CHARACTERIZATION OF *BACILLUS POLYMYXA* AMYLASE AS AN EXO-ACTING (1 → 4)-α-D-GLUCAN MALTOHYDROLASE

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1. Introduction

Robyt and French [1] have purified and characterized an amylolytic enzyme from *Bacillus polymyxa*, an enzyme first discovered by Tilden and Hudson [2]. This enzyme had characteristics of β-amylase in that it liberated β-maltose as the predominant product during action on starch, and resembled α-amylase in being able to convert amylopectin almost quantitatively into maltose, suggesting that it had the ability to bypass α-(1 → 6) branch points in the substrate. A further difference from other amylases was exhibited by the ability of the enzyme to degrade Schardinger dextrins, indicating the lack of a requirement for a non-reducing end for enzyme action. Robyt and French [1] chose the criterion of the configuration at the anomeric carbon atom in the products as being most suitable for classification of the enzyme, which they therefore considered to be a β-amylase. We have now extended the studies of *Bacillus polymyxa* amylase to show that this enzyme possesses all the characteristics of a β-amylase (EC 3.2.1.2). Thus, action on amylopectin is halted at the outermost branch points in the substrate, so that maltose and a high-molecular-weight limit dextrin are the products of enzyme action. Periodate-oxidized amylose is not significantly degraded and Schardinger dextrins are resistant to the action of the enzyme.

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2. Materials and methods

Cultures of *Bacillus polymyxa* ATCC 8523 were grown for 2 days at 30°C in the medium described by Griffin and Fogarty [3]. The amylase was purified from the culture filtrate by chromatography on DEAE-cellulose, CM-cellulose and Sephadex G-100. Full details of the purification scheme and characterization of the purified enzyme will be published later. One unit of enzyme activity is the amount which releases 1 μmole of maltose per minute from soluble starch at pH 6.9 and 37°C.

Amylopectin was prepared by butanol fractionation of potato starch [4]. Periodate-oxidized potato amylose was prepared as described previously [5]. Schardinger dextrins were prepared as described by French, Pulley and Whelan [6]. Sweet-potato β-amylase was purified as by Marshall and Whelan [7].

Reducing sugars were determined by using an alkaline copper reagent [8] calibrated against maltose hydrate. Polysaccharide concentrations were determined by enzymic degradation to glucose [9], or in the case of glucoamylase-resistant carbohydrates, by the phenol-sulfuric acid method [10]. Iodine staining measurements were carried out by addition of iodine reagent (0.02% iodine in 0.2% potassium iodide) to samples (0.1 ml) containing 0.25 mg of carbohydrate and reading the absorbance at 550 nm.

Descending paper chromatograms were developed in the solvent system ethyl acetate:pyridine:water (10:4:3, by volume). Sugars were located by using an alka-

line silver nitrate reagent [11].

Chromatography of products of enzyme action was performed at room temperature on a column (2.5 X 40 cm) of Sephadex G-50 (fine) eluted with water. Samples of volume 3.2 ml were collected automatically.

3. Experimental procedures

3.1. *Degradation of potato amylopectin by Bacillus polymyxa amylase*

A digest (100 ml) containing amylopectin (250 mg), sodium glycerophosphate buffer (pH 6.9, final concentration 25 mM), calcium chloride (5 mM) and enzyme (16 U) was incubated at 37°C. Samples were removed at intervals for determination of reducing sugars released and for determination of the iodine staining power. After incubation for 1.5 hr, by which time the iodine staining power and reducing power were no longer changing, samples (0.1 ml) from the digest were treated with pullulanase (10 μl, 0.25 U) for 2 hr, followed by measurement of the reducing power. After 3.5 hr at 37°C, enzyme was inactivated by heating the remaining portion of the digest at 100°C for 5 min, then filtered. After removal of a sample (20 μl) for examination by paper chromatography the solution was freeze-dried, re-dissolved in water (5.0 ml) and chromatographed on Sephadex G-50.

For comparative purposes, degradation of amylopectin by sweet-potato β-amylase was followed in the same manner, in this case the digests containing acetate buffer pH 4.8 (final concentration 125 mM) and without calcium chloride.

3.2. *Degradation of oxidized amylose by Bacillus polymyxa amylase*

A digest of volume 2.0 ml containing partly oxidized amylose (5.5 mg) sodium glycerophosphate buffer (pH 6.9, final concentration 25 mM), calcium chloride (5 mM) and enzyme (0.4 U) was incubated at 37°C, and samples (0.1 ml) removed at intervals for determination of liberated reducing power.

3.3. *Action of Bacillus polymyxa amylase on Schardinger β-dextrin*

Bacillus polymyxa amylase (2 U) was incubated with Schardinger β-dextrin (6.25 mg/ml) in a digest of total volume 2.0 ml, containing 25 mM sodium glycerophosphate buffer pH 6.9 and 5 mM calcium chloride. Samples (0.1 ml) were removed at intervals up to an incubation time of 2 hr, and analyzed for reducing sugars.

4. Results and discussion

Fig. 1a shows the time courses of hydrolysis of amylopectin by equal concentrations of *Bacillus polymyxa* amylase and sweet-potato β-amylase. The

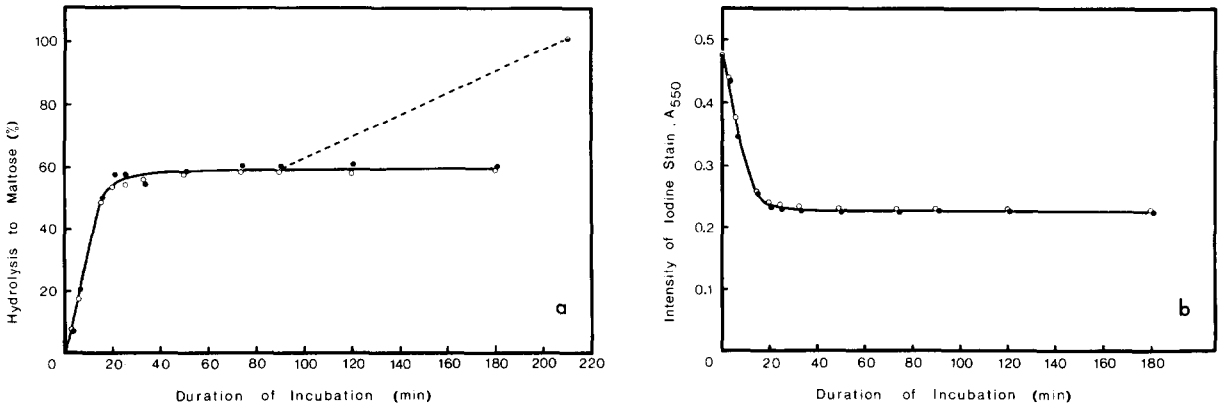

Fig. 1. Degradation of potato amylopectin by *Bacillus polymyxa* amylase (•) and sweet-potato β-amylase (0). (a) shows the conversion into reducing sugars and (b) the change in iodine staining power of the substrate. At the point indicated by the arrow, pullulanase was added to samples from the digests. For details refer to Experimental section.

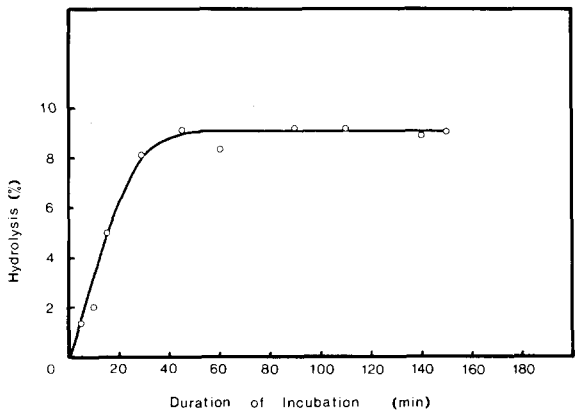

Fig. 2. Degradation of periodate-oxidized amylose by *Bacillus polymyxa* amylase. See Experimental section for details.

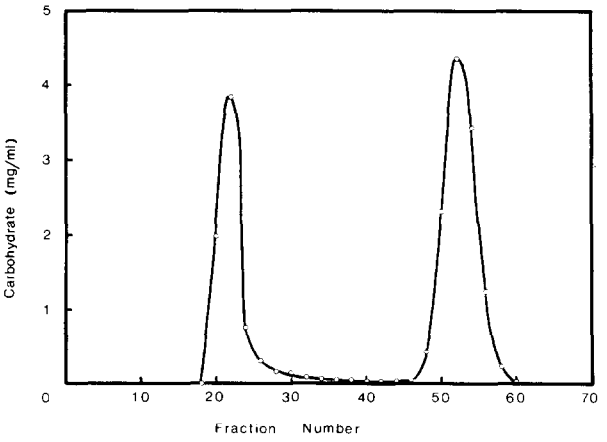

Fig. 3. Chromatography of the products resulting from action of *Bacillus polymyxa* amylase on amylopectin, on a column of Sephadex G-50. See Experimental section for details.

action of both these enzymes is closely similar, stopping at 60% conversion into maltose, but increasing to 100% conversion into maltose on addition of pullulanase, an enzyme which cleaves all the α-(1 → 6)-interchain linkages in β-amylase limit dextrin [12]. While these results are, by themselves, not proof that the *Bacillus polymyxa* enzyme has the same action pattern as the plant enzyme, it can be seen from fig. 1b that a product with about half the iodine staining power of the starting material is produced by both enzymes. This clearly indicates that action of the microbial enzyme, like that of sweet-potato and other plant β-amylases, results in production of a macromolecular limit dextrin. This is confirmed by chromatography of the products of *Bacillus polymyxa* action, on Sephadex G-50, the macromolecular product being eluted at the void volume of the column, and well separated from the low molecular weight product, identified as maltose by paper chromatography. It can thus be concluded that the action of *Bacillus polymyxa* amylase on amylopectin is closely similar to that of the sweet-potato enzyme.

The exo-nature of the microbial enzyme is confirmed by its action on partly oxidized amylose, a substrate we have found of considerable use in characterizing the action patterns of starch-degrading enzymes [13,14], and for detecting the presence of endo-enzymes in exo-enzyme preparations [5]. The small extent of hydrolysis of this substrate (9%, fig. 3) is similar to that obtained with sweet-potato β-amylase [14], and in accord with the action of the enzyme stopping at the oxidation point closest to the non-reducing end in each chain.

Incubation of our purified preparation of *Bacillus polymyxa* amylase at a concentration of 1 U/ml for 2 hr with Schardinger β-dextrin resulted in only 0.7% hydrolysis. This small extent of hydrolysis can be accounted for by the presence of traces of linear oligosaccharides in the cyclodextrin preparations (j. J. Marshall, unpublished work), and it is clear that the enzyme is without action on this cyclic dextrin. Like other β-amylases, the *Bacillus polymyxa* enzyme must require a non-reducing chain end for action. Preliminary experiments indicate that rather than being substrates, Schardinger dextrins are inhibitors of *Bacillus polymyxa* amylase. These cyclic dextrins have previously been shown to inhibit the sweet-potato enzyme [15], and recently the bacterial debranching enzyme, pullulanase [16].

This work has shown that the action patterns and products of action of *Bacillus polymyxa* and sweet-potato β-amylases are basically the same. Thus the microbial enzyme may also be classed as an exo-(1 → 4)-α-D-glucan maltohydrolase (EC 3.2.1.2). Further work is in progress to examine the detailed structure of the β-limit dextrin produced by the microbial β-amylase, and to compare the properties of sweet-potato and *Bacillus polymyxa* β-amylases.

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