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Purification and characterization of a class I fructose 1,6-bisphosphate aldolase from *Staphylococcus carnosus*

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Summary. A fructose 1,6-bisphosphate aldolase (E.C.4.1.2.13) from *Staphylococcus carnosus* DSM 20501 was purified for the first time. The enzymatic activity was insensitive to high levels of EDTA indicating that the enzyme is a class I aldolase. This enzyme exhibits good stability at high temperatures and extreme stability over a wide pH range. The K_m for fructose 1,6-bisphosphate as substrate was 0.022 mM. The *S. carnosus* aldolase is a monomeric enzyme with a molecular mass of about 33 kDa. It exhibits a relatively broad pH optimum between pH 6.5 and 9.0. Furthermore, the aldolase accepts other aldehydes in place of its natural substrate, glyceraldehyde 3-phosphate, allowing the synthesis of various sugar phosphates.

Introduction

Aldolases were first observed near the beginning of this century as an ubiquitous class of enzymes that catalyse the interconversion of hexoses and their three-carbon components (Meyerhof et al. 1936). All organisms possess aldolase enzymes and two distinct groups have been recognized (Horecker et al. 1972). Type I aldolase, found predominantly in higher plants and animals, requires no metal cofactor, and catalyses the aldol condensation through a Schiff base intermediate. This intermediate can be reduced with borohydride in the presence of dihydroxyacetone phosphate (DHAP) whereby the aldolase is inactivated. Type I aldolases are unaffected by EDTA. In contrast, class II aldolases, which are found primaraly in bacteria and fungi, are Zn^{2+}-, Ca^{2+}- or Fe^{2+}-dependent enzymes and can be inhibited by chelating agents such as EDTA.

Aldolases are the most useful enzymes for the synthesis of monosaccharides and related compounds (Akiyama et al. 1988), although only a small number of the known aldolases have been exploited for this purpose. The most thoroughly studied class I aldolase is the fructose 1,6-bisphosphate aldolase from rabbit muscle (RAMA). This tetrameric enzyme with a molecular mass of about 170 kDa accepts several aldehydes as substrates (Bednarski et al. 1989; Effenberger and Straub 1987) but is not very stable during enzymatic synthesis (Bossow-Berke 1989). Götz et al. (1980) described a very heat-stable fructose 1,6-bisphosphate aldolase from *S. aureus*, but not substrate specificity was investigated. We describe here the fructose 1,6-bisphosphate aldolase from *S. carnosus* DSM 20501, which was chosen as a safe production organism (Schleifer and Fischer 1982).

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Materials and methods

Organism and growth conditions

The bacterium used in this study, *S. carnosus* DSM 20501, was given to us by F. Götz (Institut für Mikrobielle Genetik, University of Tübingen, FRG) who also supplied information on the culture conditions. *S. carnosus* was cultivated under controlled aerobic conditions (pO_2, 5-10%) in a 200-l fermentation (Bioreaktor 300 l; Chemap, Volketswil, Swiss) at 37° C. The growth medium contained 1% glucose, 1% tryptone, 0.5% yeast extract and 0.5% NaCl in a 0.1% sodium phosphate buffer, pH 7.2. The production fermentor was inoculated with 10 l of a preculture grown in the same medium. Cells were cultivated to the end of the log phase, the culture reached an optical density at 660 nm of 16 after about 5 h. Cells were harvested by centrifugation (KA 6; Westfalia Separator, Ölde, FRG) and washed once with standard buffer (60 mM TRIS-HCl buffer pH 7.5, containing 0.1% mercaptoethanol). Approximately 3 kg wet cells were obtained.

Purification of aldolase

Crude extract. The cell paste obtained by fermentation was mixed with standard buffer to give a 40% suspension. This suspension could be stored at −20° C without any loss of activity. The cell suspension was continuously disintegrated at 2500 rpm in a water-cooled cell mill (Netzsch, LME4, Selb, FRG). The grinding chamber was filled (85%) with glass beads (0.3 mm diameter) purchased from H. Clauss (Nidderau, FRG). The supernatant obtained by centrifugation at 8000 *g* for 10 min served as the crude cell-free extract.

Ammonium sulphate fractionation. The crude cell-free extract was brought to 40% ammonium sulphate saturation at 4° C. Precipitated protein was removed by centrifugation at 20000 *g* for 30 min. The concentration of ammonium sulphate in the supernatant was adjusted to 80% saturation and precipitated protein was again removed by centrifugation. The supernatant was then brought to 100% ammonium sulphate saturation and the pH was adjusted to 5.0 with 7 M acetic acid. The precipitated protein was separated by centrifugation. Of the original aldolase activity 43% was recovered after dissolution of the sediment in standard buffer.

pH fractionation. The protein solution obtained in the ammonium sulphate fraction was cooled in ice and the pH lowered to 4.0 by dropwise addition of 7 M acetic acid under vigorous stirring. After 1 h the precipitate was removed by centrifugation at 20000 *g* for 30 min. The pH of the supernatant was lowered further to 3.5 and then to 3.0 with 6 M HCl. The supernatant at pH 3.0 obtained by centrifugation at 15000 *g* for 30 min was found to contain 31% of the original aldolase activity. This solution was diafiltered in standard buffer using an Amicon YM 10 ultrafiltration membrane (Amicon, Witten, FRG).

DEAE-Sephadex A 25 chromatography. The diafiltrated protein solution was applied to a DEAE Sephadex A 25 column (2.6 by 25 cm), previously equilibrated with standard buffer containing in addition 0.1 M NaCl. The proteins were eluted with a linear NaCl gradient (0.1–0.5 M). Aldolase was eluted at a sodium chloride concentration of about 0.19 M. The fractions containing activity were pooled, freeze-dried, and stored at −20° C for further experiments.

Assay of aldolase activity

Aldolase assay was carried out by a spectrophotometric method according to Blostein and Rutter (1963). The reaction mixture contained 60 mM TRIS-HCl buffer (pH 7.5), 0.01 M EDTA, 0.28 mM NADH, 10 μg glyceraldehyde 3-phosphate dehydrogenase (G 3PDH)/triosephosphate isomerase (Boehringer, Mannheim, FRG), 2.7 mM fructose 1,6-bisphosphate (F 1,6-BP) and enzyme solution in a total volume of 1.3 ml. One unit (U) of activity is defined as the amount of enzyme required to oxidize 1 μmol NADH per minute. A molar adsorption coefficient of 6.3 l $mmol^{-1}$ cm^{-1} was used for calculations (Boehringer 1989). The photometer cell and buffer solutions were thermostatted at 37° C.

Protein concentrations were assayed by the Bradford (1976) method using bovine serum albumin (BSA) for calibration.

Sodium dodecyl sulphate (SDS) gel electrophoresis

This was carried out by the method of Lämmli (1970). The following proteins were used (protein calibration kit for gel electrophoresis (M_r 20000–340000, Boehringer): macroglobulin [(M_r) red. 170000], phosphorylase B (M_r 97400), BSA (M_r 68000), glutamate dehydrogenase (M_r 36500) and trypsin inhibitor (M_r 20100). Purified aldolase (approximately 120 μg protein per ml) was subjected to electrophoresis. The gels were stained with Coomassie brillant blue and silver reagent (Merril et al. 1981).

Inactivation of aldolase with DHAP and $NaBH_4$

Inactivation was performed following the procedure of Lebherz and Rutter (1973). Aldolase (1 U/ml) was incubated in 20 mM DHAP (pH 6.0), then 100 mM $NaBH_4$ (in 0.02 M NaOH) was added slowly to the incubation mixture over a 30–60 min period with constant stirring. The pH was adjusted, when necessary, by addition of 2 M acetic acid. After the reaction, enzyme was separated from excess DHAP and $NaBH_4$ by gel filtration on a Sephadex G25 column. The remaining aldolase activity was assayed as described above.

Temperature stability

Samples of 12 μg purified aldolase in 1 ml of 60 mM TRIS-HCl buffer (pH 7.5) were kept in closed 1.5-ml Eppendorf tubes and heated at various temperatures in a water bath for 10 min. Thereafter the samples were cooled in an ice bath and the remaining activity determined.

Substrate specificity

DHAP was synthesized by the method of Effenberger and Straub (1987). An aqueous solution of 20 mM DHAP, 200 mM of various aldehydes and 1–4 U aldolase/ml were incubated for 5–7 h. The resulting sugar phosphates were detected with acid molybdate reagent (Hanes and Isherwood 1949) after TLC [Polygram Cel 300 20 × 20 cm from Macherey & Nagel, Düren, FRG; developed with 51.25 ml butanol:propanol:acetone:water (4:2:2:2.5) + 3.75 ml formic acid + 12 g trichloroacetic acid].

Results

Purification of S. carnosus aldolase

Purification of the F 1,6-BP aldolase is summarized in Table 1. The conventional, not optimized, procedure gave aldolase in high purity (25 U/mg protein) but low yield (20%). The enzyme from this preparation appeared to be homogeneous as judged by SDS electrophoresis and was used to determine the molecular properties of the aldolase.

Molecular mass

The subunit molecular mass of purified aldolase was determined by SDS gel electrophoresis. The logarithm of the relative electrophoretic migrations of marker proteins were plotted against the logarithm of the molecular mass yielding a calibration curve of $\ln y = a \ln x + b$ (ln, logarithm naturalis; y, molecular weight/Da; x, relative mobility/cm; a = 1,3; b = 12,9)

Table 1. Summary of *Staphylococcus carnosus* aldolase purification

Purification steps	Specific activity (U/mg)	Purification factor	Yield (%)
Crude cell-free extract	0.4	1.0	100
$(NH_4)_2SO_4$ fraction	1.6	4.6	43
pH fractionation	5.3	15.2	31
DEAE-Sephadex A 25 chromatography	25.0	71.4	21

U, units

with a correlation coefficient of −0.994. The molecular mass of the aldolase subunits was calculated from the distance of migration (6.9 cm) as approximately 33 kDa. The molecular mass of the native enzyme was also determined by gel chromatography using a Sephacryl HR 200 column (eluent: 50 mM phosphate buffer containing 150 mM NaCl, pH 7.5). The result was identical to that obtained by SDS electrophoresis, indicating that the *S. carnosus* aldolase is a monomeric enzyme.

Proof that S. carnosus aldolase is a class I enzyme

Effect of chelating agents. The EDTA inhibition test was carried out with purified aldolase. EDTA did not inhibit the aldolase from *S. carnosus* even at 0.1 M (Table 2).

Inactivation of aldolase by DHAP and $NaBH_4$. Class I fructose 1,6-bisphosphate aldolases form a Schiff base intermediate with DHAP, which can be reduced with $NaBH_4$ to yield an inactive protein. This reduced complex has no catalytic activity. After reaction with DHAP and $NaBH_4$, more than 95% of the aldolase activity was lost. In the absence of DHAP, $NaBH_4$ did not inhibit the aldolase activity (Table 2). Both results clearly show that the newly isolated aldolase from *S. carnosus* is a class I enzyme.

Table 2. Activity of *S. carnosus* aldolase in the presence of various reagents

Additives	Activity (U/ml)	Inhibiton (%)
None	1.08	—
EDTA (100 mM)	1.10	—
$NaBH_4$ (100 mM)	1.06	2
$NaBH_4$ (100 mM), DHAP (20 mM)	0.05	95

DHAP, dihydroxyacetone phosphate

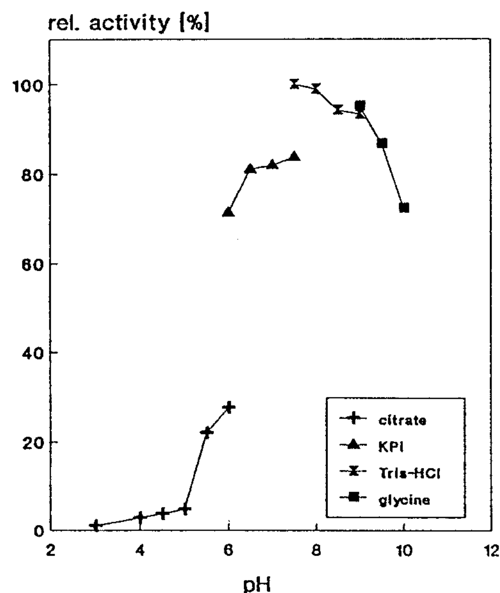

Fig. 1. pH optimum of fructose-1,6-bisphosphate (F-1,6-BP) aldolase from *Staphylococcus carnosus* determined in various buffers

pH optimum

The pH-optimum for purified aldolase was measured in citrate-, phosphate-, TRIS-HCl and glycine buffer. Like other class I aldolases, *S. carnosus* aldolase exhibited a broad pH optimum, ranging from pH 6.5 to 9.0 (Fig. 1).

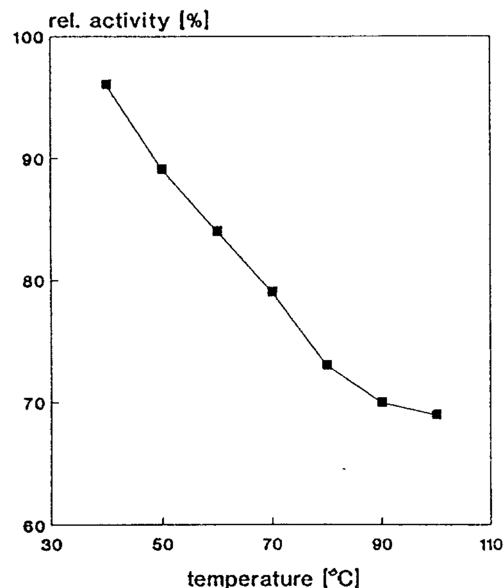

Fig. 2. Heat inactivation of F-1,6-BP aldolase from *S. carnosus*. The purified enzyme was incubated at various temperatures for 5 min. After cooling in an ice bath the samples were assayed for remaining activity

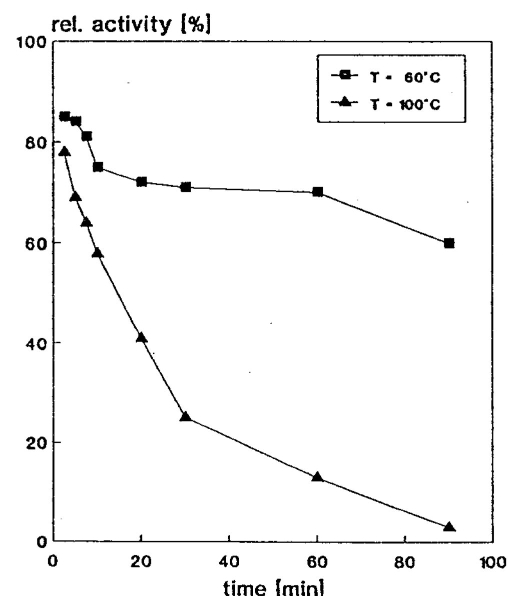

Fig. 3. Temperature (T) stability of F-1,6-BP aldolase from *S. carnosus* at 60° C and 100° C. The purified enzyme was heated at the indicated temperatures in a water bath. After cooling in an ice bath the remaining activity was determined

Heat stability

The aldolase from *S. carnosus* demonstrates remarkable resistance to heat inactivation. Figure 2 shows the rate of inactivation by incubating the purified enzyme in 60 mM TRIS-HCl buffer, pH 7.5, at various temperatures for 10 min. Figure 3 shows the rate of inactivation for various incubation times at 60° C and 100° C. The remaining activity after 90 min at 60° C was more than 70%. Complete inactivation at 100° C was observed only after more than 90 min.

pH stability

The *S. carnosus* aldolase exhibits extreme stability against acid and base (Fig. 4). The purified aldolase was titrated with HCl or NaOH to various pH values. After 5 min the aldolase activity was assayed spectrophotometrically. In the assay mixture the enzyme was immediately brought to pH 7.5. There was no detectable influence on aldolase activity by pretreatment between pH 1.0 and 12.0.

Kinetic studies and substrate specificity

The kinetic parameters, V_{max} and K_m, with F 1,6-BP and fructose 1-phosphate (F 1-P) as substrates were determined by measuring the reaction rate at various substrate concentrations. A K_m of 0.022 mM and a V_{max} of 19 μmol min^{-1} mg^{-1} were calculated for F 1,6-BP as substrate (Fig. 5) by linear regression of the Michaelis-Menten equation. A K_m of 18.8 mM and a V_{max} of 1.9 μmol min^{-1} mg^{-1} were obtained with F 1-P. Besides glyceraldehyde 3-phosphate G 3-P several other aldehydes were tested as substrates together with DHAP in the synthesis reaction. The additional aldehydes that have been identified so far as substrates are D,L-glyceraldehyde, formaldehyde, acetaldehyde and methylglyoxal.

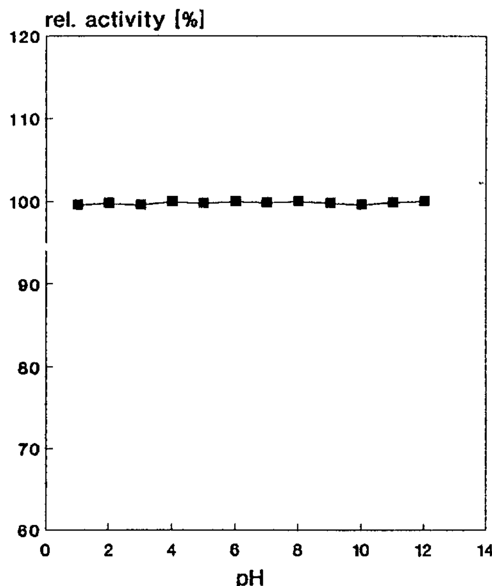

Fig. 4. pH stability of F-1,6-BP aldolase from *S. carnosus*. Purified aldolase was titrated with HCl or NaOH to the various pH values. After 5 min incubation at the indicated pH values the enzyme was diluted tenfold in standard buffer and assayed spectrophotometrically

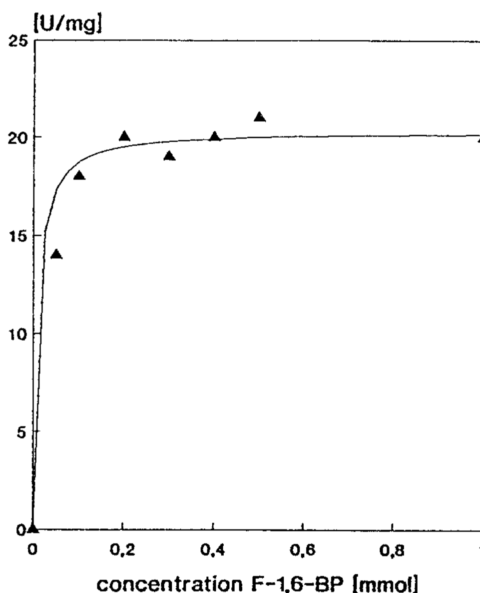

Fig. 5. Michaelis-Menten kinetics of F-1,6-BP from *S. carnosus* for fructose 1,6-bisphosphate as substrates; U, units

Discussion

Götz et al. (1980) described a very heat-stable F-1,6-BP aldolase from *S. aureus*, but did not investigate the substrate specificity. In our studies we used *S. carnosus*, which is a non-pathogenic organism usually employed as starter culture in the preparation of sausages (DFG 1987). Like the F-1,6-BP aldolase from *S. aureus*, the aldolase from *S. carnosus* was shown to be a class I enzyme. In contrast to aldolases from animal sources (RAMA for example), which are tetramers with a subunit molecular mass of 30–40 kDa, the enzymes from *S. carnosus* and *S. aureus* are monomers with a molecular mass of approximately 33 kDa.

The aldolases from *S. aureus* and *S. carnosus* exhibit extreme pH stability. This finding could perhaps be related to the monomeric structure and fast renaturation rate under assay conditions (Jaenicke 1984). The K_m for F 1,6-BP as substrate (0.022 mM) is slightly lower for the *S. carnosus* than the *S. aureus* enzyme (0.045 mM). The temperature stability of the *S. carnosus* aldolase is quite satisfactory but distinctly different from that of the *S. aureus* aldolase, which is fully active after 90 min incubation at 100° C.

It has been shown that aldolase-catalysed condensations provide a practical route to a wide variety of simple sugars and sugar derivatives (Durrwachter et al. 1986). Wong and Whitesides (1983) established the use of rabbit muscle aldolase in the organic synthesis of unnatural sugar phosphates. The advantages of these

reactions are well known (Whitesides and Wong 1985). The enzyme from rabbit muscle, however, suffers from (1) instability during synthesis of sugar phosphates and (2) activity loss in the presence of many reactor materials (Bossow-Berke 1989).

Because of its outstanding stability and good accessibility, the *S. carnosus* enzyme possesses considerable potential for catalysing asymmetric aldol condensations. The *S. carnosus* aldolase also appears to exhibit a rather wide substrate specificity for the aldehyde component similar to the enzyme from rabbit muscle. This will be investigated in greater detail in future work.

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