Production of Calcium Lactobionate by a Lactose-oxidizing Enzyme from Paraconiothyrium sp. KD-3

(Received December 11, 2007; Accepted January 25, 2008)

Hiromi Murakami, 1,* Takaaki Kiryu, 1 Taro Kiso 1 and Hiroyumi Nakano 1

1Osaka Municipal Technical Research Institute (1-6-50, Morinomiya, Joto-ku, Osaka 536-8553, Japan)

Abstract: A lactose-oxidizing enzyme was obtained from culture supernatant of a fungal strain of Paraconiothyrium sp. KD-3. The enzyme was a flavoprotein with a molecular mass of 54 kDa. The purified enzyme oxidized various monosaccharides and oligosaccharides such as D-glucose, D-galactose, D-xylene, cellobioligosaccharides and maltoligosaccharides in addition to lactose, using molecular oxygen as a good electron acceptor, to accumulate the corresponding aldonic acids and hydrogen peroxide. The Paraconiothyrium enzyme was suitable for the production of calcium lactobionate compared with a commercial hexose oxidase owing to the stability during the conversion. The enzyme converted 10–20% (w/v) lactose completely to calcium lactobionate in a 500-mL reactor under aeration, agitation and pH regulation at 5.5. The neutralization was successfully performed by the addition of 10% (w/v) slurry of calcium carbonate, while neutralization with 25% (w/v) sodium hydroxide solution inactivated the enzyme gradually. The supplement of catalase to the mixture promoted the conversion by degrading hydrogen peroxide. The immobilized enzyme, which was prepared by the adsorption to a cation exchange resin followed by the condensation with carbodiimide, oxidized 18.5% (w/v) lactose to produce calcium lactobionate in a batch reaction. Calcium lactobionate and aldonicates derived from D-galactose, D-xylene and L-arabinose showed high aqueous solubility and low calcium binding capability.

Key words: aldonic acid, lactobionic acid, lactose, calcium lactobionate, Paraconiothyrium, enzymatic oxidation

Lactose is a by-product in the dairy industry that is available cheaply, abundantly and reproducibly. Lactose generally undergoes oxidation by dehydrogenases and oxidases to form lactobionic acid (β-O-D-galactosyl-D-gluconic acid, LacA) as shown in Fig. 1. 1 The enzymes are re-oxidized with electron acceptors such as 2,6-dichlorophenolindophenol (DCIP) and molecular oxygen (O2). The primary product, lactobiono-δ-lactone (1,5-lactone), is spontaneously hydrolyzed to LacA in aqueous solutions. LacA is attractive from several practical viewpoints: LacA is used as a moisturizing agent for cosmetics, a component of a soluble antibiotic, erythromycin lactobionate, and a buffer component of preservative solution for transplantation of organs. 2 Furthermore, LacA has refreshing sweetness, and is expected to be a healthy oligosaccharide that promotes the intestinal absorption of minerals 3,4 and stimulates the selective growth of Bifidobacterium. 5

Among these examples of practical and potential usefulness, we are particularly interested in high aqueous solubility of the mineral salts of LacA. Two glycosyl moieties and a carboxyl group enable the high solubility: Calcium lactobionate dissolves at approximately 62 g/100 mL, which is almost 10–60 times higher than calcium gluconate, lactate and citrate, and 28,000 times higher than calcium carbonate, an ingredient commonly used for foods and beverages. Highly soluble calcium salts, e.g. calcium chloride, have bitter taste, whereas calcium lactobionate has no peculiar taste and a relatively high threshold concentration. With respect to safety, calcium lactobionate is registered with the U.S. Food and Drug Administration as a thickening agent for pudding mixes.

At present LacA is produced by a chemical oxidation process. The yield, however, seems to be no more than 30% 6 because of a low selectivity in the oxidation positions and of partial degradation of lactose and LacA. Biological reactions have general advantages such as high selectivity for reactions as well as substrates, high efficiency, simple reaction systems, mild reaction conditions, and avoidance of poisonous chemicals. Therefore we began the investigation on biocatalytic production of lactobionate. Some enzymes have been known to oxidize lactose. Such enzymes, cellobiose dehydrogenases (EC 1.1.99.18, CDH), 7,8 cellobiose: quinone oxidoreductases (EC 1.1.5.1, CBQ), 9 glucooligosaccharides oxidases (GOOX),10,11 and carbohydrate: acceptor oxidoreductase (COX),12 however, have not been studied from a viewpoint of calcium lactobionate production in detail. This may be due to their instability at acidic pH, and/or low specificity on O2 as an electron acceptor. We screened microorganisms that not only possessed lactose-oxidizing activity but also lacked β-galactosidase activity to decompose lactose and LacA; both seemed to be requisite for achieving high yields of LacA. 13–15 A strain of Burkholderia cepacia that exhibited the highest conversion ability among the isolates and had a membrane-bound enzyme had been applied to fermentation 13,14 and microbial conversion processes 16,17 in our earlier studies. Paraconiothyrium sp. KD-3, a fungal strain belonging to Ascomy-
Lactose is oxidized by dehydrogenases and oxidases to produce lactobiono-δ-lactone, which is then hydrolyzed to LacA.

Paraconiothyrium sp. was selected as a potent LacA producer.\textsuperscript{14,18,19} The fungus produced an extracellular oxidoreductase, Paraconiothyrium sp., which was applicable to enzyme reactions. This article reviews the conversion of lactose to calcium lactobionate by the fungal lactose-oxidizing enzyme.

Some properties of the lactose-oxidizing enzyme from Paraconiothyrium sp.

Paraconiothyrium sp. produced the enzyme in cultivation with a liquid medium containing lactose, peptone, and other nutrients.\textsuperscript{14,15} Lactose in the medium promoted the enzyme production. From the culture supernatant, the enzyme was purified by ammonium sulfate precipitation and two steps of anion exchange chromatography. The enzyme was a monomer protein with a molecular mass of 52.4 kDa in SDS-PAGE and 54 kDa in the gel-filtration method. The absorption maxima at 385 and 440 nm indicated the absence of heme. The enzyme was most active at around pH 5.5 and maxima at 420 or 500 to 600 nm indicated the absence of other Fe(III) were ineffective. This suggested the conversion of lactose, while cytochrome C, methylene blue, and DCIP, O\textsubscript{2} acted as a good electron acceptor for the oxidation of cellobiose, maltose, D-glucose, D-galactose, D-xylose and L-arabinose. With respect to the linkages at the C4 position of the reducing-end glycosyl residues was suggested by the oxidation of cellobiose, maltose, D-glucose, D-galactose, D-xylose and L-arabinose. With respect to the linkages at the C4 position of oligosaccharides, both configurations are acceptable, although the β-linkage was rather preferred. On the other hand, a strict recognition at the C2 and C3 positions was suggested by no activities on α,1,2-linked sophorose, α,1,3-linked nigerose, β,1,3-linked laminariobiose, D-mannose (the C2 epimer of D-glucose), D-allose (the C3 epimer of D-glucose), D-glucosamin, or deoxy-derivatives of D-glucose at the C2 and C3 positions. With respect to the C6 position, monosaccharides having small groups at the C6 such as D-xylose, L-arabinose, and 6-deoxy-D-glucose were effective as substrates, whereas bulky groups were unac-
Oxidation of lactose by the Paraconiothyrium enzyme and by a commercial hexose oxidase.

A hexose oxidase (D-hexose: oxygen oxidoreductase, EC 1.1.3.5) having lactose-oxidizing activity is commercially available as "SUREBake 800" (Danisco). The enzyme is originated from a red alga, and is supplied for food processing after genetic engineering. The oxidation efficiency was then compared between our fungal enzyme and the commercial algal one. A partially purified preparation of the Paraconiothyrium enzyme was obtained by the culture supernatant with an ultra-membrane filtration apparatus, and was used for the following experiments. The reactions were carried out in L-shaped tubes with reciprocal shaking. Figure 3 shows courses of the conversion of 10% (w/v) lactose. The reaction pH was controlled by solid calcium carbonate added at the half mole equivalent amount to the substrate. The conversion ratio with the algal enzyme was as high as 25 mole % in the absence of calcium carbonate, where the enzyme was active only at the beginning and inactivated by the decreased pH. In the presence of calcium carbonate, the enzyme was also inactivated immediately due to the unstable pH (pH 7–8). The Paraconiothyrium enzyme was much more stable at a wider pH region. The conversion reached approximately 75 mole % even under the pH-uncontrolled condition, where pH was decreased to 3.0. In the presence of calcium carbonate, the reaction completed within 10 h. A stoichiometric conversion of 20% (w/v) lactose within 24 h was also achieved by the fungal enzyme in the L-shaped tubes under the optimized conditions. These results suggested that the Paraconiothyrium enzyme was suited to the production of calcium lactobionate owing to the stability during the conversion compared with a commercial hexose oxidase.

Conversion of lactose to calcium lactobionate by the Paraconiothyrium enzyme in a reactor system.

The conversion was then performed in a 2-L reactor equipped with a monitoring system of pH and dissolved oxygen (DO), and a control system for aeration, agitation, heating/cooling, and sequential pumping for the addition of alkaline solution responding to pH changes (Fig. 4). The initial reaction mixture (500 mL) contained lactose...

Table 2. Some properties of enzyme from Paraconiothyrium sp. KD-3 and related sugar-oxidizing enzymes.

<table>
<thead>
<tr>
<th>Enzyme group</th>
<th>This enzyme</th>
<th>Microdochium nivale</th>
<th>Sporotrichum pulverulentum</th>
<th>Acremonium strictum</th>
<th>Phanerochaete chrysosporium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum pH</td>
<td>5.5 (O2), 4.5 (DCIP)</td>
<td>5.5 (O2)</td>
<td>6.0 (DCIP)</td>
<td>10.0 (O2)</td>
<td>5.0 (DCIP)</td>
</tr>
<tr>
<td>Anomeric specificity</td>
<td>β-form</td>
<td>ND</td>
<td>ND</td>
<td>β-form</td>
<td>β-form</td>
</tr>
<tr>
<td>Absorption driven from</td>
<td>FAD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Molecular mass (kDa)</td>
<td>54 (SDS-PAGE)</td>
<td>55</td>
<td>60</td>
<td>61</td>
<td>90</td>
</tr>
<tr>
<td>Substrate specificity (kcat/Km)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose (mM-1s-1)</td>
<td>57 (O2), 23 (DCIP)</td>
<td>N.D.</td>
<td>17–15 (DCIP)</td>
<td>210 (O2)</td>
<td>12 (DCIP)</td>
</tr>
<tr>
<td>Cellobiose (mM-1s-1)</td>
<td>69 (O2), 86 (DCIP)</td>
<td>0.21, 0.20* (O2)</td>
<td>350–330 (DCIP)</td>
<td>110 (O2)</td>
<td>140 (DCIP)</td>
</tr>
<tr>
<td>Maltose (mM-1s-1)</td>
<td>0.18 (O2), 0.16 (DCIP)</td>
<td>0.55* (O2)</td>
<td>0.26–0.27 (DCIP)</td>
<td>3.6 (O2)</td>
<td>0.0048 (DCIP)</td>
</tr>
<tr>
<td>D-Glucose (mM-1s-1)</td>
<td>0.084 (O2), 0.0023 (DCIP)</td>
<td>0.095* (O2)</td>
<td>0.0030–0.0032 (DCIP)</td>
<td>1.1 (O2)</td>
<td>0.0016 (DCIP)</td>
</tr>
</tbody>
</table>

ND, not determined; -, not detected; *, the values were obtained with recombinant enzyme.
A reactor system used for the conversion of lactose to calcium lactobionate.

A, regulation and monitoring system; B, pH electrode; C, DO electrode; D, alkaline bottle; E, electric balance; F, peristaltic tubing pump for the addition of alkaline; G, aeration pump; H, air filter, I, agitation and temperature control.

Conversion of lactose to calcium lactobionate by immobilized enzyme.

The reaction mixtures (5.0 mL) were shaken at 30°C in L-tube. Lactose concentrations were 18.5% (A) and 37% (B). Half molecule equivalent of calcium carbonate to lactose was added. The amounts of immobilized enzyme were ▲, 0.1 g (0.47 U); ●, 0.3 g (1.5 U); ■, 1.0 g (4.7 U).

Enzymatic production of other aldonic acids.

In our previous study, several aldonic acids were produced by the washed cells of B. cepacia and their calcium binding properties were measured. Similarly, effective conversions of D-galactose, D-xylose and L-arabinose to corresponding calcium aldonates were also attained by the fungal enzyme. These calcium aldonates including lactobionate were highly soluble in water. Free aldonic acids derived by the treatment with an ion exchange resin showed lower sequestering capacities compared with EDTA and citrate, and thus weak binding properties with calcium ions. Such weak calcium binding in addition to high aqueous solubility suggested the possible application of calcium aldonate to functional saccharides that promote intestinal absorption of calcium ions.
Conclusions.

Calcium lactobionate is a simple carbohydrate derived by the oxidation of the aldehyde group of lactose followed by neutralization with calcium carbonate. LacA and its mineral salts have attractive physiological functions as well as physical properties. In Japan, however, they have not been practically used as ingredients for foods, feeds or beverages so far. It is considered sugar oxidizing enzymes may be one important and possible production means for various aldolates. As demonstrated in this article, the *Paraconiothyrium* enzyme showed sufficient yields in the conversion of 10–20% lactose to calcium lactobionate in a reactor scale. However, further studies are still necessary to establish more effective and biologically safer processes before providing lactobionate to various industries.

This study was supported in part by Grants-in-Aid for Regional New Consortium Projects, Ministry of Economy, Trade and Industry of Japan. The authors wish to express their sincere thanks to Daiwa Kasei K. K. for the cooperative work and valuable advice in the production of the fungal enzyme. We also thank Takehara Industrial Chemical Cooperation for donating the stable slurry of calcium carbonate.

REFERENCES


ルカルボジイミド処理した固定化酵素を調製した。この固定化酵素はバッチ反応において18.5% (w/v) の乳糖を完全に変換することができた。D-ガラクトース、D-キシロース、L-アラビノースから、本酵素により対応するアルドノ酸を得た。これらは、いずれも高いカルシウム安定性と、カルシウムイオンに対して比較的低い結合力を示した。

＊＊＊＊＊
【質問】信州大学 工 天野
1）Heme のドメインがないが、もともと持っていないのか、またはプロセッシングされてなくなるのか？
2）電子受容体としては、O₂以外のものの比較はどうか。
【回答】
1）現在のところ不明です。なお CBQ (セロピオースキノオキシドレタクターゼ) では、プロセッシングを受けて Heme ドメインを失うものと、元来このドメインを持たないものの両方が報告されています。
2）本酵素は DCIP も電子受容体として有効ですが、O₂と DCIP では反応効率に大きな違いはありません。既報の CBH（セロピオースデヒドログナーゼ）や CBQ の場合、O₂では反応効率が大きく低下するという報告があり、その点では本酵素と相違します。
【質問】株林原生物化学研究所 西本
KD-3 株を用いた発酵法でのラクトビオン酸の製造は可能でしょうか。
【回答】乳糖を添加した培地で KD-3 株を培養しますと、培養上清にラクトビオン酸が蓄積します。しかし培養に7〜10日を要するなど効率的と考えられませんでした。
【質問】王子製紙㈱ 木村
ラクトビオン酸カルシウムのラクトビオン酸とカルシウムのモル比はどのようなになっているのでしょうか？
【回答】ラクトビオン酸は一価の塩イオン、カルシウムは二価の陽イオンですので、ラクトビオン酸：カルシウムのモル比は2:1となります。
【質問】大阪市大院・理 伊藤
1）FAD の結合数はいくつか、また反応過程での活性低下の原因が FAD の遊離ではありませんか。
2）アルドノ酸 Ca の味覚と消化管での吸収効率はいかがでしょうか。
3）反応後の生成物と未反応基質の分離は、どのように考えていますか。
【回答】
1）モル吸光係数から、酵素あたり FADI 分子と推定しています。透析や未反応の酸を長時間行っても酵素活性は顕著に低下しないため、FAD は酵素に強固に結合していると考えています。
2）遊離のラクトビオン酸は酸度と甘味を併せ持っていますが、ラクトビオン酸カルシウムは酸味も甘味もほとんどなく、無味に近い味質です。生体での消化吸収性は確認していません。従来のカルシウム剤では、シュウ酸などキレート形成能が高いものは吸収効率が悪く、逆に水溶性の高いクエン酸・リンゴ酸カルシウムなどは腸管腔濃度の上昇から、受動輸送の速度が上がり、吸収効率が良くなるといわれています。この点から、ラクトビオン酸カルシウムは有望な素材といえます。
3）反応終了後、ラクトビオン酸カルシウムと、基質成分、酵素剤由来の成分が反応液中に存在していますが、未反応の乳糖やその分解物、副生成物は存在しません。従って、脱色・脱臭のため活性炭処理を行うだけで高純度のラクトビオン酸カルシウムを得ることができます。