AN ENZYMIC METHOD FOR THE DETERMINATION OF ERYTHRITOL

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ABSTRACT

An erythritol kinase has been isolated from *Propionibacterium pentosaceum* and purified from a contaminating glycerol kinase. A spectrophotometric method has been devised whereby erythritol can be quantitatively estimated in a series of coupled reactions with enzymes. The assay method has been applied to the analysis of the erythritol produced from a number of oligo- and poly-saccharides which have been submitted to periodate oxidation, borohydride reduction, and acid hydrolysis.

INTRODUCTION

The degradation of carbohydrates by periodate oxidation, reduction, and acid hydrolysis has been extensively applied to the elucidation of the structure of oligosaccharides, polysaccharides, and glycopeptides[1–3]. These reactions produce a mixture of simple alditols, glycolaldehyde, and free sugars, if periodate-stable residues are present. Thus a (1→4)-linked glucan gives rise to glycerol from the non-reducing terminal residues, and erythritol from the (1→4)-linked residues. The identification and quantitative determination of erythritol has been achieved by column[4] or paper-chromatographic[5] separation, followed by determination of the formaldehyde liberated on further periodate oxidation[6]. In addition, erythritol has been analysed by means of gas–liquid chromatography of the free alditol[7], trimethylsilyl ether[8,9], or acetate[10,11].

The metabolism of alditols by bacteria has received considerable attention in recent years, and it has been shown that erythritol can be metabolised by a specific erythritol kinase (ATP:erythritol phosphotransferase, E.C. 2.7.1.27) from *Propionibacterium pentosaceum*[12]. This report describes the purification of erythritol kinase and its use in the determination of erythritol derived from (1→4)-linked glucans.

EXPERIMENTAL

Organisms. — *Propionibacterium pentosaceum* E 2.1 was a gift from Dr. E. Wawszkiewicz, and *P. pentosaceum* 8070 was obtained from N.C.I.B., Torry Research Station, Aberdeen. Both organisms were maintained on Bacto Fluid thioglycolate

broth, solidified with 1% agar. Glycerol kinase (GK) was purchased from C.F. Boehringer, adenosine triphosphate (ATP), phosphoenolpyruvate (PEP), pyruvate kinase (PK), lactate dehydrogenase (LDH), and nicotinamide-adenine dinucleotide, reduced (NADH) were purchased from Sigma Chemical Company.

Spectrophotometric measurements were made at 340 nm at 25° in a Unicam SP800 spectrophotometer fitted with an external recorder. Total hexose was measured by the orcinol–sulphuric acid method[13], and periodate reduction was measured spectrophotometrically[14]. D-Glucose was measured by the D-glucose oxidase method[15] and protein by a modification of the Lowry method[16]. Conditions for periodate oxidation and Smith degradation of samples were as previously described[17].

Preparation of cell-free extracts. — *P. pentosaceum* was grown at 30° in 5-litre quantities, in the medium described by Shetter[18] modified by the addition of 2.5 g of sodium thioglycolate per litre. After the final culture had been incubated for 50 h, the cells were isolated by centrifugation at 3,000 *g* for 15 min, and were re-suspended in sodium chloride (0.89%, 125 ml) and incubated for 1 h at 30°. On re-isolation of the cells by centrifugation at 3,000 *g* for 15 min, the cells were re-suspended in a minimal medium (125 ml) of the following composition: ammonium sulphate, 0.1%; K_2HPO_4, 0.1%; $MgSO_4$, 0.05%; yeast extract, 0.025%; erythritol, 0.1% adjusted to pH 7.0. Incubation was carried out overnight at 30°.

The cells were isolated by centrifugation at 3,000 *g* for 15 min and then washed five times with Tris–HCl buffer, (50 mM, pH 7.4). After the final centrifugation procedure, the cells were re-suspended in the same buffer (40 ml) and were broken by ultrasonic disintegration (M.S.E. Ultrasonic disintegrator Model 60W) over a period of 60 min at 4°. Cell debris was removed by centrifugation at 10,000 *g* for 20 min at 4°.

Streptomycin treatment. — Solid streptomycin sulphate (Glaxo Laboratories, Greenford, Great Britain) was added to the cell-free extract to a final concentration of 1.25%. The resulting suspension was kept for 10 min at 4° and then centrifuged. The supernatant fluid was decanted, and the inactive precipitate was discarded.

Ammonium sulphate treatment. — Solid ammonium sulphate (15 g/100 ml) was added to the streptomycin supernatant solution with constant stirring. After 1 h, the suspension was centrifuged, and the precipitate was discarded. Additional ammonium sulphate (15 g/100 ml) was added to the solution. After 1 h, the suspension was centrifuged, and the precipitate was taken up in 7 ml of Tris–HCl buffer (0.1M, pH 7.4).

Heat treatment. — The above solution was heated for 20 min at 55°, cooled, and then centrifuged. The resulting supernatant solution was used for enzymic assays.

Assay of erythritol kinase. — Erythritol kinase activity was assayed either by the two-part method of Holten and Fromm[12] or with a standard reaction mixture containing Tris–HCl (50 mM, pH 7.4, 2.0 ml), ATP (50 mM, 0.05 ml), magnesium chloride (0.1M, 0.05 ml), potassium chloride (M, 0.05 ml), PEP (50 mM, 0.10 ml), PK (350 units/ml, 0.02 ml), LDH (600 units/ml, 0.02 ml), NADH (10 mM, 0.05 ml), and enzyme in a final volume of 2.95 ml. The reaction was started by addition of erythritol (0.4 M, 0.05 ml). The change in extinction of NADH at 340 nm was used as

a criterion of enzymic activity. One unit of erythritol kinase activity is that amount of enzyme causing the disappearance of 1 μmole of NADH/min.

Determination of glycerol and erythritol. — Samples (0.02–0.05 ml) from the neutralised acid hydrolysates of periodate-oxidised and borohydride-reduced carbohydrates were added to the standard assay mixture to which no erythritol kinase had been added. After determination of the initial extinction, E_1, the reaction was started by the addition of glycerol kinase (85 units/ml, 0.01 ml). Extinctions were measured continuously until no further change was observed (E_2). E_2-E_1 represents the amount of glycerol in the sample. Erythritol kinase preparation (0.03 unit, 0.05 ml) was added to the cell, and extinctions were again measured until no further change was observed (E_3). E_3-E_2 represents the amount of erythritol in the sample. A suitable blank reaction was carried out at the same time, in the absence of erythritol, to compensate for any small changes in extinctions occurring during the reaction. The conversions of glycerol and erythritol were normally complete in 10 and 40 min, respectively.

RESULTS

The reactions used in the assay for erythritol are based on two coupled reactions[12,19] with enzymes:

$$\text{erythritol} + \text{ATP} \xrightarrow{\text{EK}} \text{L-erythritol 1-phosphate} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+.$$

Portions of solutions containing 0.02–0.10 μmole of erythritol were assayed by the standard procedure. Using the extinction coefficient of NADH at 340 nm as $6.22 \times 10^6 \text{cm}^2/\text{mole}$, the resulting calibration was linear up to 0.10 μmole of erythritol.

The effect on the enzymic assay of inorganic ions, aldoses, and alditols arising from the Smith degradation of polysaccharides was investigated. Borate, iodate, and sulphate ions in the concentration used in the oxidation, reduction, and hydrolysis stages had no effect on the calibration of the assay. When erythritol was replaced by D-glucose, D-glyceraldehyde, ethylene glycol, L-threitol, glycolaldehyde, formaldehyde, or *O*-β-D-glucopyranosyl-(1→2)-D-erythritol, no change in extinction was observed. No evidence of inhibition of the enzymic reaction could be demonstrated when each of the compounds (200 μg) was incubated with the enzyme in the presence of erythritol (5 μg).

The quantity of erythritol and glycerol produced from the oxidation of a series of oligosaccharides by the standard procedure is given in Table I, and the erythritol produced from similar degradations of polysaccharides is given in Table II.

DISCUSSION

The erythritol kinase from *P. pentosaceum* had previously been shown[12] to quantitatively convert erythritol and ATP into L-erythritol 1-phosphate and ADP.

TABLE I

RELEASE OF ERYTHRITOL FROM PERIODATE-OXIDISED AND BOROHYDRIDE-REDUCED OLIGOSACCHARIDES

Substrate	*Oxidation time (h)*	*Erythritol (μmoles/μmole of substrate)*		*Glycerol (μmoles/μmole of substrate)*	
		Observed	*Calc.*	*Observed*	*Calc.*
Cellobiose	108	1.04	1.0	0.97	1.0
Panose	108	0.90	1.0	1.98	2.0
Maltotriose	108	1.92	2.0	0.94	1.0
Maltotetraose	108	2.88	3.0	0.95	1.0
6^3-α-D-Glucosyl-maltotriose	108	1.85	2.0	1.95	2.0
3^2-β-D-Glucosyl-cellobiose	108	0.95	1.0	0.94	1.0
6^3-α-Maltotriosyl-maltotriose	108	3.90	4.0	1.94	2.0
O-β-D-Glucopyranosyl-(1→3 2)-D-erythritol[a]	—	0.92	1.0	—	—

[a]The periodate-oxidation and borohydride-reduction step was omitted, and the material was hydrolysed with acid. The ratio of D-glucose–erythritol was 1:0.94.

TABLE II

RELEASE OF ERYTHRITOL FROM PERIODATE-OXIDISFD AND BOROHYDRIDE-REDUCED POLYSACCHARIDES

Substrate	*(1→4)-Linkage (%)*	*Oxidation time (h)*	*Periodate (μmoles/μmole of "anhydro-D-glucose")*	*Erythritol (μmoles/μmole of "anhydro-D-glucose")*
β-D-Glucan (barley)	60–70[20]	240	0.70	0.64
Isolichenin (Iceland moss)	40[21]	240	0.41	0.37
Pullulan (*Pullularia pullulans*)	60–70[22]	240	1.38	0.59

The method used involved incubation of the substrate with the enzyme at its pH optimum of 8.6 in the presence of inhibitors to prevent further metabolism of the products. This, however, necessitates a two-stage estimation of the ADP to be used because of the inhibition of the lactate dehydrogenase by these inhibitors. In the present method, the assay of erythritol may be carried out in one operation with more highly purified enzyme extracts, since at pH 7.4, the erythritol kinase exhibits 80% of its activity[12], and ADP is quantitatively assayed under the reaction conditions[19]. Reduction of NAD^+, when incubated with cell extracts of *P. pentosaceum* and L-erythritol 1-phosphate, had been reported[23,24] to take place at pH 9.0 but not at pH 7.0. Since stoichiometric amounts of NADH are oxidised in the presence of erythritol by using the standardised reaction conditions, it would appear that further metabolism of L-erythritol 1-phosphate is minimal. With relatively crude extracts, considerable interference in the assay procedure may be obtained by con-

tinual change in extinction of NADH, in the absence of erythritol. This may be caused by the presence in the enzyme extracts of small quantities of intracellular erythritol, ADP, or, alternatively, of other NADH-linked oxidoreductases. This interference becomes negligible with enzyme fractions which have been precipitated with ammonium sulphate and subjected to heat treatment. In the analyses of carbohydrates which have been submitted to the Smith-degradation procedure, it was necessary to commence the enzymic reaction by the addition of an excess of glycerol kinase in order to remove the glycerol which is normally produced from non-reducing end-groups, since the phosphorylation of glycerol with ATP will produce an equivalent amount of ADP. Although different batches of *P. pentosaceum* cells normally produced variable quantities of glycerol kinase, this enzyme was shown to be heat labile under the purification conditions used (Table III).

TABLE III

PURIFICATION OF ERYTHRITOL KINASE FROM *Propionibacterium pentosaceum*

Enzyme fraction	*Total protein (mg)*	*Total units (EK)*	*Specific activity (EK)*	*Total units (GK)*	*Specific activity (GK)*
Crude extract	712	8.6	0.012	2.6	0.004
Streptomycin supernatant	396	8.5	0.022	2.4	0.006
Ammonium sulphate	30	4.4	0.145	0.73	0.024
Heat treatment	18	4.4	0.244	0.03	0.002

The hydrolysis of formic esters has been shown[25] to occur with high concentrations of periodate ions and at pH values of 1.0 and 7.0, respectively, with the formation of substituted hydroxymalonaldehyde. Glycerol would be released on subsequent reduction with borohydride and hydrolysis. This would result in a decrease in the theoretical amount of erythritol and an increase in the amount of glycerol produced from an oligo- and poly-saccharide containing (1→4)-linked D-glucose residues. The release of both glycerol and erythritol from the oligosaccharides under investigation corresponded to the expected values, suggesting that minimal hydrolysis of formic esters had taken place.

Under the conditions of Smith degradation, terminal hexitol residues would be expected to release formaldehyde[26]. Since the enzyme is not influenced by formaldehyde, this will not limit the use of the system for that type of structural work.

It is realised that the polysaccharides selected for study in this work may have slight variations in the ratio of (1→4)-linkages to the other structural linkages in the molecules, compared with the original reports. However, the results obtained (Table II) are in agreement with published work. For instance, the reduction and hydrolysis of the periodate-oxidised glucan from *Phytophthora cinnamomi* afforded glycerol, erythritol, and D-glucose in molar ratios of 29:10:56 compared with 23:10:66 for the original glucan[27].

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