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Note

Pentose Oxidation by Acetic Acid Bacteria Led to a Finding of Membrane-Bound Purine Nucleosidase

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D-Ribose and 2-deoxy-D-ribose were oxidized to 4-keto-D-ribonate and 2-deoxy-4-keto-D-ribonate respectively by oxidative fermentation, and the chemical structures of the oxidation products were confirmed to be as expected. Both pentoses are important sugar components of nucleic acids. When examined, purine nucleosidase activity predominated in the membrane fraction of acetic acid bacteria. This is perhaps the first finding of membrane-bound purine nucleosidase.

Key words: nucleosidase; purine-ribonucleosidase; purine-2′-deoxyribonucleosidease; D-ribose oxidation; 2-deoxy-D-ribose oxidation

In our study of enzymatic oxidation of D-aldopentoses,[1)] a novel membrane-bound enzyme, D-aldopentose 4-dehydrogenase, which oxidizes D-aldopentoses specifically at the C4-position, yielding 4-keto-aldopentoses, was found. D-Ribose was oxidized to 4-keto-D-ribonate *via* 4-keto-D-ribose.[1,2)] D-Ribose is an important sugar component of nucleic acids. It is interesting whether 2-deoxy-D-ribose is also oxidized with the membrane fraction of acetic acid bacteria. In this study, we conducted enzymatic oxidation of 2-deoxy-D-ribose by membrane enzyme of acetic acid bacteria, and two putative oxidation products, 2-deoxy-4-keto-D-ribose and 2-deoxy-4-keto-D-ribonate, were observed on a TLC chromatogram (Fig. 1). We detected the reaction products by spraying an alkaline-ethanol solution of 2,3,5-triphenyltetrazolium chloride (TTC) over a TLC plate, as described previously.[1–3)] The R*f* value of putative 2-deoxy-4-keto-D-ribonate showed a spot having higher R*f* than that of 4-keto-D-ribonate. The formation of putative 2-deoxy-4-keto-D-ribose as well as 4-keto-D-ribose was detected after shorter incubation periods. These two compounds were further oxidized to 2-deoxy-4-keto-D-ribonate and 4-keto-D-ribonate. In the case of growing cells cultured in Na-gluconate-glucose medium,[3)] to which 2-deoxy-D-ribose was added, only one TTC positive spot, corresponding to 2-deoxy-4-keto-D-ribonate, predominated on a TLC plate. Thus, with both substrates, oxidation at the C4 position took place first by D-aldopentose 4-dehydrogenase, and the aldehyde group at the C1 position was oxidized with 4-keto-D-aldopentose 1-dehydrogenase, as discussed in a previous paper,[2)] but 2-deoxy-L-ribose was not oxidized by the enzyme. Since putative 2-deoxy-4-keto-D-ribonate appeared easy to isolate from the reaction mixture, preparation, isolation, and characterization of the compound were done.

A membrane fraction was prepared from *Gluconobacter oxydans* NBRC 12528, as described previously.[2)] The reaction mixture contained 1 g of dried membrane fraction and 300 mg of 2-deoxy-D-ribose (Wako, Osaka, Japan) in 100 mL of 10 mM acetate buffer, pH 5.5. The reaction was carried out at 30 °C with gentle stirring. The reaction mixture was centrifuged at $10^5 \times g$ for 60 min to remove the membrane fraction. A clear supernatant of the reaction mixture containing putative 2-deoxy-4-keto-D-ribonate was applied to a column of Dowex 1 × 4 (1 × 10 cm. acetate form). Elution of the column was done by a linear gradient concentration of acetic acid formed of 300 mL of water and 300 mL of 0.2 M acetic acid, and 10-mL fractions were collected. Elution was checked by TLC chromatography under conditions as reported previously.[3)] One TTC positive spot, corresponding to putative 2-deoxy-4-keto-D-ribonate, appeared in the eluted chromatogram, but putative 2-deoxy-4-keto-D-ribose was not adsorbed, and passed the column. The acetic acid in the pooled fractions was evaporated under reduced pressure at 45 °C. The resulting viscous liquid was neutralized with finely powdered $CaCO_3$. After the brown color was removed by the addition of activated charcoal, the solution was evaporated until crystals appeared.

The analytical data for the oxidation products corresponding to 2-deoxy-4-keto-D-ribonate were as follows: IR ν_{max} (KBr) cm^{-1}: 3,383 (s), 2,931 (s), 2,882 (s), 1,777 (m), 1,719 (m), 1,586 (m), 1,422 (m), 1,316 (m), 1,209 (w). ^1H NMR δ_H (400 MHz, D_2O): 2.64 (d, 1H, $J = 6.6$ Hz), 2.66 (s, 1H, $J = 4.8$ Hz), 4.54 (dd, 1H, $J = 4.8$, 6.6 Hz), 4.59 (d, 2H, $J = 1.6$ Hz) ^{13}C NMR δ_C (100 MHz, D_2O): 41.13 (CH_2), 65.22 (CH_2OH), 72.63 (CHOH), 178.39 (COOH), 213.71 (C=O). HRMS: calcd. $C_5H_7O_6$ for $(M-H)^-$, 147.0293; found, 147.0269. In addition to the presence of a hydroxy

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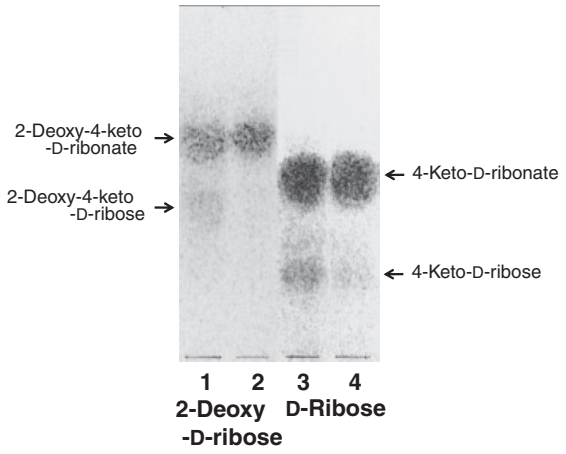

Fig. 1. TLC of Oxidation Products of D-Ribose and 2-Deoxy-D-ribose after Incubation with the Membrane Fraction of Acetic Acid Bacteria.

Incubation was carried out over 16 h at 30 °C with gentle stirring. Lanes 1 and 3, reaction mixtures after 8 h of incubation; lanes 2 and 4, 16 h of incubation.

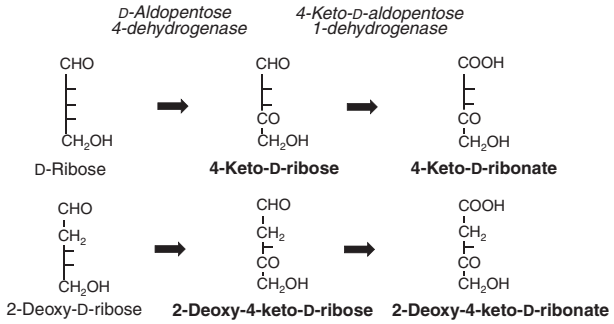

Fig. 2. Oxidation Pathways of D-Ribose and 2-Deoxy-D-ribose with the Membrane Fraction of Acetic Acid Bacteria.

group (3,383 cm^{-1}), the IR spectrum revealed the absorption of carbonyl groups (1,719 cm^{-1} for keto and 1,586 cm^{-1} for carboxy), and this was confirmed by a 13C signal at 213.7 and 178.4 ppm. The H-H COSY spectrum revealed correlations between H-2 and H-3 and the presence of an isolated methylene group at C-5. The HMBC spectrum indicated correlations of H-2 to C-1 and C-3 and of H-3 to C-1 and C-2. Based on these data, the oxidation product was identified as 2-deoxy-D-pent-4-ulosonate (2-deoxy-4-keto-D-ribonate). Thus the enzymatic preparation of 2-deoxy-4-keto-D-ribonate was conducted successfully. Combining present with a previous study,[3] the oxidation pathways of D-ribose and 2-keto-D-ribose by D-aldopentose 4-dehydrogenase and 4-keto-D-aldopentose 1-dehydrogenase is summarized in Fig. 2. Complete genome sequence of *G. oxydans* 621H is available.[4] Many membrane-bound dehydrogenases have not been assigned to genome or enzymes and to our best knowledge, the enzyme catalyzing pentose oxidation, yielding a ketone at position C4, remains to be identified.

Encouraged by these observations, our experiment was extended to determine whether the membrane enzyme is able to oxidize the pentose moiety of nucleic compounds. It is useful to check membrane-bound nucleosidase activity with the membrane fraction of acetic acid bacteria. There is no report of a nucleosidase

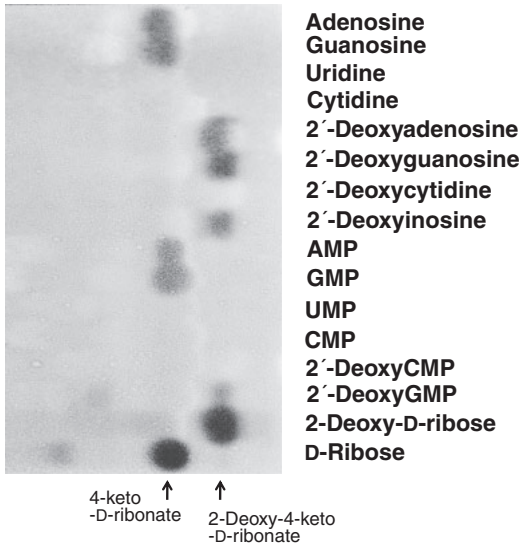

Fig. 3. TLC of Reaction Mixtures of the Membrane Fraction after Incubation with Various Nucleic Compounds.

The substrate concentrations of 2-deoxy-D-ribose and D-ribose were 5 times higher than those of the nucleic acid substrates examined.

occurring in membrane-bound form, and most studies of nucleosidases from eukaryotes and prokaryotes have dealt with cytoplasmic soluble enzymes. If membrane-bound nucleosidases exist, various biotechnological and pharmaceutical applications of them can be expected. Most membrane-bound enzymes can be regarded as an immobilized catalyst. The enzyme can be used repetitively accompanying saving cost, enhancing the concentration at a proper location, and protecting the enzyme from being destroyed. The reactivity of the membrane enzyme of acetic acid bacteria involving D-aldopentose 4-dehydrogenase and 4-keto-D-aldopentose 1-dehydrogenase was examined using different nucleic compounds. D-Aldopentose 4-dehydrogenase is a pentose oxidase, and D-ribose as well as 2-deoxy-D-ribose split from nucleosides can be measured with ease.

The membrane fraction mentioned above was used after repetition of washing and spinning-down to remove contamination of the cytoplasmic fraction. The reaction mixture contained 30 µL of membrane fraction (30 mg of protein/mL in 10 mM acetate buffer, pH 6.0), and 30 µL of 0.05 to 0.1 M of substrate. The enzyme reaction was carried out over 16 h at 30 °C with gentle stirring. The addition of TCA at 10% (v/v) to the reaction mixtures, and centrifugation gave clear supernatants. An aliquot of the supernatant was analyzed by TLC by the method described previously.[1,2] AMP was a gift from Oriental Yeast (Tokyo). 2′-Deoxy-CMP and 2′-deoxy-GMP were purchased from MP Biochemicals (Illkirch, France). The other substrates used were products from Wako (Osaka, Japan). As shown in Fig. 3, 4-keto-D-ribonate and 2-deoxy-4-keto-D-ribonate reacted positively to spraying of TTC-reagent, while no TTC-positive spots were observed for a reaction mixture containing pyrimidine nucleosides. The R*f* values of all spots from the purine 2′-deoxyribonucleic compounds were the same as that of 2-deoxy-4-keto-D-ribonate. On the other hand, the R*f* values for those from purine D-ribonucleic compounds were the same as that of 4-keto-D-ribonate. These results

indicate that the sugar moieties in all purine nucleosides examined were split first at the glycosidic bond by membrane-bound nucleosidase before pentose oxidation by membrane-bound D-aldopentose 4-dehydrogenase and 4-keto-D-aldopentose 1-dehydrogenase. When AMP and GMP were incubated with the membrane fraction, TTC positive spot corresponding to 4-keto-D-ribonate appeared on the TLC plate. Although the necessary data have not been accumulated yet, D-ribose-5-phosphate was oxidized, presumably at the C4 position, yielding a compound showing a strong reaction to TTC. It remains to be solved whether phosphoric acid moiety still exists as ester form with D-ribose at the C5 position. Membrane-bound purine nucleosidase, exemplified by *G. oxydans* NBRC 12528 in this study, looks ubiquitous in other strains of *Gluconobacter* and *Gluconacetobacter*. Two genes encoding nucleosidase, *GOX 0947* and *GOX 1593*, were found in the genome of *G. oxydans* 621H.[4] Intracellular localizations of these gene products were analyzed with the PSORTb ver 3.0.2 Program (http://www.psort.org/psortb/index.html)[5] and the TMpred Program (http://www.ch.embnet.org/software/TMPRED_form.html).[6] *GOX 0947*, annotated as inosine-uridine preferring nucleoside hydrolase, showed cytoplasmic localization, whereas *GOX 1593*, annotated as a nucleoside hydrolase, had a signal peptide, and non-cytoplasmic localization. In addition, *GOX 1593* exhibited three strongly preferred transmembrane helices in the molecule, indicating that it is perhaps a structural gene encoding membrane-bound purine nucleosidase.

The finding of a membrane-bound purine nucleosidase may be useful in determining the functions and structures of bacterial membranes. Membrane-bound D-aldopentose 4-dehydrogenase can be assayed with potassium ferricyanide or phenazine methosulfate with 2,4-dichlorophenol indophenol (PMS-DCIP) as electron acceptor. Measurement of purine nucleosidase activity was also possible by coupling with membrane-bound D-aldopentose 4-dehydrogenase of acetic acid bacteria, as will be reported elsewhere.

Acknowledgments

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