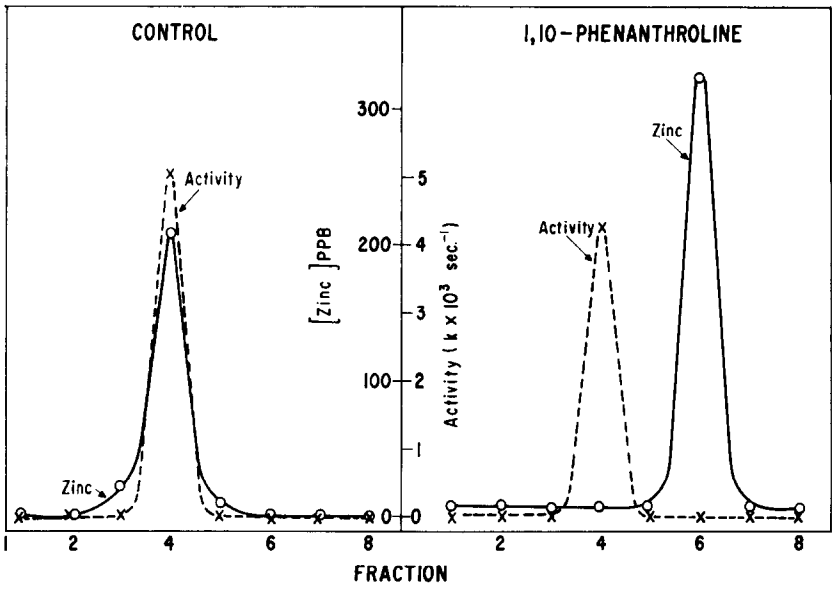

Figure 1. Removal of Zinc from B. subtilis Neutral Protease by Gel Filtration Over Sephadex G-25 Equilibrated with 1,10-Phenanthroline
($k = kcat(Eo)/Km$; 9.76×10^{-4} M FA-Gly-Leu-NH_2, pH 7.2 Hepes buffer (0.1M); other details in text).

apoenzyme peak at the void volume within 10-20 minutes of application of the enzyme to the column, depending on the size of the column.

The 1,10-phenanthroline was readily removed from the apoenzyme fraction by passage over a second Sephadex G-25 column equilibrated with pH 7.2 Hepes buffer (0.01 M) containing 0.01 M $CaCl_2$. Care was exercised to exclude contaminating metals from this second column. For the preparation of various metallo derivatives from the apoenzyme, it was not necessary to remove the 1,10-phenanthroline. Excess metal was added to the apoenzyme chelator fraction to complex all of the chelator. This could then be passed over a second column to remove the 1,10-phenanthroline without fear of contamination by other metals which are readily bound to the metal-free apoenzyme.

TABLE 1

PREPARATION OF APOENZYME BY GEL FILTRATION OVER SEPHADEX G-25 EQUILIBRATED WITH 1,10-PHENANTHROLINE

Enzyme	Zinc Content		Per Cent Activity[a]		
	Native	Apo	Native	Apo No $ZnCl_2$	Apo $+10^{-5}$M $ZnCl_2$
	PPB				
Thermolysin	700	42	100	9	100
Neutral Protease					
B. subtilis	200	<5	100	14	86
B. cereus	216	<5	100	1.5	100

[a]Activity measured using FA-Gly-Leu-NH_2 as substrate

This method also has been used with other neutral proteases. Removal of greater than 95 per cent of the zinc from thermolysin and the *B. cereus* neutral protease to yield inactive apoenzyme was readily achieved. These apoenzymes were reactivated to full activity by addition of zinc ions. Table 1 shows the zinc analysis and activity towards furylacryloyl glycyl-L-leucine amide for thermolysin, *B. subtilis* neutral protease and the *B. cereus* neutral protease. This method could be used in demonstrating the role of metal ions such as zinc in new enzymes.

In conclusion, gel filtration using chelator equilibrated columns provided a simple and rapid method for the preparation of apoenzymes.

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