VISUALIZATION OF ATPases OF *ASPERGILLUS NIDULANS* ON POLYACRYLAMIDE GELS BY LEAD NITRATE

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ABSTRACT

A simple and rapid method for the visualization of ATPases on polyacrylamide gels after electrophoresis has been developed. The inorganic phosphate liberated by ATPases from ATP gives a white precipitin with lead nitrate at the positions of ATPase activity on gels. Sodium vanadate inhibits the activity of ATPases in assay mixtures and on polyacrylamide gels.

INTRODUCTION

ATPases from a variety of sources have been visualized by staining on polyacrylamide gels[1-7]. Cytochemical methods combined with electron microscopy have also been used to study the localization of ATPases in whole cells and in isolated membranes of several bacterial species[8-10]. A general principle for cytochemical 'staining' is to get the inorganic phosphate liberated from ATP by ATPase and thereafter to precipitate the phosphate with suitable cations. There is no specific stain for this enzyme on gels; it is stained non-enzymatically with Coomassie blue, a general protein stain, after being purified free from other proteins. Current methods for purifying ATPases are based on detergent solubilization followed by density gradient centrifugation. After purification, the sample is mixed with a suitable concentration of sodium dodecyl sulphate to provide negative charges to the polypeptides which upon electrophoresis separate on a gel according to their molecular weights. The separated polypeptides are stained non-enzymatically by Coomassie brilliant blue. This procedure yields an active enzyme of high purity but takes several days. Moreover, it needs absolute purification of the ATPases, which is cumbersome. A simple but indirect method of visualization of ATPases has been used by Selvam[6]. After electrophoresis the gel is incubated with ATP solution and the phosphate liberated from ATP as a result of ATPase action is precipitated *in situ* with $CaCl_2$. The number of precipitin bands on the gels indicates the number of isoenzymes. This method of visualization of ATPases on gels is also not suitable as it takes about 5–6 h for the bands to appear. Moreover, it gives a white deposit on the whole gel, making it difficult to localize the white precipitin bands. This paper describes a simple and rapid method for the visualization of ATPases of *Aspergillus nidulans* enzymatically on polyacrylamide gels. This method has been employed to see the differential effect of vanadate on the isoenzymes of ATPase on the gels and on their specific activities in assay mixtures.

MATERIALS AND METHODS

The *riboA1, biA1* strain of *A. nidulans* was grown in Czapek–Dox medium supplemented with riboflavin and biotin in liquid shake cultures[11] (150 rpm) at 37°C for 24 h. The mycelium was collected by filtration after washing with distilled water and 0·01 M Tris–HCl buffer (pH 7.5). All the subsequent steps were carried out at 4°C. The mycelium (1 g wet wt) was homogenized using a tissue homogenizer. The homogenate was centrifuged at 800 *g* for 10 min to remove intact cells, cell debris and nuclei. The supernatant was again centrifuged at 10,000 *g* for 30 min. The resulting supernatant containing the membrane fraction was used for the present experiments. Protein was estimated by the method of Lowry *et al*[12] using bovine serum albumin as the standard.

The enzyme reaction mixture contained 1 ml of 6 mM ATP (prepared in buffer) and 1 ml of diluted enzyme sample. After incubation for 20 min at 37°C, the reaction was terminated by adding 1 ml of chilled trichloroacetic acid (10% w/v), assay tubes were centrifuged and the liberated inorganic phosphate was estimated in the supernatant by the method of Heinonen and Lahti[13]. Specific activity was expressed as μmol of Pi released per 20 min per mg of total protein.

Disc electrophoresis in polyacrylamide gel was performed by a slight modification of the method described by Davis[14], using 30% acrylamide and 0.6% bisacrylamide. Polymerization was catalysed by 0.14% ammonium persulphate and 0.22% N, N, N′, N′-tetramethylethylenediamine (TEMED). The final acrylamide percentage in the running gel was 7.5. About 200 μg of the protein sample in sucrose solution was applied onto the gel. Tris–glycine buffer (pH 8.3) was used in both the tanks. Electrophoresis was carried out at 4°C with initially a current of 0.5 mA/tube for one hour and thereafter a current of 1 mA/tube for two and a half hours. After electrophoresis the gels were incubated with ATP (50 mM in Tris-HCl buffer pH 7.5) at 37°C for 20 min. They were washed for 5–10 sec to remove ATP from the gel surface. The washed gels were dipped into tubes containing 10 mM lead nitrate (in distilled water). After half an hour white band-like precipitin appeared on the gels. If necessary, the stained gels were washed with distilled water to make the bands more clear and distinct.

RESULTS AND DISCUSSION

ATPase isoenzymes separate on the gel according to their size and charge densities. After incubation with ATP each isoenzyme liberates inorganic phosphate from ATP *in situ* on the gel. The liberated phosphate gives a white precipitate with lead nitrate. Four bands representing four isoenzymes of ATPase were observed in preparations from *A. nidulans* (figure 1a). Selvam and Shanmugasundaram[7] were able to detect only three isoenzymes of ATPase in extracts from the same strain of *A. nidulans*. They used $CaCl_2$ (50 mM) and ATP (50 mM) together in Tris–HCl buffer (pH 7.5) to liberate and precipitate inorganic phosphate on the gels. In the present investigation, an additional band (band IV), over and above the three obtained by Selvam and Shanmugasundaram[7], was obtained. They might have missed it because of its (band IV) low activity and consequently a low amount of inorganic phosphate released, which in turn could not be visibly precipitated by $CaCl_2$.

There was inhibition of the activity of ATPase in the presence of increasing concentration of vanadate up to 50 μM, at which there was about 70% inhibition (figure 2).

To see the differential effect of vanadate on the isoenzymes after electrophoresis, the gels were kept in tubes containing vanadate (25 and 50 μM) for

Figure 1a–c. Effect of vanadate on activity of ATPase isoenzymes. **a.** Control, **b.** 25 μM vanadate, and **c.** 50 μM vanadate.

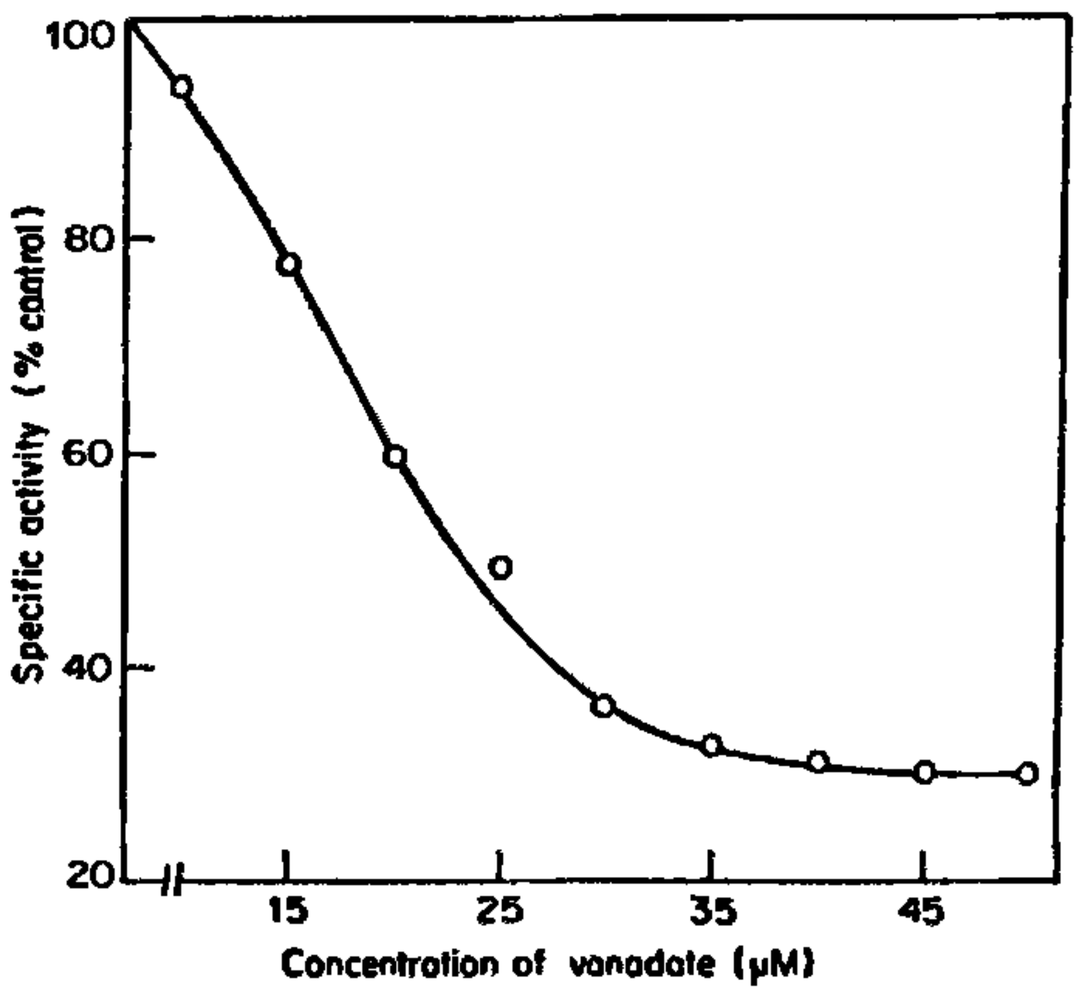

Figure 2. Inhibition of ATPase activity by sodium vanadate.

30 min. Thereafter, staining and destaining were performed as described in Materials and Methods. The inhibition of the enzymatic activities by vanadate was reflected in the intensities of the

ATPase bands on the gels. The number of isoenzymes on the control gel (no treatment with vanadate) was four (figure 1a), whereas only three and one bands were visible on gels treated with 25 and 50 μM of vanadate respectively (figure 1b, c). Thus it appears that inhibition of ATPase by vanadate in *A. nidulans* is due to a progressive inhibition of the different isoenzymes with increasing concentration. There appears to be a direct correlation between the number of isoenzymes (bands) and the specific activity of ATPase in the presence of this inhibitor.

Vanadate has been found to be a potent inhibitor of the plasma membrane ATPases of *Neurospora crassa*[15], *Saccharomyces cerevisiae*[16] and *Duniella parva*[17]. In *N. crassa* it has been reported to inhibit up to 95% of the plasma membrane ATPase at 50 μM. In *D. parva* the plasmalemmal ATPase is inhibited by about 50% at only 1 μM of this inhibitor. However, inhibition by vanadate of the activity of the isoenzymes on gels has not been shown for ATPase from these organisms.

To determine if this method of precipitation of inorganic phosphate liberated from ATPase–ATP interaction was also applicable to alkaline and acid phosphatases for the precipitation of inorganic phosphate liberated from their substrate, *p*-nitrophenyl phosphate, the same procedure was used, except that ATP was replaced by *p*-nitrophenyl phosphate and the incubation was at 30 C (the optimum temperature for the phosphatases). The phosphate liberated by the action of the phosphatases could not be precipitated. Thus the method appears to be specific for the precipitation of phosphate liberated as a result of ATPase action.

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