ENZYMATIC ACTIVITY OF TNB BLOCKED PORCINE PANCREATIC AMYLASE

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1. Introduction

Porcine pancreatic α-amylase (α-1,4-glucan-4-glucano-hydrolase, EC 3.2.1.1) contains 2 SH groups [1]. Both SH react with mercuric ions without any loss of enzyme activity. However these groups do not react with bulky thiol specific reagents such as iodo-acetamidonaphtol and DTNB : 5,5′-dithiobis-(2-nitro-benzoic acid). Addition of EDTA to amylase promotes a rapid blocking of the SH by these reagents [2–4]. In addition EDTA makes the enzyme partially accessible to trypsin [5,6]. Blocking of the 2 SH by the same reagents induces a partial or complete loss of enzyme activity [2–4]. The presence of the SH at the active center of the enzyme was thus questioned.

In the present work we report an improvement in the purification method of the enzyme. Newly purified amylase incubated with or without EDTA is highly stable. We confirm that $(TNB)_2$ amylase is still partially active. Kinetic studies of the modified enzyme indicate that the V_m is decreased by a factor of 4 while the K_m is unchanged. Finally we have shown that $(TNB)_2$ amylase has identical binding properties to polydextran gel as native amylase.

2. Materials and methods

The reaction of DTNB with SH groups was carried out at 25°C according to the method of Ellman [7]. The incubation mixture contained 10-20 μM amylase in 1 mM NaCl, 40 mM Tris–HCl buffer, pH 7.9, 0.2 mM DTNB and 10 mM EDTA. The number of reacting groups was calculated from the absorbance at 412 nm, using a molar extinction coefficient value of 13 600 for the liberated anion. The amylase content of pure preparation was measured spectrophotometrically ($E^{1\%}_{1\ cm}$ at 280 nm = 25). Amylase was assayed at pH 6.9 at 30°C by reductometry with dinitrosalicylate [8] using purified starch (Merck).

3. Results

3.1. *Purification of porcine pancreatic α-amylase*

Amylases I and II were purified as described earlier [9] except that a new step in the purification procedure was included. The ammonium sulfate precipitate was chromatographed on a Sephadex G 75 column. Porcine amylase like rat amylase [10] has an anomalous behaviour on polydextran gel. The enzyme is delayed on the column possibly by enzyme-substrate type interactions. The enzyme-gel ratio was 100 mg per 500 ml of gel. The column was buffered with 5 mM sodium phosphate at pH 8 containing 5 mM NaCl and 1 mM benzamidine. The flow rate was 0.25 V_0/hr. Amylase elutes as a single peak at 2.8 V_0. At this stage of purification the enzyme purity was checked: amylase was found homogenous by chromatography on biogel P 100 and by ultracentrifugation analysis. The specific activity is maximal. The subsequent DEAE cellulose chromatography only achieves the separation of the forms I and II of amylase. Amylases I and II are then highly stable even when incubated in the presence of EDTA. This indicates that the enzyme is free from any proteolytic contaminants which were present as traces in previous preparations [9].

3.2. *Preparation and enzymatic properties of $(TNB)_2$ amylase*

Identical results have been obtained with amylases I and II. Amylase was incubated with DTNB in stan-

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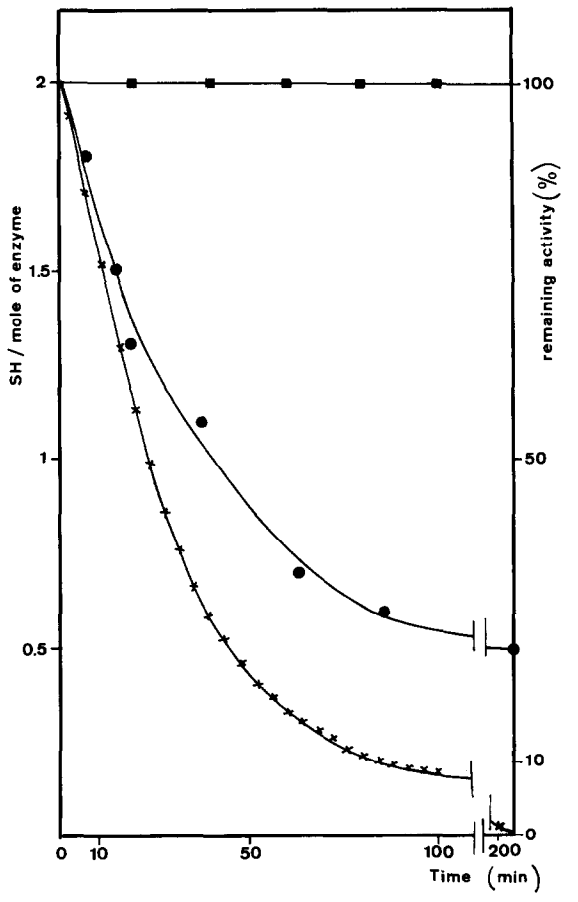

Fig.1. Blocking of amylase SH by DTNB : effect on enzyme activity. The reaction was carried out in 3 ml thermostated cuvette by measuring the increase in absorbance at 412 nm in a PMQ 2 Zeiss spectrophotometer against a blank without amylase. Aliquots of the reaction mixture were taken up at the indicated time, immediately cooled by cold buffer and assayed for amylolytic activity. (X–X–X) mole of SH remaining; (●–●–●) remaining amylase activity; (■–■–■) amylase activity from control incubation without DTNB.

dard conditions (see Techniques) until stoichiometric liberation of 2 mole of TNB^-. The modified enzyme was purified by gel filtration on biogel P 100 buffered with 5 mM sodium phosphate buffer at pH 8. The flow rate was 0.05 V_0/hr. The enzyme elutes at the same elution volume (1.4 V_0) as that of the native enzyme. After addition of 2-mercaptoethanol in excess to the purified modified enzyme, 2 mole of TNB^- per mole of enzyme were liberated from the protein. This demonstrates that the product of the reaction is $(TNB)_2$ amylase.

Blocking of the SH group by DTNB promotes a gradual decrease of enzyme activity (fig.1). The loss of activity does not parallel the blocking of the SH as previously observed [2]. $(TNB)_2$ amylase is partially active (25–35% of the fully active enzyme). No reactivation of the $(TNB)_2$ enzyme nor further inhibition was observed after 24 hr dialysis against 10 mM Tris–HCl buffer at pH 8 containing 4 mM $CaCl_2$, at 5°C. In order to obtain more information on this inhibition 2 experiments were carried out : 1) The kinetic parameters V_m and K_m were determined in native and $(TNB)_2$ amylase. 2) The affinity for polydextran gel was also compared.

In kinetic studies starch was used as a substrate at various concentrations (0.05–0.25%). Because of the

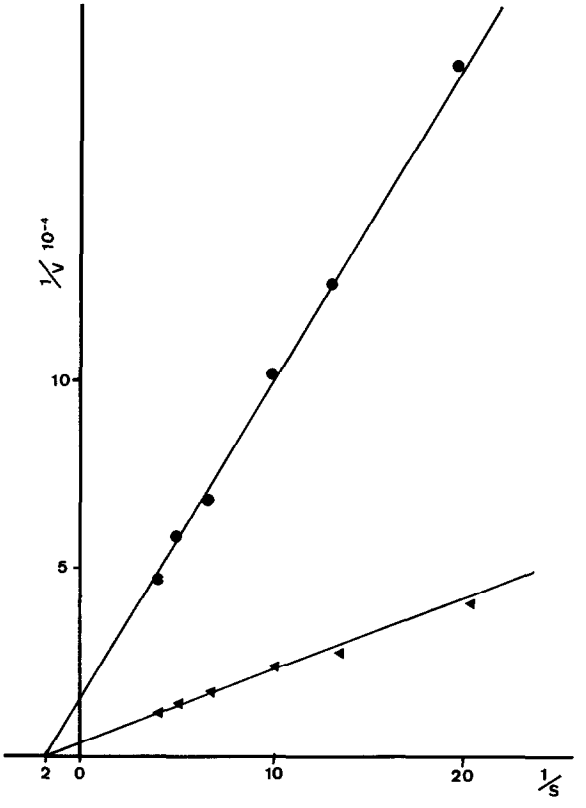

Fig.2. Lineweaver and Burk plot in the case of native and $(TNB)_2$ amylase. In the assays the starch concentration was (0.05–0.25%). The hydrolysis rates were taken from the initial slopes. (▲–▲–▲) native amylase; (●–●–●) $(TNB)_2$ amylase.

poor solubility of starch it was not possible to increase the concentration of the substrate above 0.25%. V_m and K_m were obtained from the Lineweaver and Burk plot (fig.2). The V_m of $(TNB)_2$ Amylase is 4 times less (5 800 units/mg) than the V_m of native amylase (25 000 units/mg). Interestingly amylase and $(TNB)_2$ species have the same K_m(5 mg/ml).

$(TNB)_2$ amylase prepared as indicated above was dialyzed for 24 hr against 2 X 1 liter of 10 mM Tris–HCl buffer at pH 8 in 4 mM $CaCl_2$ and chromatographed on a G 75 column equilibrated with the same buffer. The enzyme–gel ratio was as in 3.1. $(TNB)_2$ amylase elutes at 2.4 V_0. Native amylase elutes at the same volume.

4. Discussion and conclusions

Taking advantage of the affinity of amylase for polydextran gel, a perfectly stable enzyme, free of any proteolytic contaminants, has been purified. Blocking of the 2 SH groups of the enzyme by DTNB in excess give a partially active $(TNB)_2$ amylase. We have confirmed [3,4] that both SH are not essential for enzyme activity, it is possible that the blocking of both SH by DTNB induces some specific hindrance or some local change in the configuration of the protein which specifically concerns the catalytic site.

Kinetic studies indicate that the $'K'_m$ of modified amylase is unchanged. Also $(TNB)_2$ amylase is retained on polydextran gel at the same extent as the native enzyme. Both results suggest that the substrate binding site of the enzyme is not affected by the TNB blocking of the SH.

Acknowledgement

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