Purification and properties of the β-fructofuranosidase from *Kluyveromyces fragilis*

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The β-fructofuranosidase from *Kluyveromyces fragilis* was purified to one band on electrophoresis by 3 different methods. Two of the preparations were found to be impure by isoelectric focusing. This demonstrates the need for more than one criteria of homogeneity when purifying this enzyme. The enzyme was found to be a glycoprotein, stable at 50°C, with a pH optimum of 4.5. The cations Hg^{2+}, Ag^{+}, Cu^{2+} and Cd^{2+} exhibited a marked inhibition of the enzyme. Competitive inhibition was observed with the fructose analog 2,5-anhydro-D-mannitol suggesting that the enzyme is inhibited by the furanose form of fructose.

β-Fructofuranosidase *Invertase* *Inulinase* *2,5-Anhydro-D-mannitol*

1. INTRODUCTION

The β-fructofuranosidase from the yeast *Kluyveromyces fragilis* has industrial potential for use in fructose syrup production from inulin-containing plants (i.e., Jerusalem artichoke, chicory [1]). This β-fructofuranosidase has a higher specific activity against inulin than does the invertase from *Saccharomyces cerevisiae* even though both enzymes possess the ability to hydrolyze sucrose and inulin [2]. By following the S/I ratio (activity against sucrose/activity against inulin) during enzyme purification it was determined that both activities are properties of the same protein [3]. This enzyme hydrolyzes inulin by an exocleavage starting at the D-fructose end of the inulin molecule; the last linkage broken yields 1 D-glucose molecule/inulin molecule [4].

The β-fructofuranosidase from *K. fragilis* has been purified, but homogeneity has not been demonstrated [2–5].

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2. MATERIALS AND METHODS

2.1. *Enzyme production*

Kluyveromyces fragilis (ATCC 12424) was grown in 20 l in an aerated fermentor on the defined medium described in [2] with the substitution of $(NH_4)_2SO_4$ (5 g/l) and KH_2PO_4 (1.5 g/l) for the $NH_4H_2PO_4$. The $NaVO_3 \cdot 4\ H_2O$ was omitted from the medium. Inulin (10 g/l) was the carbon source and the pH was controlled at pH 4.9 by the addition of 1 N NaOH. The temperature was maintained at 30°C.

The organism was grown to stationary phase (24 h), harvested by centrifugation, washed with 20 mM potassium phosphate (pH 8.0), and then resuspended in the same buffer (29 g wet wt/l).

The enzyme was released from the cells by the addition of β-mercaptoethanol to 10 mM followed by incubation at 30°C for 45 min [7]. The cells were harvested and discarded and the resulting supernatant was concentrated with a hollow-fiber device (5000 M_r cut-off) to 2 mg protein/ml. This was then extensively dialyzed against 20 mM potassium phosphate (pH 6.0). This fraction was the crude extract.

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2.2. *Enzyme purifications*

The crude extract was treated with glutaraldehyde-activated carbon as in [8]. Activated carbon that had been washed with 6 N HCl was exposed to 1% glutaraldehyde for 3 min. This treated, activated carbon was washed with deionized water and then 2 g (dry wt) was mixed with 4 ml crude extract at room temperature for 16 h, after which both the supernate and carbon were assayed for protein and activity.

A DEAE-cellulose column (20 cm × 0.5 cm) was prepared and equilibrated with 20 mM potassium phosphate (pH 8.0). The crude extract (20 mg protein) was dialyzed against the equilibrating buffer and then adsorbed to the column. The protein was fractionated by eluting with a 200 ml NaCl gradient (0–0.3 M NaCl in equilibrating buffer).

Affinity chromatography was performed on the crude extract using a con A–agarose column as in [9].

2.3. *Electrophoresis*

Polyacrylamide disc electrophoresis was performed using a modification of the technique in [10]. The 7.7% polyacrylamide gel was fixed in 20% sulfosalicylic acid and stained with a solution of fast green (0.25%), acetic acid (10%), and ethanol (30%). Gels were de-stained electrophoretically and scanned at 630 nm with a 0.5 mm slit width to detect protein.

Isoelectric focusing was done in 5% polyacrylamide gels using a pH 2.5–5.0 ampholyte. Gels were fixed, stained, and scanned as described.

2.4. *Enzyme and protein assay*

The enzyme was assayed by incubating 10 μl properly diluted enzyme in 0.5 ml 0.1 M sucrose in 0.1 M sodium acetate (pH 4.5) at 50°C for 5 min. The reaction was stopped and reducing sugar determined by adding dinitrosalicylic acid reagent. A unit of enzyme activity, as described in [6], is defined as the amount of enzyme required to produce 1 μg reducing sugar/min from the chosen substrate at 50°C under the given pH. Protein was determined by the Lowry method.

2.5. *Determination of carbohydrate content*

Enzyme (1 ml) was placed in a 10 ml ultrafiltration apparatus and washed with 5 10-ml volumes of 0.1 M sodium acetate (pH 4.5) using a 50000 M_r cutoff membrane. The enzyme solution (1 ml) was then dialyzed overnight against 500 ml 6 M urea followed by 3 changes of deionized water. The carbohydrate content of the resulting solution was determined by the phenol–sulfuric acid method and the protein content determined by the Lowry method.

2.6. *Effect of cations on activity*

Solutions (20 mM) of the cations to be tested were made in 0.1 M sodium acetate (pH 4.5). Then 10 μl of enzyme were added to 0.25 ml of each at 50°C and incubated for 5 min at which time 0.25 ml of 0.2 M sucrose in 0.1 M sodium acetate (pH 4.5) (preincubated to 50°C) was added. Activity was determined as described.

2.7. *Determination of kinetic constants*

The K_m and V_{max} were determined from Lineweaver-Burk plots [11]. Three trials, each at a different enzyme concentration, were used to produce the Lineweaver-Burk plot.

The dissociation constant (K_i) for 2,5-anhydro-D-mannitol was determined, using sucrose as the substrate, by the graphical method in [12]. A horizontal line drawn through the reciprocal velocity axis at the point equal to $1/V_{max}$ will intersect the Dixon plot at a point where the abscissa is equal to $-K_i$. This is only valid for competitive inhibition [13].

3. RESULTS AND DISCUSSION

The β-mercaptoethanol released 60% of the cell-associated enzyme producing a crude extract which contained 26 proteins as determined by polyacrylamide disc electrophoresis. This correlates with the 22 proteins observed in thiol-released extracts [14].

When attempts were made to immobilize the crude extract to glutaraldehyde-treated, activated carbon we found that only 20% of the enzyme activity was bound to the support while 56% of the protein was bound. This resulted in an unexpected large increase in the specific activity of the remaining enzyme. Electrophoresis of the supernate from the activated carbon immobilization showed only one broad band (similar in appearance to that in [6]). This band showed β-fructofuranosidase activity when stained for invertase activity. It is not known if the removal of the contaminating pro-

teins was due to immobilization by glutaraldehyde or nonspecific adsorption to the activated carbon.

As an alternative purification, the crude extract was adsorbed to DEAE-cellulose and subsequently eluted with a salt gradient. Only one protein peak was observed on elution which corresponded to the enzyme activity. When this peak was tested by electrophoresis only one protein band was found.

A third method of purification was used where the crude extract was adsorbed to immobilized con A washed with buffer and subsequently released with α-methyl-D-mannoside. This produced a preparation showing only one protein by electrophoresis.

It would seem that we had developed 3 different methods for purifying the same enzyme. But the glutaraldehyde-activated carbon, con A affinity chromatography, and DEAE-cellulose preparations had spec. act. 8.11×10^5, 5.85×10^5 and 4.46×10^5 units/mg protein, respectively.

As a second criteria for purity we chose isoelectric focusing (IEF). The glutaraldehyde-activated carbon preparation was found to be a homogeneous protein solution (pI, ~4.0) as determined by IEF while the DEAE-cellulose preparation was resolved into 6 bands by IEF (fig.1). Similarly, the con A affinity chromatography preparation was separated into 4 bands by IEF.

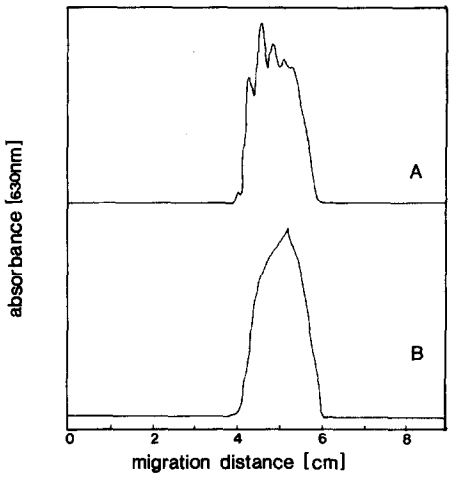

Fig.1. Gel scan of isoelectric focusing gels performed on enzyme purified by DEAE-cellulose ion-exchange chromatography (A) and glutaraldehyde-activated carbon treatment (B).

This suggests that electrophoresis cannot be relied upon as the sole criterion of purity when purifying this particular enzyme.

A uniform assay condition has not been settled upon by researchers in the field of inulin hydrolysis hampering comparative studies. Sucrose was chosen as the substrate for our enzyme studies because we found inulin activity to be substrate-limited (fig.2) at the concentrations normally employed for enzymatic assay [2,3,6]. The limited solubility of inulin also prevented a reliable determination of the K_m and V_{max} on this compound [6].

The inulin and sucrose activities are commonly regarded as properties of the same protein [3]. Following purification of the enzyme by glutaraldehyde-activated carbon treatment, we obtained an S/I ratio of 26 which corresponded with [2,3] and is consistent with the belief that both sucrose and inulin activities are properties of the same enzyme. Raffinose is hydrolyzed by the enzyme to produce fructose and melibiose. The R/I ratio (raffinose activity/inulin activity) we obtained was 3.5 which is the same as in [2] indicating the raffinose hydrolyzing activity to be a property of the same enzyme.

The enzyme was found to be a glycoprotein (66% carbohydrate, by wt) with a pH optimum for sucrose at pH 4.5. The thermostability of the enzyme has been described as both 55°C [3] and 50°C [2]. Our results suggested a slight inactivation at 55°C (fig.3) that would not be noticeable

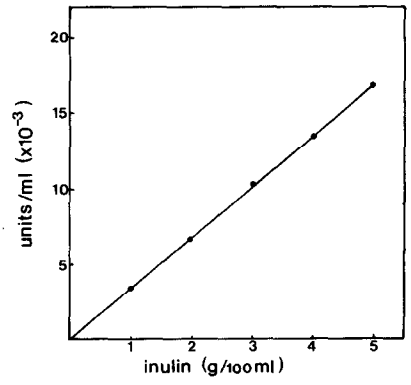

Fig.2. Unit activity *vs* inulin concentration. Each point is the average of 3 trials with the standard deviation being $\leqslant 2\%$.

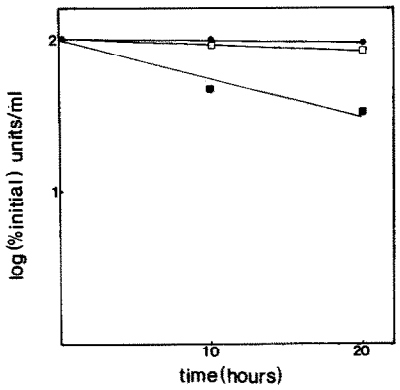

Fig.3. Thermal stability at 50°C (●), 55°C (□) and 60°C (■).

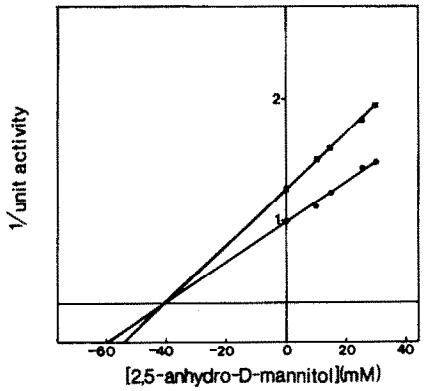

Fig.4. Dixon plot using 5 mM sucrose (■) and 7 mM sucrose (●) as substrate. The horizontal line is equal to $1/V_{max}$.

during a short incubation period. Inhibition of the enzyme by Hg^{2+} and Ag^{+} had been reported [3] but we found that Cu^{2+} and Cd^{2+} also exhibited a marked inhibition of the enzyme. $AgNO_3$ and $HgCl_2$ produced a total inhibition of the enzyme while $CuSO_4$ and $Cd(NO_3)_2$ exerted a 76% and 73% inhibition, respectively.

The K_m was determined for both sucrose and raffinose (table 1). The K_m for sucrose (pH 5.0) of 13.6 mM is similar to the value of 9.4 mM reported in [6] but the K_m for raffinose (pH 5.0) of 46.1 mM does not correspond to the 6.1 mM value described for an impure preparation [6].

Fructose is generally considered non-inhibitory for yeast invertase [15]. We have also found fructose non-inhibitory for the *K. fragilis* β-fructofuranosidase up to a concentration of 100 mM when sucrose was the substrate. 2,5-Anhydro-D-mannitol, a non-reducing fructose analog in the furanose configuration, has been shown to exert a competitive inhibition on the invertase from *Candida utilis* with a K_i of 125 mM [16]. 2,5-Anhydro-D-mannitol was found to also inhibit the β-fructofuranosidase from *K. fragilis* competitively with a K_i of 40 mM when sucrose was the substrate (fig.4). Fructose in solution is commonly found in the pyranose form (80%) over the furanose (20%) configuration [17]. Our results suggest that the enzyme is inhibited by the furanose configuration of fructose but not the pyranose configuration.

Table 1

Substrate	pH	V_{max} (units/ml)[a]	K_m (mM)[b]
Sucrose	5.0	28200	13.6 (± 0.2)
Sucrose	4.5	35400	21.0 (± 1.4)
Raffinose	5.0	17700	46.1 (± 1.6)

[a] Values obtained at 0.041 mg protein/ml
[b] Standard deviations are indicated in parentheses

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