Pergamon

PII: S0031-9422(97)00081-2

Phytochemistry, Vol. 45, No. 5, pp. 903–906, 1997

0031-9422/97 $17.00+0.00

MANNOSE METABOLIZING ENZYMES FROM THE RED ALGA *GALDIERIA SULPHURARIA*

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(*Received in revised form* 16 *December* 1996)

Key Word Index—*Galdieria sulphuraria*; Rhodophyta; metabolism; mannose; toxicity; enzymes; purification; properties.

Abstract—The unicellular thermo-acidophilic rhodophyte *Galdieria sulphuraria* is capable of growing at high rates on D-mannose, a sugar toxic to many higher plants. Mannose is introduced into metabolism through an ATP-dependent fructokinase which phosphorylates fructose (K_m = 0.21 mM) as well as mannose (K_m = 0.48 mM) but not glucose. The product, mannose-6-phosphate, is converted to fructose-6-phosphate by a mannose-6-phosphate isomerase (K_m = 1.2 mM). The fructokinase was purified by chromatography on DEAE-Fractogel and hydroxylapatite. The enzyme is a homodimer with an M_r of 75 000. The mannose-6-phosphate isomerase was purified by PEG precipitation and affinity chromatography on Affinity-Gel Blue A. The enzyme is a monomer with an M_r of 48 000. The pathway of mannose utilization in *G. sulphuraria* strongly resembles the reactions of mannose non-sensitive higher plants, while plants with mannose sensitivity lack activity of mannose-6-phosphate isomerase. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

In many higher plants the addition of exogenous mannose in concentrations as low as 1 mM leads to a characteristic toxicity syndrome. Plants show reduced growth due to decreased uptake of oxygen and of certain ions through the roots. In some plants germination processes may even cease [1]. The main reason for the toxicity of mannose is the action of unspecific hexokinases, leading to the formation of mannose-6-phosphate. In mannose sensitive plants, enzymes like mannose-6-phosphate isomerase or mannose-6-phosphate mutase, which convert mannose-6-phosphate to other intermediates, are not present or show only low activity. Therefore, mannose-6-phosphate accumulates. In consequence, the intracellular Pi concentration decreases due to sequestering in mannose-6-phosphate. Both effects may impair cell metabolism as reviewed in ref. [1].

In mannose insensitive higher plants like *Amorphophallus konjac* [2] or *Medicago sativa* [3], mannose-6-phosphate is isomerized to fructose-6-phosphate by a mannose-6-phosphate isomerase, therefore avoiding the toxicity syndrome. Alternatively, in strains of *Escherichia coli* mannose is isomerized to fructose by a mannose isomerase and fructose is phosphorylated by a fructokinase. No toxicity syndrome is known in *E. coli* [4]. The red alga *Galdieria sulphuraria* grows heterotrophically in the dark on mannose as the sole source of carbon and energy [5]. Mannose concentrations as high as 100 mM show no impairment of growth. Here, we wished to address the question, as to which of the above mentioned pathways mannose may be metabolized in *G. sulphuraria*; either by a hexokinase and a mannose-6-phosphate isomerase or by a mannose isomerase and a fructokinase. Furthermore, we wished to purify and characterize the enzymes involved in the mannose metabolism in *G. sulphuraria*.

RESULTS AND DISCUSSION

In crude extracts of *Galdieria sulphuraria*, activity of mannose isomerase, as has been known in *E. coli* strains, was not detectable, excluding a direct metabolism of mannose to fructose. Instead, mannose was phosphorylated in the presence of ATP. In crude extracts of *G. sulphuraria* mannose-6-phosphate was converted to fructose-6-phosphate by a mannose-6-phosphate isomerase (EC 5.3.1.8). Thus, *G. sulphuraria* is capable of metabolizing mannose via phosphorylation and subsequent isomerization in a way similar to higher plants [1]. The described enzymes were also present in cells either grown on glucose or mannitol as carbon source or in autotrophically grown cells.

Manno- and fructokinase activities coeluted during

Table 1. Purification of fructokinase from *G. sulphuraria*

Step	Protein (mg)	Activity* (nkat)	Specific activity* (nkat mg^{-1})	Recovery (%)	Purification (–fold)
Crude extract	149	43	0.3	100	1
1. DEAE Fractogel	7.3	29	4.00	67	13
Hydroxylapatite	0.11	1.7	15.5	4	52
2. DEAE Fractogel	0.01	0.35	35.00	1	117

* With mannose as substrate.

chromatography on DEAE Fractogel and showed bands at the same positions in activity-stained non-denaturating PAGE-gels. This indicates that both activities were likely to be due to the same enzyme. Glucose was not phosphorylated by this enzyme. Activity with fructose was *ca* 10 times higher than with mannose. The kinase showed typical Michaelis–Menten kinetics and had a lower K_m for fructose (K_m = 0.21 mM) than for mannose (K_m = 0.48 mM). Thus, the kinase was designated as a fructokinase (EC 2.7.1.4). The fructokinase was purified about 117-fold by chromatography on DEAE Fractogel and hydroxylapatite (Table 1). The native fructokinase showed an M_r of about 75 000 as estimated from density gradient centrifugation. SDS-PAGE of purified fructokinase showed one single band at 36 000 (Fig. 1). Thus, the fructokinase appears to be a homodimer. A fructokinase for *Beta vulgaris* has a very similar M_r of 72 000 with subunits of 38 000 [6]. The algal fructokinase showed different pH-activity profiles for its substrates with optima of pH 7.0 for mannose and pH 8.5 for fructose. The half maximal activity was 1 pH above and below the optimum pH, respectively. The specificity of the kinase for fructose and mannose

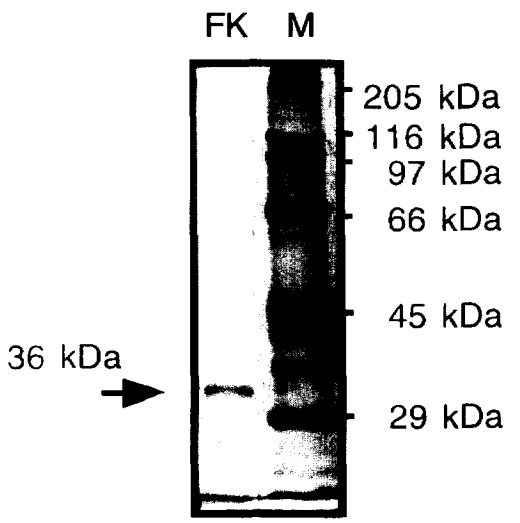

Fig. 1. SDS-PAGE of purified fructokinase from *G. Sulphuraria*. M, marker; FK, fructokinase.

and the different pH-optima for these substrates are similar for a manno-/fructokinase from *Leuconostoc mesenteroides* [7]. An optimum of pH 8.5 for fructose was also reported for fructokinases from *B. vulgaris* [6], *Glycine maxima* [8] and *Solanum tuberosum* [9]. The enzyme from *G. sulphuraria* was thermostable up to 55°. After 20 min incubation at 65° a loss of 50% activity was observed. Only ATP was used a phosphate donor by the fructokinase from *G. sulphuraria* (K_m = 0.5 mM at pH 7.5), while GTP, UTP or CTP were inactive. This stands in contrast to other fructo- and hexokinases which can use the latter nucleotides (e.g. *B. vulgaris* [6], *Spinacia oleracea* [10], *Dendrophthoe falcata* [11] or *Ricinus communis* [12]).

The mannose-6-phosphate isomerase of *G. sulphuraria* was very unstable *in vitro*. About 90% of the activity in a crude extract was lost after about 24 hr at 4°. We succeeded in purifying an active mannose-6-phosphate isomerase about 70-fold using PEG-precipitation and affinity chromatography on Affinity-Gel Blue A (Table 2). Non-denaturating PAGE of the purified native enzyme indicated an M_r of 48 000. SDS-PAGE gave a single band of 48 000 (Fig. 2). Therefore, the enzyme must be a monomer. Mannose-6-phosphate isomerases from the alga and from *Saccharomyces cerevisiae* [13] and *Medicago sativa* [14] show very similar M_r. The native mannose-6-phosphate isomerase from *Cassia coluteoides*, however, has a higher M_r of 74 500 [14]. The enzyme from *G. sulphuraria* showed optimal activity at pH 6.5–7.0 with half maximal activities at pH 6.0 and 7.5. The K_m with mannose-6-phosphate was 1.2 mM, similar to values obtained from *Amorphophallus konjac* [2], *S. cerevisiae* [13] and *C. coluteoides* [14].

Although the reaction sequence of mannose metabolism is basically the same in *G. sulphuraria* as in higher plants like *M. sativa*, *C. coluteoides* or *A. konjac*, there are differences. In higher plants very active hexokinases phosphorylate glucose, fructose and mannose [1, 10–12, 15] and are, therefore, less specific than the algal enzyme. In *G. sulphuraria* glucose is phosphorylated by a specific membrane-bound glucokinase (Gross, W., unpublished data). The mannose-6-phosphate isomerase in mannose-sensitive higher plants has a much lower activity than the

Table 2. Purification of mannose-6-phosphate isomerase *G. sulphuraria*.

Step	Protein (mg)	Activity (nkat)	Specific activity (nkat mg^{-1})	Recovery (%)	Purification (−fold)
Crude extract	165	100	0.6	100	1
PEG supernatant	20	100	5.0	100	8
Affinity-Gel Blue A	2.2	93	42	93	70

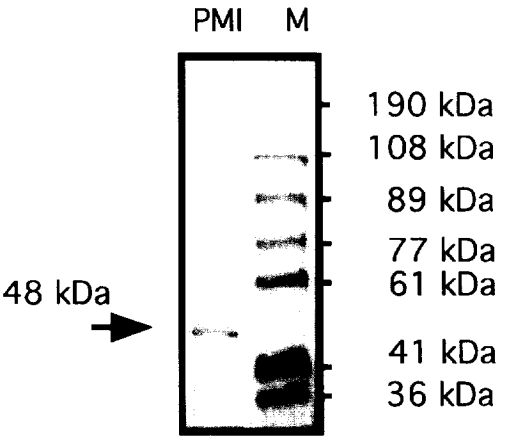

Fig. 2. SDS-PAGE of purified mannose-6-phosphate isomerase from *G. sulphuraria*. M, marker; PMI, mannose-6-phosphate isomerase.

hexokinases, resulting in accumulation of mannose-6-phosphate and depletion of Pi [1]. The fructokinase and mannose-6-phosphate isomerase of *G. sulphuraria*, in contrast, have equally high activities and thereby ensure that no toxic accumulation of mannose-6-phosphate or phosphate deprivation occurs.

EXPERIMENTAL

Materials. Fractogel TSK-650(s) was obtained from Merck (Germany) and Affinity-Gel Blue A from Amicon (Sulzbach/Taunus, Germany). All experiments were performed with *Galdieria sulphuraria* (Galdieri) Merola strain 074 from the culture collection of Naples (Italy) [16]. Cells were grown in liquid culture autotrophically [5] or in the dark in mineral media containing 50 mM D-mannose, D-glucose or D-mannitol [5].

Preparation of enzymes. The prepn of crude extracts has been described previously [17]. *Purification of fructokinase.* Proteins from a crude extract were applied onto a Fractogel TSK-650(s) column equilibrated with 10 mM Mops buffer, pH 7.0 (buffer A). The column was washed with buffer A and eluted with a gradient of 0 to 200 mM KCl in buffer A. Frs with fructokinase activity were pooled and applied to a hydroxylapatite column which had been equilibrated with 20 mM K–Pi buffer, pH 7.5 (buffer B). The column was washed with buffer B and proteins were eluted with a gradient of 20–300 mM K–Pi buffer, pH 7.5. Frs containing fructokinase activity were loaded onto a second Fractogel TSK-650(s) column. This column was equilibrated, washed, and eluted as described for the previous Fractogel TSK-650(s) column. Frs containing fructokinase activity were used for enzyme characterization.

Purification of mannose-6-phosphate isomerase. A crude extract was brought to 25% [w/w] PEG, the ppt. removed by centrifugation (20 min, 36 000 *g*) and the supernatant applied to an Affinity-Gel Blue A column. Following incubation for 1 hr at room temp., the mannose-6-phosphate isomerase was eluted with buffer A.

Enzyme assays. Fructokinase activity was assayed by coupling the production of fructose-6-phosphate to glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase. The reaction mix. of 1 ml contained 8.5 μmol Mops, pH 7.0, 2.1 μmol $MgCl_2$, 0.3 μmol ATP, 0.25 μmol NADP, 15 nkat glucose-6-phosphate isomerase, 15 nkat glucose-6-phosphate dehydrogenase, 2.5 μmol fructose, and enzyme. For mannokinase activity the assay mixture contained, in addition, 15 nkat of mannose-6-phosphate isomerase. The reaction was started with mannose instead of fructose. Mannose 6-phosphate isomerase activity was assayed according to [18]. Mannose isomerase activity was assayed using the reaction mixt. for fructokinase with mannose instead of fructose as substrate. Activities were monitored at 340 nm. Protein concns were estimated as described in either ref. [19] or ref. [20]. All K_m values were measured at pH 7.5.

Electrophoresis. Samples for SDS-PAGE were desalted by gel filtration and lyophilized. The dried samples were dissolved in sample buffer [21]. SDS-PAGE was carried out in 10% (w/w) polyacrylamide gels as described in ref. [21]. After electrophoresis, proteins were visualized by silver staining as described in ref. [22]. For PAGE under non-denaturating conditions the same solns as for SDS-PAGE were used except that SDS was omitted. Zymograms were carried out as described in ref. [23].

M_r *estimation.* The M_r of native fructokinase was determined by density gradient centrifugation of a 1:2 diluted crude extract as described in ref. [17]. The M_r of the native mannose-6-phosphate isomerase was determined by comparison of mannose-6-phosphate isomerase specific zymogram bands in non-dena-

turating PAGE-gels of 6, 7, 8 and 9% (w/w) polyacrylamide, as described in ref. [24].

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