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# A NOVEL ENZYME, TAGATOSE KINASE, FROM *MYCOBACTERIUM BUTYRICUM*

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**A novel enzyme catalyzing the phosphorylation of D-tagatose to D-tagatose 6-phosphate with ATP has been identified in extracts of dulcitol-grown *Mycobacterium butyricum* The enzyme was purified 100-fold with 29% recovery It required $Mg^{2+}$, $Mn^{2+}$ or $Fe^{2+}$ and showed maximum activity at pH 7 5 The molecular weight as determined by Sephadex G-100 filtration amounted to 63 000 The apparent Michaelis constants for D-tagatose and ATP were 0 8 and 1 0 mM, respectively Apart from tagatose, none of 14 common sugars including fructose and sorbose served as a substrate The enzyme preparations were not very sensitive to SH group inhibitors and heavy metals but rapidly lost activity on heating above 50°C**

## Introduction

As described in the literature specific NAD-dependent bacterial dehydrogenases can catalyze the formation of D-tagatose and its $C_6$-phosphate ester from dulcitol [1] and dulcitol 6-phosphate [2 3], respectively L-Arabinose isomerase from *Aerobacter aerogenes* [4] is also able to convert D-galactose into D-tagatose, slowly However, there are only incidental data on the metabolism of tagatose itself Early attempts to demonstrate the phosphorylation of tagatose in animal tissues were unsuccessful [5] On the other hand, there is subsequent information that bovine liver fructokinase is able to phosphorylate fructose as well as tagatose and sorbose at $C_1$ [6]

We have found a very specific kinase, tagatose kinase, in dulcitol-grown non-pathogenic strains of mycobacteria, which has been proved to phosphorylate D-tagatose to D-tagatose 6-phosphate with ATP as a phosphoryl group donor It seems that tagatose kinase has not been described as yet

The present report deals with the partial purification and characterization of tagatose kinase from *M butyricum*

## Materials and Methods

### *Special chemicals*

D-Tagatose, ATP, phospho*enol*pyruvate and crystalline lactate dehydrogenase and pyruvate kinase were from Sigma (U S A ), crystalline DNAase from Koch-Light (U.K ), NADH from Reanal (Hungary), dulcitol from Lachema (Czechoslovakia), DEAE-cellulose DE-52 from Whatman (U K ) and Sephadex G-100 from Pharmacia (Sweden)

### *Organism and growth conditions*

*M butyricum* was used throughout the study The strain was obtained from the Department of Plant Physiology, University of Lublin Cells were grown statically for 6 days at 37°C on the surface of a liquid medium [7] containing per l 25 g dulcitol/5 g galactose/10 g glutamic acid/2 g citric acid/0 5 g $KH_2PO_4$/0 5 g $MgSO_4$ 7 $H_2O$/0 05 g ferric ammonium citrate/0 02 g $ZnSO_4$ 7 $H_2O$ The medium was adjusted to pH 6 8 with KOH prior to sterilization The sugar solution was autoclaved separately

*Enzyme assays*

D-Tagatose kinase catalyzes an irreversible phosphorylation according to Eqn 1,

$$\text{D-tagatose} + \text{ATP} \xrightarrow{Mg^{2+}} \text{D-tagatose 6-phosphate} + \text{ADP} \quad (1)$$

The kinase activity could be measured either by the rate of disappearance of tagatose (Assay I) or formation of ADP (Assay II)

*Assay I* This assay was used to determine the enzyme activity in less purified preparations which contained NADH oxidase and ATPase The method is based on removal of phosphorylating sugar by means of $ZnSO_4$ and $Ba(OH)_2$ treatment [8] following by the estimation of free sugar extracted from the incubation mixture This method was used thoughout the purification procedure The routine assay mixture contained in 0 5 ml 30 μmol Tris-HCl buffer, pH 7 5/2 μmol $MgCl_2$/5 μmol tagatose/2 μmol ATP/ enzyme After incubation at 37°C for two equal time intervals, 0 1-ml samples were transferred to 1 ml water, then 0 25 ml 5% $ZnSO_4$ solution was added followed by 0 25 ml 0 3 N $Ba(OH)_2$ solution The suspension was mixed, centrifuged and 1-ml portions of supernatant were withdrawn for tagatose determination As the reaction was linear with time, 1 unit of enzyme activity was defined as 1 μmol tagatose utilized/min under assay conditions Specific activity was expressed as 1 unit/mg protein

*Assay II* Enzyme activity in the final preparations was assayed by ADP generation procedure which utilizes a coupled reaction sequence catalyzed by pyruvate kinase and lactate dehydrogenase The activity was monitored spectrophotometrically at 340 nm with the incubation mixture which contained in 1 7 ml 100 μmol Tris-HCl buffer, pH 7 5/2 μmol $MgCl_2$/2 μmol phospho*enol*pyruvate/0 2 μmol NADH/3 μmol tagatose/enzyme/non-limiting amounts of pyruvate kinase and lactate dehydrogenase When concomitant ADP was exhausted (after about 4 min), the reduction started with tegatose addition

*Determination of molecular weight*

A Sephadex G-100 column (1 5 × 90 cm) equilibrated with 50 mM phosphate buffer (pH 7 2) were calibrated with protein markers myoglobin (17 800), trypsin (24 000), hemoglobin (64 500) and aldolase (149 000)

*Methods*

Protein was determined by the method of Lowry et al [9] with crystalline bovine serum albumin as standard Tagatose was estimated with resorcinol hydrochloride color reaction [6] Paper chromatography of sugars was performed with Whatman No 1 filter paper, water-saturated phenol and *n*-butanol/pyridine/water (3 2 1 5) as solvents Reducing spots were detected with alkaline silver nitrate spray [10], ketose with resorcinol [11] and orcinol [12] reagents, phosphate esters with molibdate test [13] Paper electrophoresis was carried out using pyridine/acetic acid/water (15 50 2 500), pH 3 9, as a medium [14] Alditol acetates and trimethylsilyl derivatives of sugars were prepared subjected to gas chromatography by standard methods [15,16]

## Results and Discussion

*Purification of tagatose kinase*

Unless otherwise stated, all operations were performed at 0–4°C and all potassium phosphate buffers (pH 7 2) contained 1 mM EDTA and 5 mM 2-mercaptoethanol

*Step I Crude extract* The cells of *M butyricum* were harvested, washed with cold water on several layers of cheese-cloth, blotted with filter paper, weighed and stored at −15°C for several days before kinase extraction Frozen cells (25 g) were thawed in 120 ml 50 mM phosphate buffer and sonicated in 25-ml portions twice for 5 min in a MSE apparatus (100 W, 24 kHz) under cooling with ice Cell debris was removed by centrifugation at 16 000 × *g* for 45 min The crude extract was incubated with DNAase (2 mg) at room temperature for 30 min before further fractionation

*Step 2. First $(NH_4)_2SO_4$ precipitation* The crude extract (125 ml) was brought to 35% saturation with solid $(NH_4)_2SO_4$ After standing for 30 min in an ice bath, the inactive material was discarded by centrifugation at 16 000 × *g* for 30 min To the supernatant, solid $(NH_4)_2SO_4$ was added upto 70% saturation and the resulting precipitate was collected by centrifugation The pellet was resuspended in a minimal

amount of 50 mM phosphate buffer (fraction AS-1)

*Step 3 First Sephadex G-100 filtration* The non-dialyzed fraction AS-1 (about 17 ml) was applied to a Sephadex G-100 column (2 5 × 85 cm) equilibrated with the above mentioned buffer Fractions of 5 5 ml were collected at a flow rate of 25 ml/h

*Step 4 Second $(NH_4)_2SO_4$ precipitation* The pooled active fractions from the preceding steps were treated with solid $(NH_4)_2SO_4$ The protein precipitating at 50–65% saturation were collected by centrifugation The pellet was resuspended in a minimal volume of the buffer (fraction AS-2)

*Step 5 Second Sephadex G-100 filtration* The non-dialyzed fraction AS-2 (about 2 ml) was applied to a second Sephadex G-100 column (1 5 × 90 cm) Fractions of 3 ml were collected at a flow rate of 10 ml/h

*Step 6 DEAE-cellulose column chromatography* The pooled active fractions from the preceding step were loaded on a DEAE-cellulose column (1 × 3 cm) previously equilibrated with 10 mM phosphate buffer. After preliminary washings of the column with the buffer, the enzyme was eluted with a 200-ml linear gradient of NaCl from 0 to 0 5 M The tagatose kinase fractions were pooled, diluted with the same volume of 10 mM phosphate buffer, loaded on a small DEAE-cellulose column and concentrated by elution with 0 5 M NaCl

The purification procedure is summarized in Table I As one can see, 106-fold increase in specific activity with 29% recovery is achieved

*The characterization of tagatose kinase*

*Metal ion requirement* In coincidence with other kinases, tagatose kinase requires $Mg^{2+}$ for activity At 6 mM ATP and 6 mM tagatose concentrations the maximum activity was found in the presence of 3 mM $MgCl_2$ Higher concentrations of $MgCl_2$ gradually inhibited the reaction $Mg^{2+}$ can be partially substituted for $Mn^{2+}$ (63%) and $Fe^{2+}$ (24%) resulting from $MnCl_2$ and $FeSO_4$ (2 5 mM), respectively Other metal compounds, i e , $CaCl_2$, $CoSO_4$, $NiSO_4$, $CdSO_4$ and $ZnSO_4$ are not activatory

*Effect of pH* Fig 1 shows the dependence of the tagatose kinase activity on pH It is evident that the enzyme has a steep pH optimum of 7 5

*Substrate specificity* The enzyme displayed a high degree of specificity towards D-tagatose, and did not act on a variety of other sugars and sugar alcohols including D-glucose, D-mannose, D-galactose, D-fructose, D-galactosamine D-2-deoxy-galactose, dulcitol, L-sorbose, L-rhamnose, D-ribose, D-xylose, D-lyxose, D-xylulose and glycerol (5 mM final concentrations) The $K_m$ values for D-tagatose and ATP as determined spectrophotometrically with Assay II, at 22°C, amounted to 0 8 and 1 0 mM, respectively (Lineweaver-Burk presentation)

It is important to underline that the purified preparations are not contaminated with fructokinase as occurs in mycobacteria as a separate enzyme [17] In fraction AS-1 both enzymes can be partially separated by filtration on a Sephadex G-100 column (Fig 2)

TABLE I

PURIFICATION OF TAGATOKINASE FROM *MYCOBACTERIUM BUTYRICUM*

Enzyme activity was measured with Assay I 1 unit enzyme activity was defined as the amount of the enzyme forming 1 μmol tagatose ester/min at 37°C under standard assay conditions

| Purification step | Total volume (ml) | Total protein (mg) | Total units | Specific activity (units/mg) | Recovery (%) |
|---|---|---|---|---|---|
| 1 Crude extract | 125 | 1 150 | 42 6 | 0 037 | 100 |
| 2 Fraction AS-1 | 17 | 799 | 41 5 | 0 052 | 97 |
| 3 First Sephadex G-100 eluate | 70 | 189 | 27 4 | 0 145 | 64 |
| 4 Fraction AS-2 | 1 9 | 80 | 22 4 | 0 280 | 53 |
| 5 Second Sephadex G-100 eluate | 12 | 25 | 15 0 | 0 600 | 35 |
| 6 DEAE-cellulose eluate | 20 | 3 2 | 12 5 | 3 920 | 29 |

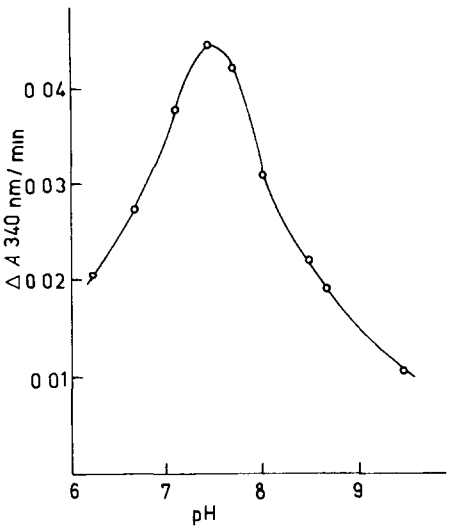


Fig 1 Effect of pH on D-tagatose phosporylation 0 13 M Tris-HCl buffer and Assay II were used

*Effect of inhibitors* The tagatose kinase is not very sensitive to the action of typical inhibitors When particular compounds were added directly to the incubation mixture, the following inhibition was obtained *p*-chloromercuribenzoate (2 mM), 15%, *p*-chloromercuriphenylsulfate (2 mM), 21%, $HgCl_2$ (2 mM), 32%, $ZnSO_4$ (1 mM), 38% and $CuSO_4$ (1 mM), 50% The enzyme was not inhibited by *N*-ethylmaleimide (1 mM), iodoacetate (1 mM), NaF (1 mM), EDTA (1 mM) or *o*-phenanthroline (1 mM)

*Molecular weight* The molecular weight of the enzyme was estimated by filtration on a Sephadex G-100 column with proteins of known molecular weight The molecular weight of tagatose kinase was found to be 63 000

*Stability* Fig 3 depicts an inactivation profile of the enzyme at various temperatures The results indicate that tagatose kinase s sensitive to heat and undergoes rapid destruction above 50°C No loss of activity occurred when the final preparation was stored at room temperature for 1 day in 0 2 M phosphate buffer (pH 6 3–8 0)

*Reaction products* To identify tagatose ester, its preparation was performed from the tagatose kinase incubation mixture The incubation mixture, which contained in 30 ml 1 6 mmol Tris-HCl buffer, pH 7 5/1 mmol $MgCl_2$/2 5 mmol ATP/3 mmol tagatose/ enzyme/step 6, 30 mg protein were incubated at 37°C for 5 h During this period 167 μmol tagatose were converted To the mixture, 80 ml ethanol were added and the precipitate discarded by centrifugation The supernatant was evaporated to dryness at 50°C and the residue dissolved in water (50 ml) The solu-

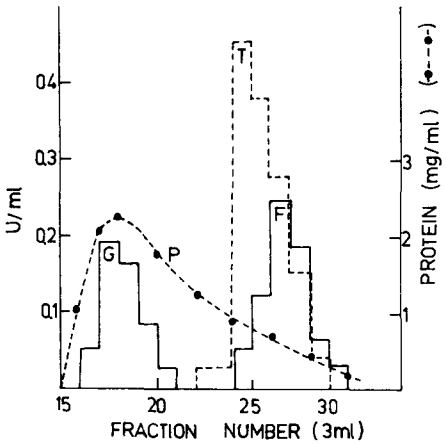


Fig 2 Separation of sugar kinases on a Sephadex G-100 column Fraction AS-1 (1 5 ml, 60 mg protein) was applied to the column (1 5 X 85 cm) Symbols G, glucokinase, T, tagatose kinase, F, fructokinase and P, protein

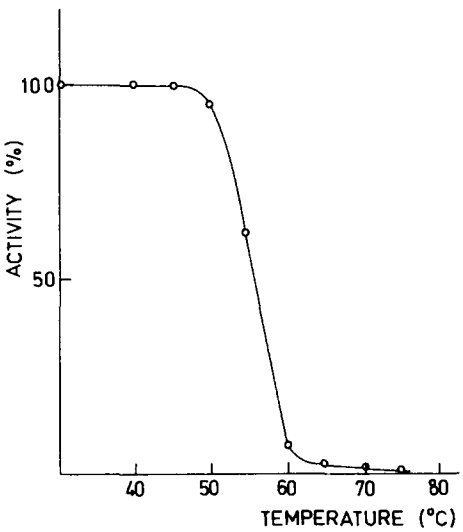


Fig 3 Heat-sensitivity of tagatose kinase Samples of a final enzyme preparation (0 13 ml, 100 μg protein) in 20 mM phosphate buffer (pH 7 2) were incubated for 1 min at various temperatures, then cooled and activity assayed using a spectrophotometric method

tion was put on a Dowex-1/$HCOO^-$/resin column (1 × 12 cm), washed with water and eluted with a linear 200-ml gradient of $HCOONH_4$ (pH 8 2) from 0 to 0 4 M The ester appeared in eluate from 130 to 185 ml. Concentrated eluate (8 ml) was treated with barium acetate solution (25%, 1 5 ml) and the precipitate discarded by centrifugation To the supernatant, 50 ml ethanol were added and kept cold overnight The precipitate was collected, suspended in water, treated with Dowex-50 ($H^+$) and neutralized with KOH 71 μmol tagatose ester were found in the final preparation

The ester migrated during electrophoresis towards anode and could be detected with alkaline silver nitrate [10], resorcinol hydrochloride [11], orcinol trichloroacetate [12] and molibdate [13] sprays It possesses reductive properties and its acid-stability resembles fructose 6-phosphate The ester was split with yeast acid phosphatase and phosphate [18] and the tagatose [6] released was estimated The ratio of $P_i$ to tagatose was found to be 1 00 0 91 Tagatose was subsequently identified by means of gas chromatography [15,16] A sample of tagatose ester was oxidized with periodate according to Jeanloz and Forchielli [19] The identification of glycolaldehyde phosphate [20,21] as a periodate oxidation product has strengthened the supposition that the phosphorylation takes place at $C_6$ of tagatose In a coupled test with pyruvate kinase and lactate dehydrogenase, ADP was found as a second tagatose kinase reaction product The stoichiometry of the tagatose kinase reaction is shown in Table II

The results vote for the conclusion that tagatose kinase phosphorylates D-tagatose at $C_6$ and thus should be classified as ATP D-tagatose-6-phosphotransferase The enzyme represents a part of the dulcitol degradation pathway in saprophytic mycobacteria [22] which is distinct from the tagatose 6-phosphate route of lactose catabolism in *Staphylococcus aureus* [23] The results are in agreement with our previous report [24] in which phosphorylation of tagatose was suggested

TABLE II

THE STOICHIOMETRY OF THE TAGATOSEKINASE REACTION

Experimental conditions as described in the text Tagatose 6-phosphate was calculated from the difference between tagatose, initially added, and tagatose found after incubation

| Compound | Amount added or produced (μmol) |
|---|---|
| Tagatose | 3 15 |
| Tagatose 6-phosphate | 2 93 |
| ADP | 3 37 |

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