Detection of Nonspecific Phosphodiesterase in Polyacrylamide Gels[1]

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Abstract

Nonspecific phosphodiesterase purified from bovine spleen was separated by electrophoresis in polyacrylamide gel at pH 4.3. The gels were scanned in a spectrophotometer, incubated with bis-p-nitrophenyl phosphate, treated with ammonium hydroxide and scanned again. It was possible to both localize and quantitate the enzyme activity in the gel.

Introduction

One of the puzzling features of DNase II is its intimate association with an activity against bis-p-nitrophenyl phosphate (PNPP) at pH 5.7. This close association has persuaded Bernardi (1) and others (6, 7) that the two activities are ascribable to a single protein. In order to test this hypothesis, we have conducted a number of studies (3, 5, 8, 9) and have succeeded in obtaining a nonspecific phosphodiesterase (PDE) free of DNase activity. The zymogram method described below was developed so that the purification could be monitored.

Materials and Methods

PDE was prepared from bovine spleen by extraction with water, acidification to pH 3 overnight, adjustment to pH 4.5, clarification at 15,000-34,000 xg, and dialysis against running distilled water followed by chromatography on CM-cellulose (9). The column eluate was concentrated, dialyzed again, and loaded onto a 110-ml Ampholine column, pH 3-10. After focusing for 40 hours the fractions containing PDE but not DNase were collected and refocused in a pH 8-11 gradient. The PDE focused at pH 9.2.

Gel electrophoresis was performed in 5 mm discs of 7.5% polyacrylamide in the anodic system of Davis (2) or the cathodic systems of Reisfeld *et al.* (4). The latter was used for the zymograms. Thin layers of gel on microscope slides have also been used. The disc gels were scanned in a Gilford spectrophotometer at 280 and 400 nm prior to incubation in PDE substrate. The zymograms were incubated in PDE substrate (PNPP, acetate buffer, pH 5.7 and Tween 80 [3]) at 37° and developed by addition of 2 N ammonium hydroxide. This stopped the reaction and developed the color. The gel was transferred immediately to the quartz gel boat of the Gilford linear transport for scanning. Duplicate gels were stained for visualization of protein with Amido Swartz 10B: Coomassie brilliant blue.

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Results

When gels are scanned before incubation in PDE substrate the only absorption at 400 nm is in the region of the dye marker, whereas several protein peaks and the marker appear following scanning at 280 nm (Fig. 1). After incubation in PNPP and development with ammonium hydroxide at least one peak of PDE activity is visible at 400 nm. The incubation causes only slight changes in the gel size although it is difficult to reposition the gel exactly in the boat. Compensation for the shift is possible because the dye marker absorbs at both 280 and 400 nm before incubation in PNPP and the individual traces can be aligned by use of the marker. The stained gels can be scanned for protein at 540 nm. The 540 nm and 280 nm scans of the stained and unstained gels respectively compare favorably with the naked eye scan of the stained gel.

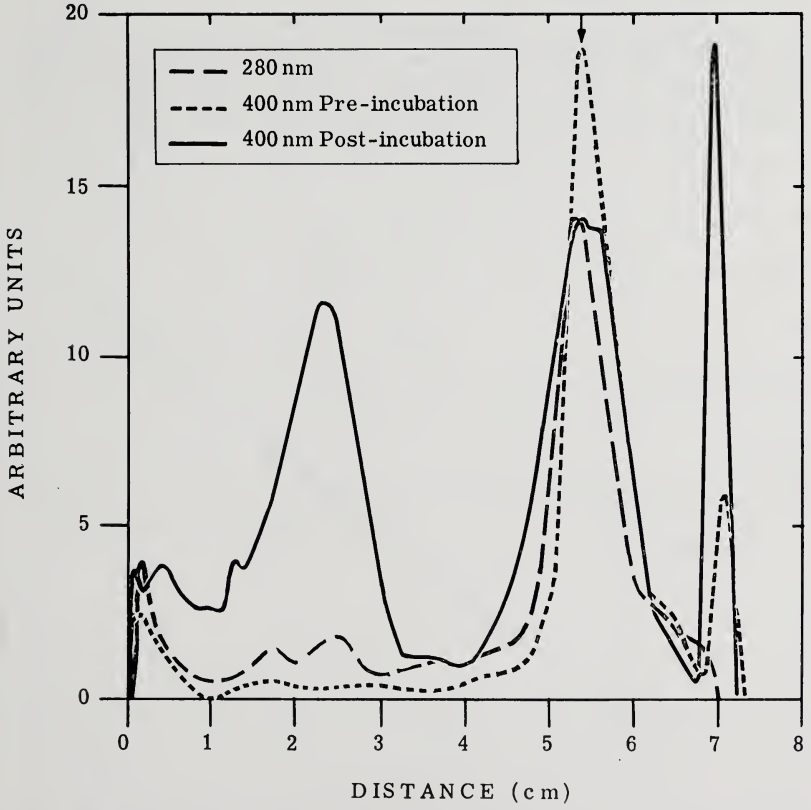

FIGURE 1. *Variation of absorbance with gel length before and after treatment with nonspecific phosphodiesterase substrate. Gels were scanned at 280 and 400 nm before incubation in nonspecific phosphodiesterase substrate and at 400 nm after incubation. The scans were aligned by superposition of the dye marker (arrow). The absorbance is shown in arbitrary units as the ordinate and the position of the absorbing material in the gels as the abscissa.*

To determine the variation of color intensity with time, a series of zymograms was incubated as described for 10, 20 and 60 min. The gels were developed and scanned and the height of the 400 nm peak corresponding to PDE determined. The reaction was linear to 20 min but damped off at 60 min.

The variation of absorbance at 400 nm with protein concentration was determined after incubation for 20 minutes. The absorbance, as seen in Fig. 2, increases linearly over at least a 3-fold range.

Discussion

Zymograms have contributed to an understanding of polymorphism in enzymes, and studies of isozyme patterns in health and disease are numerous (10). These methods utilize differences in mobility of activity in the gel to localize enzyme variants. The quantity of enzyme is determined roughly by comparison, using the naked eye, of the intensity of staining of adjacent areas.

The studies presented here show that PDE activity can be localized by reaction with PNPP. Furthermore the reaction rate has been shown to be a linear function of time over a 20 min period and of protein concentration over at least a 3-fold range. This means that PDE can

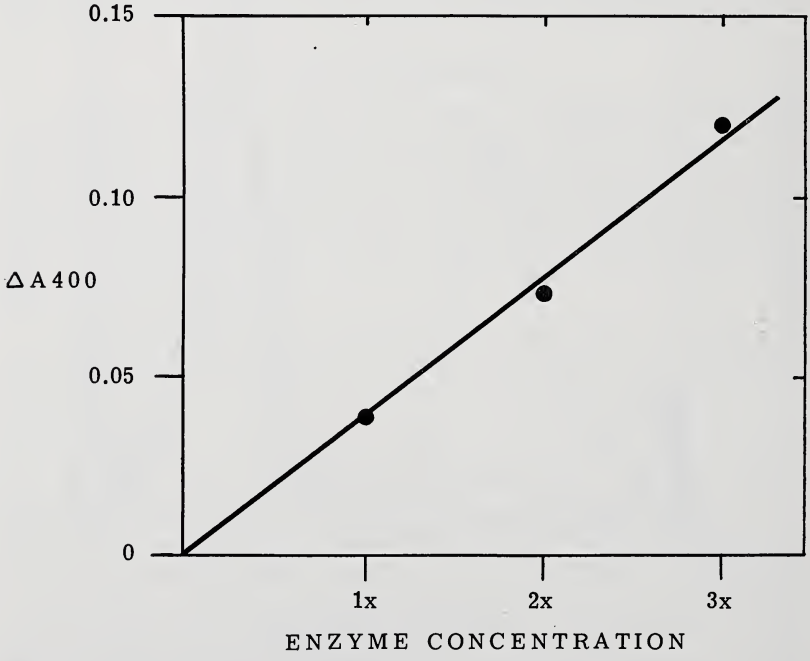

FIGURE 2. *Variation of enzyme activity with concentration. Either 1, 2 or 3 μl of a solution of nonspecific phosphodiesterase containing 0.003 units/ml was loaded on polyacrylamide gel discs and the proteins separated by electrophoresis. After incubation with substrate the gels were scanned as in Figure 1. The difference in A_{400} of the peak and the base line was determined and plotted as the ordinate (ΔA_{400}).*

be quantitated in the gel and the ratio of isozymic activities, if found, compared. As quantitation of both protein and PDE activity can be done, the specific activity (enzyme units/A_{280}) can be calculated. It is also possible that the enzyme can be recovered from an unstained, undeveloped disc, although this has not yet been accomplished. A recent paper (11) describes a method similar to ours for quantitation of human tissue esterases. Quantitation is more complicated, and requires integration of the area under the spectrophotometer trace, rather than measurement of peak height as in our method.

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