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Note

Enzymatic Synthesis of Galactosylkojic Acid with Immobilized β-Galactosidase from *Bacillus circulans*

Mohd. Ali HASSAN,* Fadhilah ISMAIL,* Shuichi YAMAMOTO,** Hidenori YAMADA,*** and Kazuhiro NAKANISHI†

Department of Biotechnology, Faculty of Engineering, Okayama University, Tsushima, Okayama 700, Japan
**Faculty of Food Science and Biotechnology, Universiti Pertanian Malaysia, 4340 UPM Serdang, Selangor, Malaysia*
***Department of Chemical Engineering, Faculty of Engineering, Yamaguchi University, Tokiwadai, Ube 755, Japan*
****Department of Bioengineering Science, Faculty of Engineering, Okayama University, Tsushima, Okayama 700, Japan*
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Galactosylkojic acid, in which the hydroxymethyl group of the kojic acid moiety is β-galactosylated, was synthesized by using a transgalactosidation reaction with an immobilized β-galactosidase from *Bacillus circulans*. With 5% kojic acid and 20% lactose as the substrates, the yield with respect to the initial amount of kojic acid was around 24% as a maximum. The inhibitory effect of galactosylkojic acid towards the oxidation reaction of L-β-(3,4-dihydroxyphenyl)alanine (L-DOPA) with tyrosinase was similar to that of kojic acid.

β-Galactosidase (EC 3.2.1.23) catalyzes the hydrolysis as well as transfer reaction; that is, the galactose part forming a glycon part in the substrate molecule can be transferred to water (hydrolysis) or to some hydroxylic acceptor (transfer reaction).[1] We have previously isolated two β-galactosidases from *Bacillus circulans*;[2] one of them, designated as β-galactosidase-2, was quite high in transfer activity, while the other one designated as β-galactosidase-1 was low. We also show that β-galactosidase-1 exhibits a high transfer activity similar to that of β-galactosidase-2 when immobilized on sintered porous silica gel with a glutaraldehyde treatment.[3]

In this study, we synthesized galactosylkojic acid from kojic acid and lactose by using immobilized β-galactosidase-1. Glycosylation has been recognized as a powerful tool to improve such physical properties as solubility[4] and biological activities.[5] We also identified the structure and studied some properties of the compound.

β-Galactosidase-1 from *Bacillus circulans*, which was a major component of the partially purified enzyme preparation obtained from Daiwa Kasei K. K. (Osaka, Japan) was purified to homogeneity by polyacrylamide gel electrophoresis, using the method reported previously.[6] The purified enzyme was immobilized onto a sintered porous ceramic support having an amino group (SM10-C2, NGK Insulators, Ltd., Nagoya, Japan). This support was first treated with 3% glutaraldehyde, and then washed with a 0.1 M sodium phosphate buffer at pH 6.0. The support thus treated was suspended in an enzyme solution and incubated at 4°C for 17 h while gently shaking to bind the enzyme to the support. The enzyme that had covalently bound to the support was again treated with 3% glutaraldehyde at 4°C for 3 h. The immobilized β-galactosidase-1 showed high transfer activity similar to that of β-galactosidase-2 (data not shown). The specific activity of the immobilized enzyme was evaluated to be about 100 units/g (wet) by assying with 4.56% (w/v) lactose as the substrate.[7] Details of the preparation method and characteristics of the immobilized enzyme will be reported elsewhere. We carried out our reaction at 40°C while vigorously shaking, using kojic acid and lactose (monohydrate) with various concentrations as the substrates. Figure 1 showed the time-course for galactosylkojic acid formation, using 5% (w/v) kojic acid and 2.5% (w/v) lactose as the substrates at a 0.1 g (wet)/ml immobilized enzyme concentration. The concentrations of kojic acid and galactosylkojic

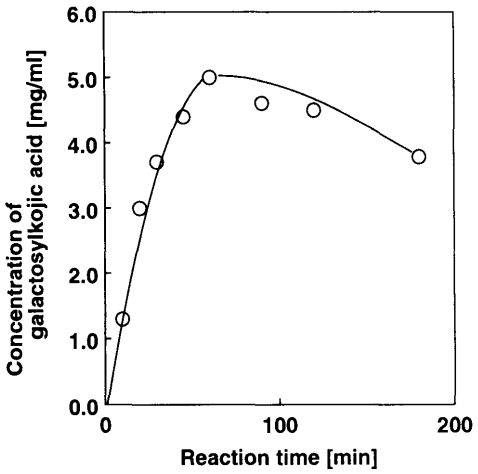

Fig. 1. Time-Course for Galactosylkojic Acid Formation.
The reaction was carried out at 40°C with vigorous shaking, using 5% (w/v) kojic acid and 2.5% (w/v) lactose (monohydrate) as the substrates at 0.1 g (wet)/ml of immobilized enzyme concentration. The concentration of galactosylkojic acid was determined by HPLC as described in the text.

Table Maximum Yield of Galactosylkojic Acid

Substrate		Yield of galactosylkojic acid (%)[b]
Kojic acid (%)	Lactose (%)[a]	
1.0	1.0	4.0
2.5	1.0	5.0
5.0	1.0	5.2
5.0	2.5	10.0
5.0	5.0	12.0
5.0	10.0	18.0
5.0	20.0	24.0

[a] Percentage of lactose monohydrate.
[b] Yield with respect to the initial amount of kojic acid.

† To whom correspondence should be addressed.
Abbreviation: L-DOPA, L-β-(3,4-dihydroxyphenyl)alanine.

acid were determined by HPLC (LC-6A, Shimadzu Corp., Kyoto, Japan) equipped with a YMC-Pack NH_2 column (6 × 150 mm, YMC Co. Ltd., Kyoto, Japan) at 280 nm. The elution buffer was a mixture of acetonitrile and a 0.1 M sodium phosphate buffer, pH 6.0 (30/70 (v/v)), and was fed at a flow rate of 0.8 ml/min. As shown in Fig. 1, the concentration of galactosylkojic acid that was formed increased to around 5 mg/ml after 60 min of incubation and then gradually decreased when the hydrolytic reaction of galactosylkojic acid was predominant, since most of the lactose had been consumed. This is a typical feature of a transfer reaction catalyzed by hydrolyzing enzymes. Besides galactosylkojic acid, oligogalactosylkojic acid-like compounds were also observed, although their amounts were much less than that of galactosylkojic acid. In the Table, the maximum yield of galactosylkojic acid formed with respect to the initial amount of kojic acid is summarized for various combinations of substrate concentration. The maximum yield of galactosylkojic acid was around 25%, with 5% kojic acid and 20% lactose as the substrates.

Galactosylkojic acid was purified in order to identify the structure and to investigate some properties. Purification was done by passing the acid through a preparative HPLC column (YMC-Pack NH_2, 20 × 250 mm), using a mixture of acetonitrile–water (40 : 60, v/v) at a flow rate of 3 ml/min.

We determined the structure of the product by analyzing the FAB-mass spectrum and ^{1}H-NMR spectrum. The negative FAB-mass spectrum of the product revealed a predominant peak at m/z of 303, consistent with an M − 1 molecular ion peak and confirming the product to be a galactoside of kojic acid. The ^{1}H-NMR spectrum of the product [^{1}H-NMR δ (D_2O) ppm: 3.52–3.76 (5H, m), 3.72 (2H, s), 3.91 (1H, t, J = 2.5 Hz), 4.47 (1H, d, J = 7.6 Hz), 6.68 (1H, s), 8.07 (1H, s)] also supported this 1 : 1 stoichiometry. A β-anomer was deduced from the coupling constant of the anomeric proton at 4.47 ppm (J = 7.6 Hz). Furthermore, the UV spectrum of the product was dependent on pH and very similar to that of kojic acid. The maximum wavelengths for both kojic acid and the compound obtained in this study were around 268 nm and 312 nm in the acidic and alkaline pH regions, respectively. This result indicates that the acidic hydroxyl group of the kojic acid moiety was free. From these data, the product was identified as galactopyranosyl-β-*O*-2-methyl-5-hydroxy-γ-pyrone, in which the hydroxymethyl group of the kojic acid moiety is β-galactosylated as shown in Fig. 2.

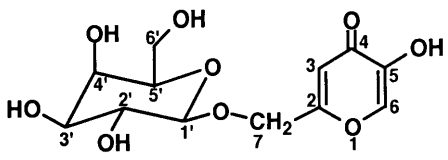

Fig. 2. Structure of Galactosylkojic Acid.

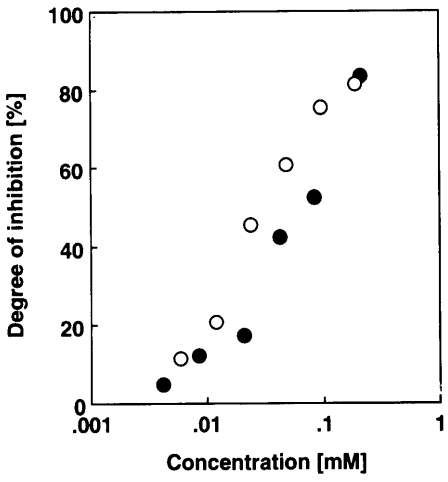

Fig. 3. Relationship between the Inhibitor Concentration and Degree of Inhibition towards Tyrosinase Activity.

To a mixture of 2 ml of 6 mM L-DOPA dissolved in a 50 mM potassium phosphate buffer at pH 6.8 and 0.9 ml of a solution containing kojic acid (○) or galactosylkojic acid (●), 0.1 ml of a tyrosinase solution was added at 30°C. The increase in the optical density at 475 nm was followed with a spectrophotometer, and the initial velocity was evaluated from the linear part of the resulting curve. The degree of inhibition (%) was calculated as 100 × {1 − (initial velocity in the presence of the inhibitor)/(initial rate in the absence of the inhibitor)}.

Kojic acid is known to possess such biological functions as antimicrobial activity, metal-chelating activity and inhibiting activity toward tyrosinase.[4,8-10] Because of its inhibiting activity, kojic acid is used for preventing melanosis in pink shrimp and is sometimes added to cosmetics. We measured the inhibiting activity of kojic acid and galactosylkojic acid towards tyrosinase. Two milliliters of 6 mM L-DOPA (Wako Pure Chemical Industries, Osaka, Japan) dissolved in a 50 mM potassium phosphate buffer at pH 6.8 was mixed with 0.9 ml of a solution containing the inhibitor at an appropriate concentration. To this mixture, 0.1 ml of 10 mg/ml of tyrosinase (Tyrosinase-5000, Wako Pure Chemical Industries) was added, and the reaction was allowed to proceed at 30°C in a glass cell. The increase in optical density at 475 nm was followed with a spectrophotometer (UV-160, Shimadzu Corp., Kyoto, Japan) and the initial velocity was evaluated from the slope of the linear part of the curve. In Fig. 3, the degree of inhibition defined by Eq. (1) is plotted against the concentration of the inhibitor:

$$\text{degree of inhibition (\%)} = 100 \times \left(1 - \frac{\text{initial velocity in the presence of the inhibitor}}{\text{initial velocity in the absence of the inhibitor}}\right) \quad (1)$$

Galactosylkojic acid had similar or slightly less inhibiting activity compared to kojic acid as shown in Fig. 3. Galactosylkojic acid also showed coloration behavior quite similar to that of kojic acid in the presence of ferric chloride ($FeCl_3$).[4] One ml of a solution containing 5 μM galactosylkojic acid or 5 μM kojic acid was mixed with 0.1 ml of 0.5% (w/v) ferric chloride. The resulting absorption spectrum was similar for both the compounds, showing a maximum wavelength of 500 nm. Galactosylkojic acid is a compound which was first synthesized enzymatically. The synthesis of glucosylkojic acid (glucopyranosyl-α-*O*-2-methyl-5-hydroxyl-γ-pyrone), in which the hydroxymethyl group of the kojic acid moiety is α-glucosylated by using the transfer reaction of α-amylase, from *B. subtilis* X-23 has recently been reported.[4] As a donor saccharide, maltotriose, maltopentaose, and soluble starch were each effective. With 2% kojic acid and 10% soluble starch as the substrate, the highest yield of 29.8% was obtained. However, the yield was only several percent when disaccharide (maltose) was used as a donor. Galactosylkojic acid may be similar to glucosylkojic acid in its coloration behavior,[4] solubility and so on, although the inhibiting activity of glucosylkojic acid towards tyrosinase is not known. For a practical application, the synthesis of galactosylkojic acid with the immobilized β-galactosidase-1 from *B. circulans* seems to be more advantageous, since lactose may be more easily available than maltotriose, maltopentaose, or soluble starch. Furthermore, an immobilized enzyme with high activity and stability can be utilized. It will be interesting to discover if there is any difference in the other biological activities between galactosylkojic acid and glucosylkojic acid, which should be further investigated.

References

1) K. Wallenfels and O. P. Malhotra, *Adv. Carbohydrate Chem.*, **16**, 239–298 (1961).
2) Z. Mozaffar, K. Nakanishi, R. Matsuno, and T. Kamikubo, *Agric. Biol. Chem.*, **48**, 3053–3061 (1984).
3) Z. Mozaffar, K. Nakanishi, and R. Matsuno, *Appl. Microbiol. Biotechnol.*, **25**, 426–429 (1987).
4) T. Nishimura, T. Kometani, H. Takii, Y. Terada, and S. Okada, *J. Ferment. Bioeng.*, **78**, 37–41 (1994).
5) I. Yamamoto, N. Muto, E. Nagata, T. Nakamura, Y. Suzuki, *Biochim. Biophys. Acta*, **1035**, 44–50 (1990).
6) Z. Mozaffar, K. Nakanishi, and R. Matsuno, *Appl. Microbiol. Biotechnol.*, **25**, 224–228 (1986).
7) K. Nakanishi, R. Matsuno, R. Torii, K. Yamamoto, and T. Kamikubo, *Enzyme Microb. Technol.*, **5**, 115–120 (1983).
8) A. Beelik, *Adv. Carbohydrate Chem.*, **11**, 145–183 (1956).
9) T. Kotani, I. Ichimoto, C. Tatsumi, and T. Fujita, *Agric. Biol. Chem.*, **40**, 765–770 (1976).
10) R. Saruno, F. Kato, and T. Ikeno, *Agric. Biol. Chem.*, **43**, 1337–1339 (1979).