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Carbohydrate Research 288 (1996) 127–134

CARBOHYDRATE RESEARCH

A novel enzymic determination of maltobionate

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Received 10 November 1995; accepted 25 March 1996

Abstract

A novel enzymic determination of maltobionate with three enzymes (a new enzyme, maltobionate α-D-glucohydrolase [EC 3.2.1.-], gluconate kinase [EC 2.7.1.12], and 6-phosphogluconic dehydrogenase [EC 1.1.1.44]) is described. It fulfilled the need for an accurate, specific, and rapid assay of maltobionate, as shown by the procedure, the linearity of calibration curve, and the analytical precision, and was simple as compared to an HPLC approach. There was a good correlation ($r = 0.998$) between the results obtained by the enzymic and HPLC methods. As an application, the concentrations of maltobionate in the culture broth of *Pseudomonas taetrolens* IFO 3460 were determined by the proposed method, affording the maltobionate production from maltose in the course of cultivation of this strain. © 1996 Elsevier Science Ltd.

Keywords: Maltobionate; Maltobionate α-D-glucohydrolase; Gluconate kinase; 6-Phosphogluconic dehydrogenase; *Pseudomonas taetrolens*

1. Introduction

Maltobionic acid (4-*O*-α-D-glucopyranosyl-D-gluconic acid) is an oxidation product of maltose, which can be prepared efficiently by bromine oxidation [1] or electrolytic oxidation [2]. The biochemical formation of maltobionate by microorganisms, for example the proper strains of the genus *Pseudomonas*, has been demonstrated [3], and it has been reported that the economical and industrially viable processes provide the production of maltobionate in quantity, in good yield, and in high purity [4–7]. The maltobionate is in use as a food additive, with both an acid taste like citric acid etc. and a sweet taste of disaccharide [8].

Although maltobionate has been determined by paper chromatography [9], ion-ex-

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0008-6215/96/$15.00
PII S0008-6215(96)00086-9

change chromatography [10,11], and HPLC [6], an enzymic method has not yet been developed for the determination of maltobionate in foodstuffs or culture broth.

Recently, we have isolated from soil a bacterial strain (alkalophilic *Bacillus* sp. N-1053) which produced a new enzyme, classified as EC 3.2.1.-, maltobionate α-D-glucohydrolase, being highly specific for maltobionate [12]. In order to establish a convenient determination of maltobionate, we devised a novel enzymic endpoint assay with this enzyme. After hydrolytic cleavage of maltobionate to β-D-glucose and D-gluconate, the resultant D-gluconate could be estimated with the coupled enzymes, gluconate kinase and 6-phosphogluconic dehydrogenase (see also ref. [13]). Although new enzymic methods for the determination of D-gluconate with D-gluconate 2-dehydrogenase [EC 1.1.99.3] and 5-keto-D-gluconate 5-reductase [EC 1.1.1.69], respectively, have been reported by Ameyama et al. [13], these approaches are not suitable for the maltobionate determination. The optimum pH of about 7.0 of maltobionate α-D-glucohydrolase is extremely different from those of the dehydrogenase (pH 5.0) and the reductase (pH 10.0).

In our proposed two-reagent system, endogenous constituents (D-gluconate and 6-phospho-D-gluconate) are eliminated before determination, making it possible to quantitate maltobionate using the following reactions. There are two reactions in the "first step" and three reactions in the "second step".

First step (elimination of D-gluconate and 6-phospho-D-gluconate):

$$\text{D-Gluconate} + \text{ATP} \xrightarrow{\text{gluconate kinase}} \text{6-Phospho-D-gluconate} + \text{ADP}$$

$$\text{6-Phospho-D-gluconate} + \text{NADP}^+ \xrightarrow{\text{6-phosphogluconic dehydrogenase}} \text{Ribulose-5-phosphate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

Second step (determination of maltobionate):

$$\text{Maltobionate} + \text{H}_2\text{O} \xrightarrow{\text{maltobionate α-D-glucohydrolase}} \text{β-D-Glucose} + \text{D-Gluconate}$$

$$\text{D-Gluconate} + \text{ATP} \xrightarrow{\text{gluconate kinase}} \text{6-Phospho-D-gluconate} + \text{ADP}$$

$$\text{6-Phospho-D-gluconate} + \text{NADP}^+ \xrightarrow{\text{6-phosphogluconic dehydrogenase}} \text{Ribulose-5-phosphate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

The increase of NADPH in the second step, as measured by the change in extinction at 340 nm, is proportional to the amount of maltobionate present.

2. Experimental

Materials.—Sodium maltobionate was prepared from maltose by oxidation with bromine using the slightly modified method of Glattfeld and Hanke [1]. Maltose monohydrate was purchased from Nacalai Tesque Inc., Japan, and ATP and NADP were obtained from Sigma Chemical Co., USA. All other chemicals were of the highest grade generally available.

Maltobionate α-D-glucohydrolase [EC 3.2.1.-] was purified to apparent homogeneity

from a cell-free extract of alkalophilic *Bacillus* sp. N-1053 [12]. This enzyme hydrolyzed maltobionate more effectively than disaccharides such as maltose and maltitol, and no enzyme activity was detected towards sucrose, lactose, isomaltose, and *p*-nitrophenyl α-D-glucopyranoside, whereas α-glucosidases [EC 3.2.1.20] and glucoamylases [EC 3.2.1.3] from various origins showed weak or no activities towards maltobionate, compared with the activity towards maltose. Some other properties were: K_m value for maltobionate, 1.63 mM; optimum pH, about 7.0; optimum temperature, about 55 °C. The maltobionate α-D-glucohydrolase could be stored at −20 °C for at least several months without any significant loss of activity in 20 mM phosphate buffer (pH 7.5) containing 5% glycerol and 0.005% 2-mercaptoethanol until use. Gluconate kinase [EC 2.7.1.12] from *Escherichia coli*, 6-phosphogluconic dehydrogenase [EC 1.1.1.44] from yeast, and glucose oxidase [EC 1.1.3.4] from *Aspergillus niger* were also purchased from Sigma Chemical Co. α-Glucosidase [EC 3.2.1.20] from *Saccharomyces* sp. and peroxidase [EC 1.11.1.7] from horseradish were from Toyobo Co., Ltd., Japan.

Preparation of reagents.—A 0.8 mM sodium maltobionate standard solution (distilled water) was freshly prepared. Reagent A was prepared to contain 330 U gluconate kinase, 2,500 U 6-phosphogluconic dehydrogenase, 6.25 mmol ATP, 6.25 mmol $MgCl_2$, and 1.88 mmol NADP per L in 50 mM HEPES–NaOH buffer (pH 7.5). Reagent B was prepared to contain 40 kU maltobionate α-D-glucohydrolase, 5% glycerol, and 0.005% 2-mercaptoethanol per L in 50 mM HEPES–NaOH buffer (pH 7.5).

Procedure for the determination of maltobionate.—Maltobionate was enzymically assayed by the procedure described in Scheme 1 using a Hitachi Model 557 Double Wavelength Double Beam Spectrophotometer (Hitachi Ltd., Japan) equipped with a cuvette holder keeping a constant temperature.

The amount of maltobionate was calculated by the following formula:

$$\text{maltobionate (mmol/L)} = \frac{(Es1 - Es0 \times 0.9) - (Eb1 - Eb0 \times 0.9)}{(Est1 - Est0 \times 0.9) - (Eb1 - Eb0 \times 0.9)} \times 0.8$$

where 0.9 is the factor for correction of the reaction volume and 0.8 represents the maltobionate standard solution in mmol/L.

HPLC method.—To compare with the proposed enzymic method, maltobionate was also analyzed by HPLC. The operating conditions were as follows: column, TSKgel Amido-80 (4.6 × 250 mm, Tohso Co., Japan); solvent, 70:30 acetonitrile–water containing 0.1% phosphoric acid; flow rate, 1.0 mL/min; column temperature, 30 °C; injection volume, 30 μL. The effluent was monitored at 210 nm. The detection limit for this method was 0.01–0.02 mmol/L and its time taken for an individual run was 15 min.

Determination of maltose.—The determination of maltose was carried out by measuring D-glucose formed on hydrolysis of maltose as follows. To 0.1 mL of a properly diluted sample, 1.0 mL of a reaction reagent containing 10 kU α-glucosidase, 6 kU glucose oxidase, 0.7 kU peroxidase, 10 mmol phenol, and 0.5 mmol 4-aminoantipyrine per L of 50 mM phosphate buffer (pH 7.5) was added. After 20 min of incubation at 37 °C, the increase in absorbance at 505 nm was measured with a UV-Visible Recording Spectrophotometer (model UV-240, Shimadzu Co., Japan). The amount of maltose was calculated by comparison with the absorbance of a standard solution of maltose (0.8 mmol/L).

Procedure for the determination of maltobionate

	Microcuvette no.		
	I	II	III
1. Pipette into the microcuvettes:			
Sample	0.1 mL	-	-
Standard solution (0.8 mmol/L)	-	0.1 mL	-
Distilled water	-	-	0.1 mL
Reagent A	0.8 mL	0.8 mL	0.8 mL
2. Mix and measure the absorbances at 340 nm against distilled water after incubation at 37 °C for 5 min			
A_{340}	Es0	Est0	Eb0
3. Add into the microcuvettes:			
Reagent B	0.1 mL	0.1 mL	0.1 mL
4. Mix and measure the absorbances at 340 nm against distilled water after reaction at 37 °C for 10 min			
A_{340}	Es1	Est1	Eb1

Scheme 1.

Production of maltobionate by cultivation.—The biochemical production of maltobionate was done by cultivation of a strain of *Pseudomonas taetrolens* IFO 3460 with maltose. This strain was grown and maintained on nutrient agar slants containing 1.0% polypepton, 0.2% yeast extract, and 0.1% $MgSO_4 \cdot 7H_2O$ (pH 7.0). The bacteria were cultured aerobically at 30 °C for about 2 days in a Sakaguchi flask containing 100 mL of the medium (100 g maltose monohydrate, 0.6 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 10 mL corn steep liquor, 2.0 g urea, and 25 g $CaCO_3$ per L, no adjustment of pH). The oscillation rate was 140 opm. Various samples containing maltobionate formed were obtained from the culture broth in the course of cultivation and stored at −20 °C until analysis.

3. Results and discussion

Assay conditions.—Maltobionate was quantitatively determined by the developed novel method; maltobionate α-D-glucohydrolase specifically hydrolyzes maltobionate [12] and gluconate kinase is absolutely specific to D-gluconate [14]. Optimal incubation conditions in the first step using reagent A were arranged according to the principle of D-gluconate measurement [15]. The sufficient amounts of two coupling enzymes, gluconate kinase and 6-phosphogluconic dehydrogenase, were found to be 0.25 and 2.0 U/mL of assay mixture, respectively, and the optimal concentrations of ATP, $MgCl_2$, and NADP were 5.0, 5.0, and 1.5 μmol/mL of assay mixture, respectively. The incubation time in the first step needed only 5 min for the eliminating reaction. The optimal amount of maltobionate α-D-glucohydrolase and the reaction time for the determination of maltobionate were examined using the maltobionate standard solution and varying amounts of maltobionate α-D-glucohydrolase (1.25–5.0 U/mL of assay mixture). In Fig. 1 the reaction curves are shown, which are obtained after the addition of this enzyme in the second step. The sufficient amount of maltobionate α-D-glucohydrolase in reagent B was 4.0 U/mL of assay mixture and the reaction was complete within 10 min. Thus, the final procedure for the determination of maltobionate was established as outlined in the Experimental section.

Reliability of the measurement.—The linearity of a calibration curve constructed from aqueous maltobionate standard solutions was assessed. When net absorbance change was plotted vs. maltobionate concentration, the relation was linear up to 1.3 mmol/L, the linear regression equation being $y = 0.598x + 0.00858$ mmol/L (correlation coefficient, 0.999). The lowest detection for this assay was about 0.02 mmol/L. To determine within-run and between-run precision (CV values), two maltobionate solu-

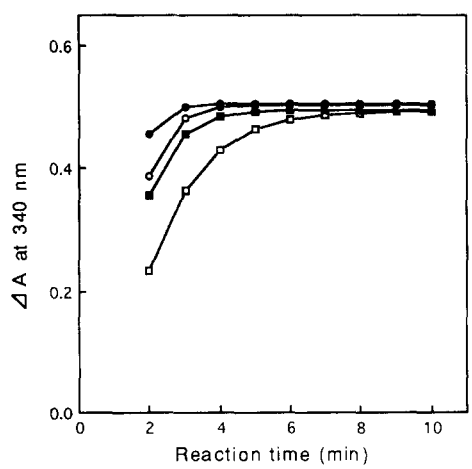

Fig. 1. Reaction curves for determination of maltobionate. The reaction curves of the maltobionate standard solution (0.8 mmol/L) were obtained with various amounts of maltobionate α-D-glucohydrolase (□: 1.25 U; ■: 2.5 U; ○: 3.75 U; and ●: 5.0 U; per mL of assay mixture) in the second step of the procedure as described in the Experimental section.

Table 1
Within-run and between-run precision

	Maltobionate solution (mmol/L)	ΔA at 340 nm		CV (%)
		Mean	SD	
Within-run	0.25	0.156	0.00148	0.95
($n = 5$)	1.0	0.614	0.00212	0.35
Between-run	0.25	0.155	0.00267	1.72
($n = 5$)	1.0	0.615	0.00886	1.44

tions (0.25 and 1.0 mmol/L) were assayed 5 times each. The within-run precision (CV) for maltobionate solutions was less than 1.0% and the between-run precision (CV) for daily maltobionate analyses of aqueous solutions was less than 2.0% (Table 1). The precision of this method was satisfactory, and was similar to those of many enzymic methods applied to food chemistry and clinical chemistry. There was no inhibitory effect of substances such as saccharides (D-glucose, lactose, and sucrose, etc.), organic acids (citrate, succinate, and oxalate, etc.) and metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, and Fe^{2+}, etc.) on the maltobionate α-D-glucohydrolase activity [12]. Therefore, it seemed that the assay system coupled with this enzyme was reliable for the determination of maltobionate without any significant interference.

Comparison of the enzymic method with the HPLC method.—Although several chromatographic methods have been used for the determination of maltobionate, these required an expensive apparatus, and were time-consuming and inappropriate for use in a large number of analyses. On the other hand, a comparison of those data obtained by each method has not been reported yet. The enzymic method was therefore compared

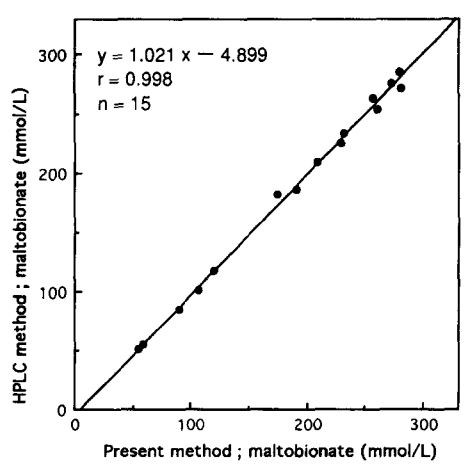

Fig. 2. Correlation between maltobionate concentrations in the culture broth obtained by the present and HPLC methods.

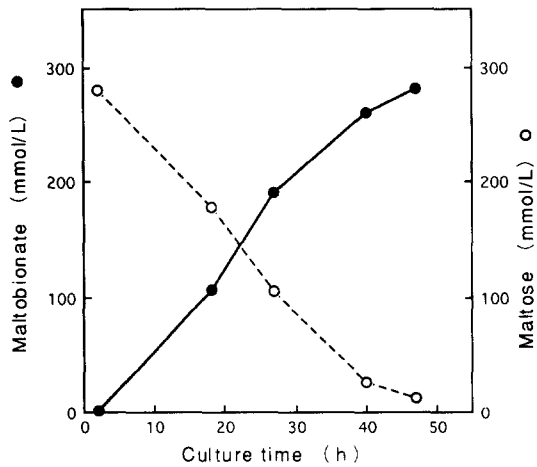

Fig. 3. Production of maltobionate from maltose. The cultivation of *Pseudomonas taetrolens* IFO 3460 was carried out as described in the Experimental section.

with an HPLC method, using 15 samples of culture broth as shown in Fig. 2. A good correlation ($r = 0.998$) was obtained between the two methods and the regression equation for these data was $y = 1.021x - 4.899$ mmol/L, indicating that the proposed method fulfilled the need of an accurate and specific assay of maltobionate, and that it was simple as compared to previous HPLC methods.

Determination of maltobionate in the culture broth.—Although the proper strains of the genus *Pseudomonas* have been well known to oxidize maltose directly to maltobionic acid without cleaving enzymically the glucosidic linkage [3], there have been few reports on the simultaneous determination of both maltobionate formed and residual maltose in the course of cultivation. In order to confirm the relationship between the production of maltobionate and the consumption of maltose, the amounts of the two disaccharides in the culture broth of *P. taetrolens* IFO 3460 were determined by the respective enzymic methods. In Fig. 3 the production of maltobionate from maltose in the course of cultivation is shown. When the formation of maltobionate was almost finished after about 50 h of cultivation, little or no residual maltose was present, and no D-glucose or D-gluconate was detected in the culture broth (data not shown). Thus, it was demonstrated that maltose was directly oxidized in the course of cultivation of *Pseudomonas taetrolens* IFO 3460 to give an equimolar yield of maltobionate.

In conclusion, it is likely that the enzymic method proposed here will be valuable and useful for the determination of maltobionate in foodstuffs or culture broth, etc.

Acknowledgements

We wish to thank Drs N. Saito, E. Nakano, K. Mizusawa, and N. Yamaji for their support and encouragement.

References

[1] J.W.E. Glattfeld and M.T. Hanke, *J. Am. Chem. Soc.*, 40 (1918) 973–992.
[2] H.L. Frush and H.S. Isbell, in R.L. Whistler and M.L. Wolfrom (Eds.), *Methods in Carbohydrate Chemistry*, Vol. 2, Academic Press, New York and London, 1963, pp 14–15.
[3] F.H. Stodola and L.B. Lockwood, *J. Biol. Chem.*, 171 (1947) 213–221.
[4] T. Miyake and Y. Sato, British Patent 1 316 971 (1973) 5 pp; *Chem. Abstr.*, 79: 64567.
[5] J.B.M. Meiberg, P.M. Bruinenberg, and B. Sloots, European Patent 384 534 (1990) 4 pp; *Chem. Abstr.*, 114: 41054.
[6] S. Maruo, Y. Gouren, and H. Yamashita, Japanese Patent (Jpn. Kôkai Tokkyo Kôho) 6-7184 (1994) 5 pp; *Chem. Abstr.*, 120: 321534.
[7] N.M.A. El-Shayeb and H.A. El-Minofi, *Egypt J. Microbiol.*, 29 (1995) 159–164.
[8] T. Miyake and Y. Sato, French Patent 2 072 692 (1971) 9 pp; *Chem. Abstr.*, 77: 47008.
[9] H.R. Knull and J.H. Pazur, *J. Chromatogr.*, 55 (1971) 425–428.
[10] O. Samuelson and L. Thede, *J. Chromatogr.*, 30 (1967) 556–565.
[11] R.G. Jones, A.R. Law, and P.J. Somers, *Carbohydr. Res.*, 42 (1975) 209–216.
[12] Y. Shirokane, A. Arai, and R. Uchida, *Biochim. Biophys. Acta*, 1207 (1994) 143–151.
[13] M. Ameyama, K. Matsushita, E. Shinagawa, and O. Odachi, in J.N. BeMiller, D.J. Manners, and R.J. Sturgeon (Eds.), *Methods in Carbohydrate Chemistry*, Vol. X, John Wiley & Sons, New York, 1994, pp 69–73.
[14] S.S. Cohen, in S.P. Colowick and N.O. Kaplan (Eds.), *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, pp 350–354.
[15] H. Möllering and H.U. Bergmeyer, in H.U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, Vol. 3, 2nd ed., Verlag Chemie, Weinheim/Academic Press, New York and London, 1974, pp 1243–1247.