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PURIFICATION AND PROPERTIES OF ALDOSE 1-EPIMERASE FROM *ASPERGILLUS NIGER*

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Aspergillus niger **ATCC 6274 was selected as an aldose 1-epimerase (EC 5.1.3.3) producer from 45 stock cultures of *A. niger*. The aldose 1-epimerase was purified 115-fold to apparent homogeneity from cell extracts with a yield of 2.6%. The molecular weight was calculated to be 260 000 and that of the subunit to be 130 000. The enzyme preparation was active at pH 5–7. The K_m value was 50 mM and the V value was 1200 units/mg toward α-D-glucose. This enzyme catalyzed mutarotation of the following substrates; α-D-glucose, β-D-fructose, β-L-arabinose and β-D-galactose. The time required for glucose determination with a glucose oxidase reagent was significantly shortened by the addition of aldose 1-epimerase.**

Introduction

Aldose 1-epimerase was found as a promotion factor of glucose oxidation during the purification of glucose oxidase from *Penicillium notatum* [1,2] because glucose oxidase acts on only β-D-glucose. The existence of this enzyme was reported in mammalian tissues [3] and plants [4].

Some properties of the enzyme from *P. notatum* [5], green pepper [4] and hog kidney [6] were studied, but none of these preparations were purified to homogeneity.

β-D-Glucose comprises about two-thirds of the equilibrium state of D-glucose. When a glucose-determination reagent containing glucose oxidase is used, two-thirds of D-glucose is oxidized quickly as a true substrate; the rest of the glucose is only oxidized after α-D-glucose spontaneously mutarotates to a β-form and this requires a fairly long time. In order to determine D-glucose in a short time with an automatic analyzer, the addition of the epimerase is necessary.

We screened strains for aldose 1-epimerase activity among our stock strains of *A. niger*, a glucose oxidase producer, selected one strain, *A. niger* ATCC 6274, purified the enzyme and studied its properties.

Materials and Methods

Determination of enzyme activity with a glucose determination reagent

The glucose determination reagent (Diacolor, Toyo Spinning Co., Osaka) contained 5 units glucose oxidase, 1.25 units peroxidase, 25 μg 4-aminoantipyrin, 250 μg diethylaniline and 3 mg Tris-HCl in 1 ml 0.1 M acetate buffer (pH 5.8). 2 ml reagent, 0.5 ml enzyme sample and 0.5 ml freshly-prepared α-D-glucose (0.2 mg/ml) were mixed in a cuvette and placed in a double-beam spectrophotometer (ultraviolet-210A, Shimadzu Corp.) equipped with the temperature-controlled bath at 30°C. Usually the reaction was started within 1 min after dissolving α-D-glucose. Color development was recorded at 558 nm (chart speed, 6 cm/min). A control experiment (for natural mutarotation) was carried out with buffer A (0.1 M acetate buffer (pH 5.8)/50 mM Tris-HCl) instead of an enzyme sample. The enzyme activity was calculated from the difference of the oxidation rates between a sample and a control, and one unit of enzyme activity was defined as the amount of enzyme to promote mutarotation of 1 μmol D-glucose in 1 min. The range of enzyme activity measurement was $5–25 \cdot 10^{-3}$ units/ml. This unit is lower

than that obtained with a polarimeter as described later, but mainly used in this report because of convenience and reliability of the method.

Determination of mutarotase activity with a polarimeter

0.2 ml enzyme and 1.8 ml freshly-prepared α-D-glucose (11.1 mg/ml) were mixed, introduced into a cell (light path, 10 cm) of a polarimeter (DIP-191 Japan Spectroscopic Corp. Ltd.) and the mutarotation was recorded via a digital-analog converter. The enzyme activity was calculated from the difference of rate constants (assuming first-order kinetics) between an enzyme sample and a control.

Protein concentration was estimated by assuming 1 A_{280} unit to be 0.5 mg protein/ml.

Screening of strains

For 45 stock strains of *A. niger,* spore suspensions were prepared by mixing the slant culture with 5 ml water. 2 ml of each spore suspension were seeded into 100 ml medium (3 g sucrose/5 g potato starch/0.2 g yeast extract/0.2 g $NaNO_3$/0.05 g $MgSO_4 \cdot 7H_2O$/1 g $CaCl_2 \cdot 2H_2O$ (pH 6.0)). The flasks were incubated on a rotary shaker at 30°C for 2–4 days depending on their growth. The mycelia were collected by filtration, washed with water and chilled acetone (−20°C) a few times, and then dried in a desiccator under vacuum at 4°C. For these samples, the mycelial yield was 2–8 mg/ml culture broth. The dried mycelia were ground in a mortar, extracted with 10 ml buffer A, and the supernatant was used as an enzyme sample after centrifuging the extract at 15 000 × *g* for 15 min.

Mass cultivation of A. niger

An agar slant of *A. niger* was mixed with 15 ml sterilized water. A 5 ml spore suspension was seeded into 100 ml medium as described above. After 2 days cultivation, a 5-ml aliquot of the seed culture was seeded into each of 30 flasks, but starch was omitted from the medium and 1 g $CaCO_3$ was added. After 1 day cultivation, the whole preculture (about 3 l) was seeded into a 100-l fermentor (Marubishi Co. Ltd.) containing 70 l medium, the composition of which was the same as that of the initial medium except for the omission of starch. The fermentor was operated for 24 h at 30°C, with an agitation speed of 240 rev./min and an aeration rate of 30 l/min. The medium pH was controlled at 5–5.5 by adding 10 N KOH and foaming in the fermentor was regulated by adding silicon oil. The mycelia were collected by filtration through double layers of gauze and washed with buffer A.

Purification of aldose 1-epimerase

Extraction of enzyme. 4.2 kg wet mycelia were obtained after cultivating in a 100-l fermentor. The mycelia were suspended in 3 l buffer A and homogenized in Waring blendor for 3 min. This suspension was disintegrated twice in a Dyno-Mill (Shinmaru Enterprises Corp., Switzerland) with glass beads. The crude enzyme solution was obtained after centrifuging the cell lysates at 8000 rev./min for 20 min.

$(NH_4)_2SO_4$ fractionation. The precipitate forming at 40–80% satn. $(NH_4)_2SO_4$ was dissolved in 200 ml buffer A and dialyzed overnight against buffer A. (three changes). The precipitate formed was removed by centrifugation.

Heat treatment. The above enzyme solution was incubated in a water bath at 60°C for 10 min and the precipitate formed was removed by centrifugation. Though almost half of the enzyme activity was lost by this step, most of protein was removed and the specific activity increased about 7-times. This step can be carried out after Sephacryl S-300 chromatography with less than 5% loss of the activity, but it was preferable to remove much other protein earlier in this step. Without this heat treatment the homogeneous enzyme preparation was not obtained after the Sephacryl S-300 chromatography.

DEAE-Sephadex A-50 column chromatography. The above enzyme was charged on a DEAE-Sephadex A-50 column (φ 6 × 60 cm), washed with 100 ml buffer A, and 300 ml buffer A 0.1 M NaCl, and eluted with 2 l buffer A containing a linear 0.1–0.5 M NaCl gradient (20-ml fractions). The enzyme was eluted at about 0.3 M NaCl. The active fractions were pooled (240 ml) and concentrated to 15 ml by precipitation with 75% satn. $(NH_4)_2SO_4$.

Sephacryl S-300 column chromatography. The enzyme was applied to a Sephacryl S-300 column (φ 2.5 × 70 cm), and eluted with buffer A, (5-ml fractions). The enzyme was eluted after a big protein peak. The six active fractions were subjected to polyacrylamide gel electrophoresis and three fractions

showed a single band. These were pooled and used as the purified enzyme in further experiments.

Chemicals

The gels for the enzyme purification, DEAE-Sephadex A-50, Sephacryl S-300, Sephadex G-200 were products of Pharmacia Fine Chemicals. The marker proteins for gel filtration and SDS-polyacrylamide gel electrophoresis were purchased from Boehringer-Mannheim GmbH and the molecular marker, *Thermus* RNA polymerase B, was from Mitsubishi Oil Chemicals. Aldose 1-epimerase from hog pancreas was from Sigma Chemical Corporation.

Results and Discussion

After the determination of those enzyme activities among 45 strains the highest mutarotase activity, $4.4 \cdot 10^{-3}$ units/ml culture broth, was obtained with strain ATCC 6274. For comparison, *P. notatum* [1] was cultured and $1.5 \cdot 10^{-3}$ units activity/ml were seen. By this acetone drying, the efficiency of enzyme extraction was one-third of that by Dyno-Mill disruption, but the enzyme was stable to acetone treatment. During screening of microorganisms, a number of samples had to be treated with small amount of cells and this method was convenient. In further experiments, *A. niger* ATCC 6274 was used.

A summary of the purification steps is given in Table I. Aldose 1-epimerase was purified 115-times as for specific activity, with a yield of 2.6% from the crude cell extract. Without heat-treatment, the enzyme was not purified to homogeneity. The purified enzyme was subjected to polyacrylamide gel electrophoresis at pH 9 [7] with parallel runs, one gel was stained with amino black and a single protein band was observed at a relative mobility of 0.43 (Fig. 1). The other gel was sliced into 32 pieces of 2 mm in length, the enzyme in each gel piece was extracted with 1 ml buffer A at 5°C overnight. The enzyme activity was observed in the 13th and 14th fractions from the top, which corresponded to the protein band.

Properties of aldose 1-epimerase

Molecular weight. The purified enzyme was subjected to Sephadex G-200 column (ϕ 2.6 X 100 cm) chromatography [8] with marker proteins (ferritin, 54 000; beef liver catalase, 240 000; rabbit muscle aldolase, 158 000; *Bacillus amyloliquefaciens* α-amylase (Daiwa Chem. Corp.), 96 000; bovine serum albumin, 67 000; hen egg albumin, 45 000) and its molecular weight was estimated to be 260 000. This molecular weight was very high, compared to those for *P. notatum* [5], green pepper [4] and hog kidney [6] which were reported to be 70 000, 50 000 and 53 400, respectively, by density gradient centrifugation.

The purified enzyme was subjected to SDS-polyacrylamide gel electrophoresis [9] with marker proteins: bovine serum albumin, 68 000; soybean trypsin inhibitor, 21 500; *Escherichia coli* RNA polymerase β'-subunit, 165 000; β-subunit, 155 000; α-subunit, 39 000; *Thermus thermophilus* RNA polymerase β'-subunit, 180 000; β-subunit, 140 000; x-subunit; 100 000, α-subunit, 42 000; z-subunit, 39 000. The gel concentration was lowered to 7.5% in order to increase the mobility. In this electrophoresis this enzyme showed a single band (Fig. 1) and the subunit molecular weight was estimated to be 130 000. From these results this enzyme would appear to be a homodimer.

Optimal pH. The rate of natural mutarotation is

TABLE I

PURIFICATION OF ALDOSE 1-EPIMERASE

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude enzyme	3 500	2 800	111	0.040	100
$(NH_4)_2SO_4$ fractionation	257	1 140	72.8	0.064	65
Heat treatment	244	78	34.2	0.44	31
DEAE-Sephadex	15	5.1	15.1	3.0	14
Sephacryl S-300	15	0.62	2.84	4.6	2.6

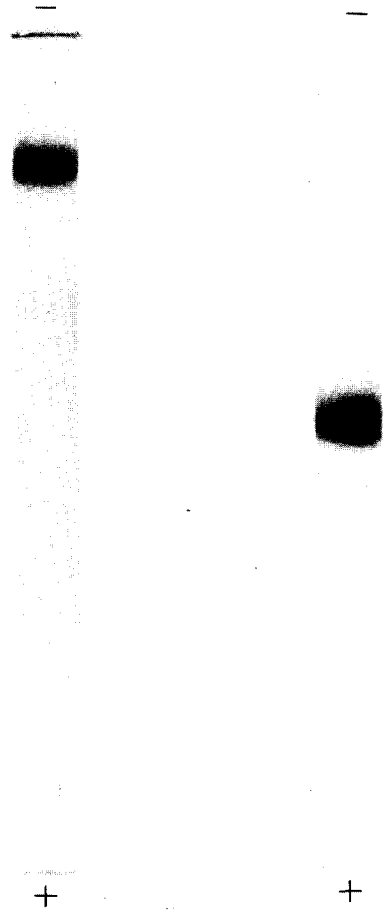

Fig. 1. Electrophoresis patterns of purified aldose 1-epimerase. Left, SDS-polyacrylamide gel electrophoresis (7.5% gel); right, polyacrylamide gel electrophoresis (pH 9).

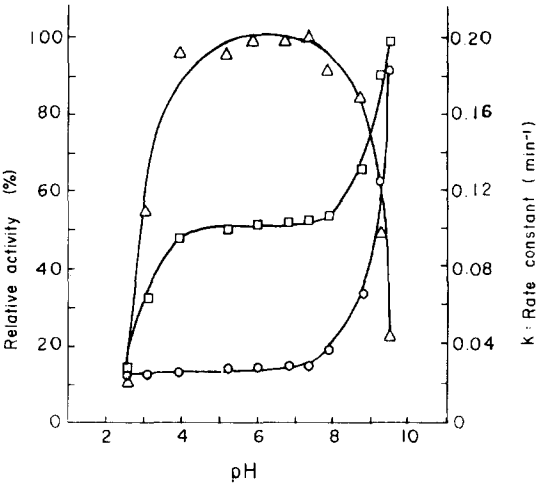

Fig. 2. Effect of pH on the aldose 1-epimerase activity. Rate constants of spontaneous mutarotation (a) and catalyzed mutarotation (b) were measured at different pH values using a polarimeter. The activities were calculated as the difference of (b) – (a). Activity (△———△), K with enzyme (□— —□) and K without enzyme (○———○).

high at an alkaline pH. To determine the optimal pH, we used the same buffer system of 50 mM Tris/30 mM acetate and its pH was adjusted with 1 M NaOH or 1 M HCl. The changes of ionic strength did not make so much difference because only 5% of the activity decreases when 0.5 M NaCl was added to the reaction at pH 5.8. Phosphate buffer was not used because this buffer strongly promotes spontaneous mutarotation [10]. The rate constants of spontaneous mutarotation and mutarotation catalyzed by the enzyme were determined with a polarimeter (Fig. 2). The optimal pH range was 5–7.

pH stability. The enzyme pH was adjusted to 2–3.5 with 50 mM glycine/HCl buffer, to 3.5–6.0 with 50 mM acetate buffer, to 5.5–8.0 with 50 mM phosphate buffer, to 7.5–9.0 with 50 mM Tris-HCl, and to 9–11 with 50 mM bicarbonate buffer. After keeping the solutions at 20°C for 24 h, the pH values were readjusted to pH 6 with 1 M HCl or 1 M NaOH, and residual activity was measured by using a glucose oxidase system. No inactivation was observed during storage at pH 5.5–9, half of the activity was lost at pH 3, two-thirds of the activity was lost at pH 10.5 and all the activity was lost at pH 2 and 11.

Thermal stability. The enzyme was kept for 30 min at different temperatures and residual activity was determined. Activity loss was not observed on keeping the enzyme at lower than 55°C. The following activity losses were observed: 5% at 60°C; 25% at 65°C; 95% at 75°C. The enzyme was heat-inactivated at 75°C following first-order kinetics, with a half-life of 6.9 min.

Kinetic constants. The effect of α-D-glucose concentration on the mutarotation rate was studied. The initial rate was determined with a polarimeter, and the K_m value was estimated to be 50 mM (Fig. 3). This K_m was higher than the 20 mM for hog pancreas [6] and green pepper [4]. The V value was 1200 units/mg protein, which was lower than the 5000 units/mg protein for hog pancreas [6]. The turnover number was calculated to be 2600 mol/mol per s

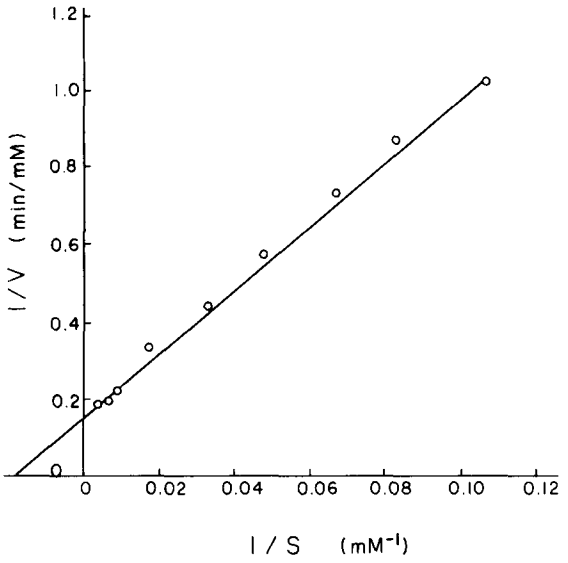

Fig. 3. Effect of initial glucose concentration on the initial reaction rate of aldose 1-epimerase. The initial rates were calculated as described in the legend of Fig. 2 with various concentrations of glucose.

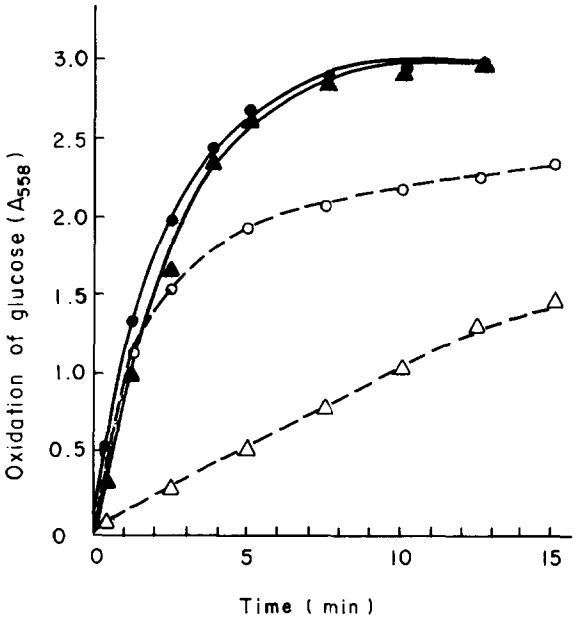

Fig. 4. Effect of aldose 1-epimerase addition to α-D-glucose and D-glucose in the equilibrium state on glucose determination with a glucose oxidase system. A half unit of mutarotase was added to a standard reaction system. The equilibrium state is abbreviated as Eq. Glucose (Eq) with enzyme (●——●), α-glucose with enzyme (▲——▲), glucose (Eq) without enzyme (○- - - - -○) and α-glucose without enzyme (△- - - - -△).

assuming a molecular weight of 130 000.

Enzyme activity with a glucose oxidase system was only 0.5% of that with a polarimeter because of the low substrate concentration. But the activity with the former system was correlated with the amount of enzyme.

Substrate specificity. Spontaneous mutarotation and that by the enzyme toward various sugars were determined (Table II). Fructose, arabinose, xylose and galactose had a large spontaneous mutarotation. By addition of the enzyme the mutarotation of glucose and galactose was greatly promoted. The relative rates toward fructose, arabinose, galactose and glu-

TABLE II

SUBSTRATE SPECIFICITY OF ALDOSE 1-EPIMERASE

Substrate	Reaction rate constant		Relative rate (b)−(a) (%)	Stimulation (b)/(a) − 1 (%)
	Spontaneous (a) (min^{-1})	Catalyzed (b) (min^{-1})		
α-D-Glucose	0.026	0.086	100	230
α-D-Xylose	0.099	0.118	31	19
α-D-Galactose	0.038	0.101	105	166
β-D-Fructose	0.378	0.459	135	21
β-Maltose	0.026	0.034	13	31
β-Lactose	0.025	0.031	10	24
β-L-Arabinose	0.120	0.185	108	54
β-D-Cellobiose	0.019	0.028	15	47

cose were high. These results were similar to those for *P. notatum* [5] and hog kidney [3].

Application of aldose 1-epimerase to a glucose oxidase system

Time courses of color development in determinations of α-D-glucose and D-glucose in the equilibrium state are shown in Fig. 4. α-D-Glucose was oxidized in a linear fashion and it takes more than 3 h to complete the reaction [6]. Two-thirds of the D-glucose in the equilibrium state was oxidized quickly, but all the glucose was not oxidized in 30 min. On addition of the enzyme, most of the glucose was oxidized quickly within 8 min. We conclude that it is possible to determine glucose in a short time by the addition of the enzyme. This enzyme is active in a broad pH region and heat-stable, therefore it is favorable to use glucose determination reagents with this enzyme.

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References

1 Bentley, B. and Neuberger, A. (1949) Biochem. J. 45, 584–590
2 Keilin, D. and Hartree, E.F. (1952) Biochem. J. 50, 331–348
3 Keston, A.S. (1954) Science 120, 355–356
4 Bailey, J.M., Fishman, P.H. and Pentchev, P.G. (1967) J. Biol. Chem. 242, 4263–4269
5 Bentley, R. and Phate, D.S. (1960) J. Biol. Chem. 235, 1219–1224
6 Li, L.K. and Chase, A.M. (1964) Fed. Proc. 23, 162
7 Davis, B.J. (1965) Annu. N.Y. Acad. Sci. 121, 404–427
8 Andrews, P. (1965) Biochem. J. 124, 581–590
9 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 224, 4406–4412
10 Chase, A.H., Lapedes, S.A. and Von Meier, H.C. (1963) J. Cell. Comp. Physiol. 61, 181–193