relatively great reactivity toward dansyl chloride of the lysine residue in rennin suggests an alternative function, *viz.*, that it may be part of a positively charged site which binds to an anionic part of the substrate, as proposed for lysine in ribonuclease[16]. More definite knowledge on this point may be gained from a study of the amino-acid sequence near the dansylated lysine.

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Received June 6th, 1966
Revised manuscript received September 13th, 1966

Biochim. Biophys. Acta, 132 (1967) 188–190

BBA 63230

Purification of two alkaline phosphatases from *Aspergillus nidulans*

It has been shown previously that under conditions of limiting phosphate, *Aspergillus nidulans* produces two electrophoretically distinct alkaline phosphatases (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1). The activity of phosphatase I is under the control of at least nine genetic loci whereas no mutations have ever been isolated which affect the activity of phosphatase II (refs. 1,2). Kinetic growth studies have suggested that the synthesis of phosphatase I is repressible by high levels of inorganic phosphate while phosphatase II appears to be a constitutive enzyme[3]. The genetic and growth studies indicate strongly that the two phosphatases are distinct enzymes; however, very little is known about the biochemical nature and physical structure of these enzymes. This paper describes a procedure for chemically separating the two phosphatases. Electrophoretically homogeneous preparations of phosphatase I have been obtained. Evidence is presented that the two enzymes have different pH optima and molecular weights.

Phosphatase activity was measured by observing the absorbance change at

410 mμ for a 2-min interval at 30° in a total volume of 1 ml containing an appropriate dilution of the enzyme and the following in μmoles: Tris chloride (pH 9.5), 50; *p*-nitrophenylphosphate (Sigma), 10; $MgCl_2$, 10. One unit of enzyme activity was defined as the release of 1 μmole of *p*-nitrophenol per min. Protein was measured by the LOWRY *et al.*[4] modification of the phenol method.

A biotin-requiring strain (*bil*) of *A. nidulans* was grown in 2-l flasks containing 1500 ml limiting phosphate glucose medium with 30 μg biotin supplement[1]. After 60 h growth in a New Brunswick Gyrotory Shaker, the mycelium was harvested on a Buchner funnel and suctioned dry. Approximately 500 g of mycelium were resuspended in 400 ml of 0.025 M sodium veronal and extracts were prepared by homogenizing this mixture at 4° in a Waring Blendor. After centrifuging at 9000 × *g* for 30 min, four parts of supernatant were shaken with one part of chloroform for 15 min. The precipitate was spun down at 2000 × *g* and the chloroform treatment was repeated a second time. Ammonium sulfate was added to the resulting supernatant and the fractions precipitating between 53% and 68% of saturation and between 68% and 90% of saturation were redissolved in 0.01 M veronal buffer (pH 7.4). Both ammonium sulfate fractions were centrifuged at 12 000 × *g* for 30 min and then desalted on a Sephadex G-50 column equilibrated with an 0.01 M $MgCl_2$ solution in 0.01 M veronal buffer (pH 7.4). Magnesium appeared to be essential for full enzyme activity and all solutions described below contained 0.01 M $MgCl_2$.

Phosphatase I was obtained by treating the 68% to 90% ammonium sulfate material with chloroform 3 times and then applying the supernatant to a Cellex aminoethyl-cellulose column (1 cm × 10 cm) equilibrated with 0.05 M NaCl in 0.01 M veronal buffer (pH 7.4). The column was washed with 100 ml of 0.05 M NaCl in 0.01 M veronal buffer (pH 7.4) and the enzyme was eluted with 0.15 M NaCl in the same buffer. The enzyme material was then applied directly to a Cellex DEAE-cellulose column

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURE

Fraction	*Vol. (ml)*	*Phosphatase I and II*		
		Protein (mg)	*Total activity (units)*	*Specific activity (units/mg)*
(1) Crude extract	850	2500	1400	0.56
(2) Centrifugation	690	965	540	0.56
(3) Chloroform	665	830	490	0.59

Fraction	*Vol. (ml)*	*Phosphatase I*			*Vol. (ml)*	*Phosphatase II*		
		Protein (mg)	*Total activity (units)*	*Specific activity (units/mg)*		*Protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*
(4) Ammonium sulfate	42	26.9	324	12.0	40	11.6	145	12.5
(5) Chloroform	35	20.3	230	11.3	—	—	—	—
(6) Aminoethyl-cellulose	53	9.2	120	13.1	44	4.2	56	13.3
(7) DEAE-cellulose	40	2.5	77	30.8	20	0.7	40	57.3

(1 cm × 10 cm) previously equilibrated with 0.1 M NaCl in 0.01 M veronal (pH 7.4). Sequentially, 100 ml each of 0.1, 0.15 and 0.2 M NaCl in the same buffer were applied to the column. Phosphatase I was eluted with the 0.2 M NaCl solution.

Phosphatase II was obtained by absorbing the 53% to 68% ammonium sulfate fraction on to a Cellex aminoethyl-cellulose column (1 cm × 10 cm) previously equilibrated with 0.05 M NaCl in 0.01 M veronal (pH 7.4). This enzyme was also eluted with a 0.15 M NaCl solution. The enzyme material was desalted on a Sephadex G-50 column equilibrated with 0.01 M veronal (pH 7.4) and applied to a Cellex DEAE-cellulose column previously equilibrated with 0.05 M NaCl in 0.01 M veronal (pH 7.4). Sequentially, 100 ml of each 0.1 and 0.15 M NaCl in the same buffer were applied to the column. Phosphatase II was eluted with the 0.15 M NaCl solution.

The results of the purification procedure are summarized in Table I. Starch gel electrophoresis of the separate stages of the purification procedure revealed that the two alkaline phosphatases are almost completely separated from each other by the salt treatment. The cellulose columns were used to remove the traces of the contaminating alkaline phosphatase, the acid phosphatases and other extraneous material. Starch-gel electrophoresis indicated that the final material contained only the appropriate alkaline phosphatase. Although the two alkaline phosphatases can be completely separated from each other on the DEAE-cellulose column, the use of ammonium sulfate and the aminoethyl-cellulose column steps resulted in the recovery of more highly purified material.

Ultraviolet spectra of both the phosphatase I and II preparations indicated absence of nucleic acids. Acrylamide electrophoresis[6] of the phosphatase I material revealed only a single protein band which coincided with phosphatase activity. However, the phosphatase II material gave five protein bands on acrylamide. Only one of the five protein bands had phosphatase II activity; the other four bands are either inactive breakdown products of the enzyme or contaminating proteins.

Fig. 1 shows the pH optima of phosphatase I and II. Phosphatase I has two pH optima, one at 8.0 and a second above 10.0. Phosphatase II has a single pH optimum at 9.5. Neither enzyme has any appreciable phosphatase activity below pH 5.5.

Table II gives an estimate of the molecular weights of the alkaline phosphatases

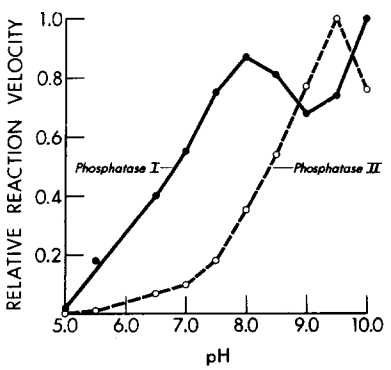

Fig. 1. Effect of pH on the rate of purified alkaline phosphatase I and II against *p*-nitrophenylphosphate. Incubation mixtures contained 0.05 mM Tris, 0.05 mM sodium acetate, 0.01 mM nitrophenylphosphate, 0.01 mM $MgCl_2$ and 5 μg phosphatase I or II in a final volume of 1 ml. Measurements were made at 30°.

TABLE II

MOLECULAR WEIGHT OF THE PHOSPHATASE ENZYMES

1 ml of purified phosphatase I or II (0.1 mg/ml) in 0.05 M Tris (pH 7.5) together with catalase (0.2 mg/ml) and ceruloplasmin (1 mg/ml) was placed directly on a 30 ml, 5 to 20% sucrose gradient and centrifuged at 4° for 40 h in a SW 25.1 bucket rotor at 25 000 rev./min. Ceruloplasmin was measured at 610 mμ; catalase was assayed by following the method of MARTIN AND AMES[5]. Neither purified preparation showed any heterogeneity for phosphatase activity.

"Standard"	*Phosphatase I*	*Phosphatase II*
Catalase	180 000	145 000
Ceruloplasmin	190 000	155 000
Average	185 000	150 000

which is based on the method of MARTIN AND AMES[5]. Catalase (mol.wt. = 250 000) and ceruloplasm (mol.wt. = 160 000) were used as standards. The approximate molecular weight of phosphatase I is 185 000, while that of phosphatase II is 150 000. The variation for the two standard proteins may either be due to a difference in shape or an inaccuracy in reporting the molecular weight.

It is now apparent that the two genetically and electrophoretically distinct alkaline phosphatases of *A. nidulans* are indeed different enzymes. They can be readily separated from one another and have decidedly different pH optima and molecular weights. Genetic and electrophoretic analysis of the phosphatases in this organism have led to the proposal that the two alkaline phosphatases have polymeric structures[1]. The large molecular weights of these enzymes are consistent with this view.

This work was supported in part by a grant from the National Institute of Health (RO1 GM 12711-01) and by grants from the Health Research Council (I-453 and U-1775).

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Received October 17th, 1966