Biochimica et Biophysica Acta, 661 (1981) 158–163
Elsevier/North-Holland Biomedical Press

BBA 69359

PHOSPHOFRUCTOKINASES FROM *LACTOBACTERIACEAE*

II. PURIFICATION AND PROPERTIES OF PHOSPHOFRUCTOKINASE FROM *STREPTOCOCCUS THERMOPHILUS* *

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(Received February 11th, 1981)

Key words: Phosphofructokinase; Heat stability; Kinetic properties; (Strep. thermophilus)

Phosphofructokinase (ATP : D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) from *Streptococcus thermophilus* has been purified. It is a tetramer composed of identical subunits of molecular weight 36 000 and exhibits Michaelis-Menten kinetics. Compared to the phosphofructokinases from taxonomically related bacteria, the enzyme from *S. thermophilus* is more stable at high temperatures. In addition, it has been demonstrated that the phosphofructokinases from lactobacteria and also from *Bacillus stearothermophilus* show immunologic cross-reaction. In spite of the significantly different kinetic properties and the different thermostability of these enzymes, this finding indicates great structural resemblance.

Introduction

Bacterial phosphofructokinases (ATP : D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) consist of a group of enzymes with similar molecular weights which are about half that of phosphofructokinases from higher organisms [1–3]. In spite of the relatively low molecular weight of the subunits, bacterial phosphofructokinases exhibit a diversity in kinetic and physical properties [3–8] which has not been demonstrated in higher organisms. In bacteria phosphofructokinases occur which exhibit allosteric behaviour and end-product inhibition [1–5,7]. Certain phosphofructokinases also exhibit heat stability [7,8].

In our laboratory, the phosphofructokinases from the *Lactobacteriaceae* family have been extensively studied [3,9,10]. The kinetic properties of the phosphofructokinases vary greatly as was previously exemplified for the allosteric phosphofructokinase from *L. acidophilus* and for the non-allosteric enzyme from *L. plantarum* [3]. This study reports the characteristics of a thermostable variant of phosphofructokinase from *Streptococcus thermophilus*. It has been further shown that within the *Lactobacteriaceae* family the phosphofructokinases so far examined exhibited immunologic cross-reaction and therefore share common antigenic determinants which indicate great structural resemblance in spite of the kinetic and physical varieties.

Materials and Methods

Materials. Biochemicals were obtained from Boehringer Mannheim, F.R.G. The other chemicals and the growth media were from Merck, Darmstadt, F.R.G. Cibacron blue Sephadex was prepared by the method of Kopperschläger et al. [11]. Phosphofructokinases from *L. plantarum* and *L. acidophilus* and antibodies against phosphofructokinase from *L. plantarum* were prepared as described previously [3].

Assays. Routine assays for phosphofructokinase activity were performed in the following system: 50

* The preceding paper in this series is Ref. 3.

mM triethanolamine-HCl (pH 7.6) 4.0 mM $MgCl_2$, 4.0 mM ATP, 5.0 mM fructose 6-phosphate, 0.3 mM NADH, 60 μg/ml fructose-1,6-bisphosphate aldolase, 5 μg/ml trisephosphate isomerase, and 40 μg/ml glycerol-1-phosphate dehydrogenase. Temperature was maintained at 25°C. Protein concentrations were determined according to Lowry et al. [12].

Cultivation of bacteria. Cultures of *S. thermophilus* were a gift from the Eidgenössisches Institut für Milchforschung, Bern-Liebefeld, Switzerland. The bacteria were grown in the following medium: whey, obtained from a local dairy, was brought to pH 4.0 by the addition of acetic acid, sterilized and centrifuged after cooling. Then, 10 g peptone and 3 g yeast extract were added to 1 l supernatant and the pH was adjusted to pH 7.0 by the addition of triethanolamine. This medium was sterilized again. 5 ml of a stock culture of the bacteria were used for the inoculation of 1 l medium and the bacteria were grown at 55°C. *B. stearothermophilus* was cultivated as described by Sidler and Zuber [13]. *Lactobacilli* were grown on a selective medium [14] as described previously [3].

Purification of phosphofructokinase from S. thermophilus. The purification of phosphofructokinase from *S. thermophilus* followed the procedure developed earlier for the purification of the enzyme from *L. plantarum* [3] with the following modifications: protamine sulphate (180 mg/nkat phosphofructokinase) was added to the extract from the bacteria and the precipitated phosphofructokinase was separated by centrifugation (3 000 × *g*, 45 min). The enzyme was extracted from the precipitate with 0.5 M NaCl, cleared by centrifugation (20 000 × *g*, 25 min), and dialyzed overnight against 40 l 20 mM phosphate buffer (pH 6.5). During dialysis, the enzyme was precipitated again and could be separated by centrifugation. The precipitate was dissolved in 0.2 M NaCl and stirred with the 0.6-fold vol. of CM-Sephadex (soaked in water) for 20 min in order to remove residual protamines. The phosphofructokinase did not bind to the ion exchanger and after dialysis against 20 mM phosphate buffer (pH 6.5), the phosphofructokinase-containing filtrate was applied to a chromatography column filled with Cibacron blue coupled to Sephadex G-200. The enzyme was eluted from this column by 10 mM ATP. This eluate was then subject to chromatography on DEAE-Sephadex A-50 which was equilibrated with 20 mM phosphate buffer and was eluted with a linear gradient from 20 to 200 mM phosphate (pH 6.5).

The data of a typical preparation are summarized in Table I. Homogeneity of the preparation was proved by SDS-polyacrylamide gel electrophoresis [15] and by analytical ultracentrifugation.

Molecular weight determinations. The determinations of molecular weights on polyacrylamide gels in the presence of SDS and by sedimentation equilibrium centrifugation were performed as described previously [3].

Kinetic experiments. Kinetic analyses were performed in 0.1 M triethanolamine-HCl buffer in the presence of 5.0 mM $MgCl_2$, 0.3 mM NADH, 10 μg aldolase/ml, 5 μg glycerol-1-phosphate dehydrogenase/ml, and 2 μg triosephosphate isomerase/ml and varying concentrations of either fructose 6-phosphate or ATP and a fixed concentration of the other substrate. At 50°C, the phosphofructokinase reactions were run for 2 min, stopped with 1 M $HClO_4$ and the samples were then assayed for fructose 1,6-bisphosphate, after neutralization, using the coupling enzyme system described above.

TABLE I

PURIFICATION OF PHOSPHOFRUCTOKINASE FROM *S. THERMOPHILUS*

	Volume (ml)	Activity (μmol · min^{-1})	Protein (mg)	Specific activity (μmol · min^{-1} · mg^{-1})	Yield (%)
Extract	365	3 040	5 865	0.52	100
Protamine sulphate treatment	400	2 832	1 176	2.41	86
Cibacron Blue Sepharose chromatography	2 200	2 288	158	14.5	70
DEAE-Sephadex chromatography	380	1 525	11.4	134	46

Kinetic data were evaluated as described previously [10].

Results

Molecular and kinetic properties of phosphofructokinase from S. thermophilus

Phosphofructokinase from *S. thermophilus* migrated as a single band on polyacrylamide electrophoresis in the presence of SDS. The molecular weight of the protomers was estimated to be 36 000 (data not shown). The molecular weight of the native enzyme has been determined by sedimentation equilibrium centrifugation as 148 400 (data not shown). Thus, the enzyme appears to be a tetramer composed of four identical subunits.

The activity of phosphofructokinase from *S. thermophilus*, as determined in the presence of a saturating ATP concentration (5.0 mM) by extrapolation to the rate in the presence of an infinite fructose 6-phosphate concentration, was optimum at pH 7.0 (25°C). At pH 8.5, V decreased to 76% and at pH 5.5 to 61% of that measured at the pH optimum. The substrate saturation curves for fructose 6-phosphate and ATP strictly followed Michaelis-Menten kinetics between pH 5.5 and 8.5. ADP, fructose 1,6-bisphosphate, glucose 1,6-bisphosphate, 2- and 2-phosphoglycerate, or phospho*enol*pyruvate had no apparent effect on the activity when present at a concentration of 1 mM.

Table II summarizes apparent values for V and the Michaelis constants for ATP and fructose 6-phosphate at three different temperatures. The apparent K_m values for ATP were the same at 25, 37 and 50°C. V and K_m values for fructose 6-phosphate were examined in the presence of 5.0 mM ATP, a concentration that was practically saturating. The data given in Table II for these values are therefore approximates of the true constants. At 50°C, V was about 3-times higher than at 25°C; but the K_m for fructose 6-phosphate also increased by a factor of 2.4. In the presence of 0.5 mM fructose 6-phosphate and 5 mM ATP, the ratio of the phosphofructokinase rates at 50 and 25°C is only 1.5. In the presence of lower fructose 6-phosphate concentrations, the accelerating effect of rising temperature on the phosphofructokinase activity is still smaller.

TABLE II

APPARENT VALUES FOR V AND K_m FOR FRUCTOSE 6-PHOSPHATE AND ATP AT THREE DIFFERENT TEMPERATURES (pH 7.0)

Temperature:	25°C	37°C	50°C
V (μmol · min^{-1} · mg^{-1})	174	299	534
K_m^{app} for fructose 6-P [a] (mmol/l)	1.44	2.15	3.44
K_m^{app} for ATP (mmol/l) [b]	0.23	0.28	0.24

[a] In the presence of 5.0 mM ATP.
[b] In the presence of 2.0 mM fructose 6-phosphate.

Thermostability

Fig. 1A shows the activity of purified phosphofructokinases from three lactobacteria after 3 min heating at different temperatures. The allosteric enzyme from *L. acidophilus* was completely inactivated at 66°C, the non-allosteric enzyme from *L. plantarum* at 79°C and the phosphofructokinase from *S. thermophilus* required 90°C for complete inactiva-

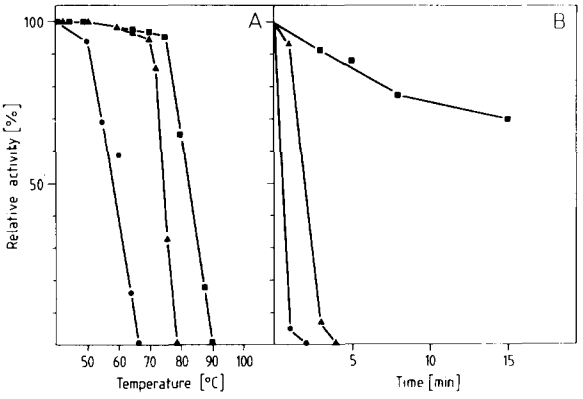

Fig. 1. Heat stability of purified phosphofructokinases from *Lactobacteriaceae*. A. Stability of phosphofructokinase activities after 3 min heating to various temperatures. B. Time dependence of the inactivation of phosphofructokinases at 75°C. Samples of the enzymes in 0.1 M triethanolamine-HCl buffer (pH 7.6) were warmed to 40°C in thin-walled glass tubes and then immersed in a water bath kept at the given temperature. After the incubations, the samples were cooled on ice and assayed for enzyme activity at 25°C. The activity was referred to the initital activity. Source of the phosphofructokinases: *S. thermophilus* (■———■), *L. plantarum* (▲———▲), *L. acidophilus* (●———●).

tion. The higher thermostability of phosphofructokinase from *S. thermophilus* is also obvious when the time dependence of inactivation was investigated at a constant temperature of 75°C (c.f. Fig. 1B). The *L. acidophilus* enzyme was completely inactivated within 2 min and the *L. plantarum* enzyme within 4 min, whereas the phosphofructokinase from *S. thermophilus* exhibited relatively high thermostability. This enzyme lost only 30% of its activity within 15 min at 75°C.

Immunologic comparison of some microbial phosphofructokinases

The immunologic relationship between the phosphofructokinases from lactobacteria is demonstrated by the Ouchterlony double-diffusion test depicted in Fig. 2. The center well contained an antiserum against the phosphofructokinase from *L. plantarum*. In addition to precipitating the corresponding antigen, the serum also precipitated the phosphofructokinases from *L. acidophilus* and *S. thermophilus*. The spurs of the precipitation lines, however, clearly demonstrate that there were antigenic determinants on the *L. plantarum* enzyme which were not carried by the phosphofructokinases from *L. acidophilus* and *S. thermophilus*. In addition, this indicates that there were some antigenic determinants recognized by the antiserum on phosphofructokinase from *S. thermophilus* which were not present on the enzyme from *L. acidophilus*.

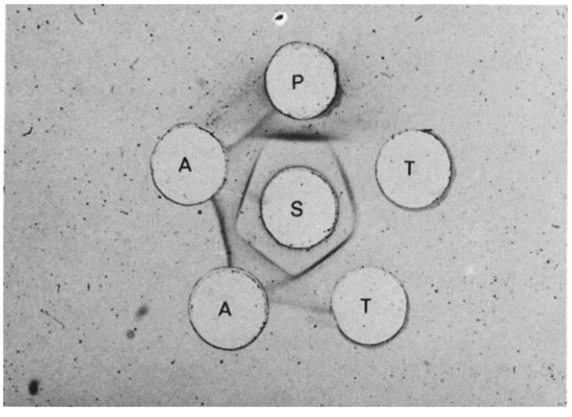

Fig. 2. Immunologic double-diffusion test of an antiserum against phosphofructokinase from *L. plantarum* with phosphofructokinases from *S. thermophilus* (wells T), *L. acidophilus* (wells A) and *L. plantarum* (well P). The antiserum was placed in the center well (S). The immunoplates were incubated for 14 h at 4°C, washed with 0.1 M phosphate buffer (pH 7.6) and stained with Lichtgrün [19].

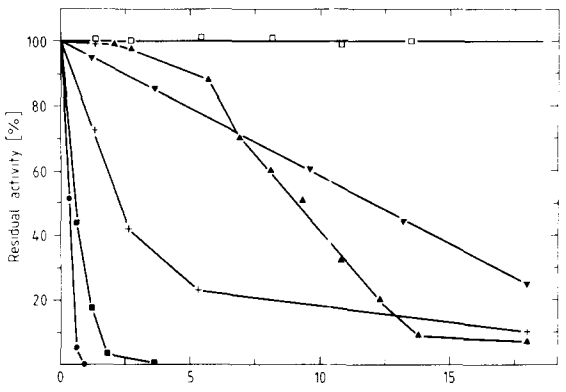

Fig. 3. Reaction of phosphofructokinases from different bacteria with an antiserum against phosphofructokinase (PFk) from *L. plantarum*. About 5 nkat phosphofructokinase were dissolved in 0.2 ml 0.9% NaCl or 0.9% NaCl mixed with various amounts of the antiserum and incubated for 30 min at 20°C. After 3 min centrifugation at 18000 × *g*, the residual enzyme activities were determined in the supernatants. The source of the phosphofructokinases was: *L. plantarum* (purified enzyme, ●———●), *S. thermophilus* (purified enzyme, ■———■), *L. helveticus* (extract, +———+), *L. acidophilus* (purified enzyme, ▼———▼), *B. stearothermophilus* (extract, ▲———▲), *E. coli* (extract, □———□). Controls were simultaneously run with the serum of a non-immunized sheep, but in no case were significant differences in the enzyme activities found as compared to incubations in the absence of sheep serum.

A broader comparison of the immunologic cross-reaction between different phosphofructokinases is shown in Fig. 3. Phosphofructokinases from bacteria and yeast were incubated in the presence of the antiserum against phosphofructokinase from *L. plantarum*, centrifuged in order to sediment precipitated antigen-antibody complexes, and then the enzyme activity was determined in the supernatants. Only small amounts of antibodies were required to remove the activities of the phosphofructokinases from *L. plantarum* and *S. thermophilus* indicating the close relationship between these enzymes. Other phosphofructokinases from the *Lactobacteriaceae* family, such as the allosteric phosphofructokinase from *L. acidophilus* and the non-allosteric phosphofructokinase from *L. helveticus* required higher anti-

body concentrations for partial inhibition and the enzyme could not be quantitatively precipitated under the conditions of the experiments. Interestingly enough, the thermostable phosphofructokinase from *B. stearothermophilus* [4] could be almost quantitatively precipitated by the antiserum, whereas the *E. coli* and yeast phosphofructokinase showed no detectable reaction.

Discussion

It has been shown previously that phosphofructokinase from *L. plantarum* and *L. acidophilus* are structurally related in spite of their markedly different kinetic and regulatory properties [3]. The present study has dealt with the phosphofructokinase from a moderate thermophile bacterium of the *Lactobacteriaceae* family. This enzyme has the same molecular weight as the enzymes from the lactobacilli but is more stable against heat denaturation. Its kinetic properties resemble that of *L. plantarum*, but the apparent K_m for fructose 6-phosphate is significantly higher. The parallel increase of V and K_m for fructose 6-phosphate apparently renders the enzyme less sensitive against temperature changes within the range of substrate concentrations which are supposed to be physiological. The enzyme is not inhibited by ATP as was observed with the phosphofructokinase from *L. acidophilus* nor by phospho*enol*pyruvate as were some other bacterial phosphofructokinases [4,5].

The double-diffusion test suggested a close structural relationship between the three enzymes from the lactobacteria. The phosphofructokinase from *S. thermophilus* has antigenic determinants in common with the *L. plantarum* enzyme and also shares some of these determinants with the enzyme from *L. acidophilus*. Since the precipitation line of *L. acidophilus* phosphofructokinase did not form a spur with that of *S. thermophilus* phosphofructokinase, the enzyme from the *Streptococcus* appears to have had more antigenic determinants in common with the *L. plantarum* enzyme than the allosteric phosphofructokinase from the *Lactobacillus*. This is interesting in light of the fact that no immunological relationship has been found between the L-lactate dehydrogenase from *L. acidophilus* and another streptococci [16].

The immunological comparison of phosphofructokinase from different sources also revealed a close relationship between the phosphofructokinases from *Lactobacteriaceae* and *B. stearothermophilus*. This comparison is of interest from different points of view: on one hand, the phosphofructokinase from the extreme thermophile bacterium *B. stearothermophilus* is different from the other bacterial phosphofructokinases mentioned in this study not only in its high thermostability but also by the fact that it is allosterically inhibited by phospho*enol*pyruvate. On the other hand, the primary [17] and tertiary [18] structures of this enzyme have been worked out recently.

The close immunologic relationship between the *B. stearothermophilus* enzyme and other bacterial phosphofructokinases confirms that the main characteristics of the structure of the thermophilic allosteric phosphofructokinase are representative also for other phosphofructokinases. These include the non-allosteric enzyme from *S. thermophilus* with moderate thermostability described in this study and the non-allosteric thermolabile phosphofructokinase from *L. plantarum* as well as the allosteric phosphofructokinase from *L. acidophilus* which is different to the enzyme from *B. stearothermophilus* with respect to its main allosteric inhibitor, ATP.

Acknowledgements

We are indebted to Professor H. Zuber and Dr. W. Sidler, ETH Zürich, for generously providing us with *B. stearothermophilus*, and to the Eidgenössisches Institut für Milchforschung, Bern-Liebefeld, for the gift of cultures of *S. thermophilus*. We thank Professor H. Sund and Mrs. U. Markau for the performance of the ultracentrifugational analysis and Professor B.G. Harris, NTSU, Denton, TX, for his help during the preparation of the manuscript. The expert technical assistance of D. von Nell is gratefully acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft and the Fonds der chemischen Industrie, Frankfurt am Main.

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