Isolation and purification of an acid phosphatase from baker's yeast (Saccharomyces cerevisiae)

Most yeasts contain at least 2 non-specific phosphatases. Several investigators[1-5] have shown that the acid phosphatase (EC 3.1.3.2) of baker's yeast with a pH optimum between 3 and 4 is localized in the cell wall. The alkaline yeast phosphatase (EC 3.1.3.1) with a pH optimum between 9 and 10 is situated in the cytoplasm[1-3].

In order to understand how these non-specific phosphatases, with optimal enzymatic activity at entirely different pH values, exert their catalytic action, we have embarked upon a comparative investigation of their mechanism of action at the molecular level.

For this purpose both enzymes first have to be isolated and purified. The isolation and purification of the acid phosphatase has now been achieved and forms the subject of this report.

To obtain a good starting material commercial baker's yeast ("Koningsgist") is cultivated (21 h, 27°) in a medium[5] poor in phosphate, as first described by SUOMALAINEN, LINKO AND OURA[3]. In this way an approximately 40-fold enrichment in acid phosphatase can be achieved. From this phosphatase-enriched yeast the enzyme is isolated by shaking for 2 min with glass beads (No. 12), keeping the temperature below 18°, in a MERKENSCHLAGER apparatus[6]. After centrifugation of the yeast homogenate at 15 000 × g for 1 h the inactive sediment is discarded, and the supernatant is stored at −15° overnight[7]. A considerable amount of inactive cell material precipitates and can be removed by sedimentation, leaving all the activity in the clear yellow cell-free extract.

From this extract the enzyme is concentrated by precipitation with 50% (v/v) aqueous ethanol at −2°. An ethanol fractionation follows, in which an inactive precipitate is removed at 25% ethanol, while the acid phosphatase is again precipitated from the supernatant at 50% ethanol (bright yellow preparation: "ethanol precipitate").

This preparation is filtered through Sephadex G-75 gel, producing several discrete peaks. Fractions of the first peak, containing all the activity, are pooled and concentrated by means of precipitation at 50% ethanol. The colorless preparation
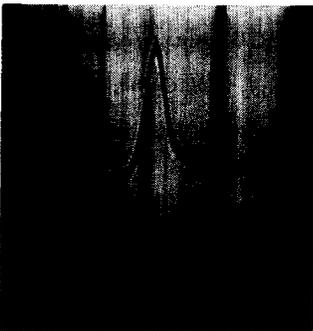

Fig. 1. Sedimentation pattern of acid phosphatase preparations in 0.1 M acetate buffer, (pH 4.2). Time, 96 min after reaching 56 100 rev./min. Temperature, 7°. Upper: before gradient centrifugation (Sephadex filtrate); lower: after gradient centrifugation.

thus obtained, "Sephadex filtrate", exhibits 2 main components in the analytical ultra-centrifuge (Fig. 1). The fast component contains all the acid phosphatase activity, while carbohydrate is present in both.

Many procedures were attempted in order to separate the 2 components. It was ultimately achieved by ultracentrifugation on a sucrose gradient (5–20% (w/v) sucrose, centrifugation at 90 600 × g for 21 h). All the activity is localized in 1 peak, from which fractions are collected, concentrated and dialyzed against 0.1 M acetate buffer (pH 4.2). As can be seen in Table I, gradient centrifugation removes a great deal of carbohydrate and little protein.

The preparation now obtained, "gradient centrifugate", usually shows a single peak in the analytical ultracentrifuge (Fig. 1). Homogeneity is confirmed by electro-phoresis on polyacrylamide gel at pH 4.0. A slow-moving band contains the activity. All proteins moving more rapidly at this pH, and present in earlier steps of the purifi-cation, have disappeared. This is also the case upon electrophoresis at pH 8.6.

The pH-stability range of the enzyme is not very broad, namely from pH 3.5 to 5.0 for the cell-free extract and from pH 3.0 to pH 5.4 for the gradient centrifugate. At lower or higher pH irreversible inactivation takes place.

TABLE I

SUMMARY OF THE PURIFICATION PROCEDURE

One enzyme unit is defined as the amount which will hydrolyze 1 μmole p-nitrophenyl phosphate per min in 0.1 M acetate buffer (pH 3.8), at 30°. Specific activity is related to total nitrogen (N) and also to carbohydrate, expressed as glucose (Glc).

Preparation	Volume (ml)	Total activity (units)	Yield (%)	Specific activity	
				(units/ mg N)	(units/ mg Glc)
Commercial baker's yeast (533 mg/ml)	150	$4.0 \cdot 10^2$		0.22	0.083
Phosphatase-enriched yeast (476 mg/ml)	420	$4.2 \cdot 10^4$	100	8.3	3.8
Cell-free extract	1060	3.2	76	19	12
Ethanol precipitate	63	2.9	69	280	62
Sephadex filtrate	2.5	2.3	55	470	57
Gradient centrifugate	8	1.2	29	490	106

While the ethanol precipitate preparation can be stored at −15° for months, the Sephadex filtrate and the gradient centrifugate are irreversibly inactivated by freezing and thawing. All preparations keep well at room temperature and at +2°.

Estimation of the Michaelis constant K_m with p-nitrophenyl phosphate and with β-glycerophosphate as substrates, both for commercial baker's yeast and for the gradient centrifugate ($K_m = 1.6 \cdot 10^{-3}$ M, $1.3 \cdot 10^{-3}$ M, respectively, with p-nitro-phenyl phosphate; $5.0 \cdot 10^{-3}$ M, $3.1 \cdot 10^{-3}$ M, respectively, with β-glycerophosphate), seems to indicate that the purified phosphatase is indeed the enzyme originally present in the yeast, but more substrates and modifiers will have to be tested to make sure.

The molecules in the gradient centrifugate are large: the sedimentation co-efficient is about 15 S. This preparation still contains carbohydrate. Upon estimation we found about equal amounts of carbohydrate and protein (calculated as 6.25 times

total N). After hydrolysis of the gradient centrifugate (4 h, 100°, 1 M HCl) qualitative analysis by thin-layer chromatography on Kieselgel G (ref. 8) indicates the presence of mannose as the main carbohydrate component. No other sugars could be detected in this way.

Work is in progress to establish whether the carbohydrate moiety is required for the stability and/or the activity of the enzyme.

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Isolation of a xylanase from a commercial cellulase preparation

A pure xylanase (β-1,4-xylan xylanohydrolase, EC 3.2.1.8) was needed for the degradation of soluble wheat flour pentosans[1]. Xylan-splitting enzymes are present in many fungal enzyme preparations high in cellulase and hemicellulase activities[2]. For structural studies on complex polysaccharides, however, only purified enzymes should be used with known substrate specificities. Of a number of commercial enzyme preparations tested for xylanase activity, the 'cellulase crude' obtained from Miles Chemical Co., Clifton, N.J. (U.S.A.) showed the highest activity. This preparation was therefore used as starting material for the isolation of a xylanase. In addition to xylan the following substrates were hydrolyzed by the crude enzyme: CM-cellulose, pectin, starch, araban and proteins.

Water-soluble CM-xylan prepared by carboxymethylation of wheat straw xylan[3] was used as a substrate. The hydrolysis was followed by determination of the reducing groups liberated using the 3,5-dinitrosalicylic acid–phenol reagent[4]. One xylanase unit corresponds to the amount of enzyme that liberates 1 μmole of reducing group (as xylose) per min at 25° under optimal conditions.

Separation of a practically pure xylanase from the crude preparation was achieved by $(NH_4)_2SO_4$ precipitation, chromatography on hydroxyapatite and gel filtration as follows: 10 g of crude cellulase (Miles Chemical Co.) (4.3 units/mg) were