Although α- and β-linked 3-deoxy-D-manno-octulosonic acid (KDO) is found in lipopolysaccharides (LPSs) of Gram-negative bacteria (1–3), it is found in lipopolysaccharides of Gram-negative bacteria (1–3). In addition, α-galactopyranosyl chloride of the per-O-acetylated methylester of KDO with dimethylformamide; TLC, thin-layer chromatography; DMF, N,N-dimethylformamide; μ, μ-ketoside of KDO; β-μ-ketoside of KDO; MS, mass spectrometry.

Materials

KDO (ammonium salt) was prepared by alkaline hydrolysis of oxalacetic acid and n-arabinose (7). The following were purchased from commercial sources: oysters (C. virginica) were from P&J Oyster Company (New Orleans, LA); KDO, octyl-Sepharose, and phenylmethylsulfonyl fluoride were from Sigma; precocital silica gel-60 and 60 F254 TLC plates, silica gel, and Fractogel EMD SP-650(S) were from E. Merck (Darmstadt, Germany); and Sepheryl S 200-SF and Con A-Sepharose were from Pharmacia LKB Biotechnology. Re-LPS from Escherichia coli K12, D31 m4, lot 5 (KDO content 11.7%); Re-LPS from Salmonella minnesota R595, lot 30457B (KDO content 11.3%) and Rd-LPS from S. minnesota R7, lot 3 (KDO content 16.5%) were from List Biological Laboratories (Campbell, CA). DeOa-LPS prepared from Re-LPS R595 by mild hydrazinolysis as described previously (8) was a gift from Dr. S. C. Szu (National Institutes of Health, Bethesda, MD).

General Methods

Melting points were determined with a Fisher-Johns apparatus and were not corrected. NMR spectra were recorded at 25 °C with a Bruker AMX-300 spectrometer at 300 and 100 MHz for 1H NMR and 13C NMR, respectively. Mass spectra were recorded with a VG 70-S mass spectrometer in a chemical ionization mode (reagent gas, NH3) or a fast atom bombardment-positive mode (matrix: 3-nitrobenzyl alcohol). TLC was carried out on precoated silica gel 60 F254 plates, silica gel, and Fractogel EMD SP-650(S) were from E. Merck (Darmstadt, Germany); and Sepheryl S 200-SF and Con A-Sepharose were from Pharmacia LKB Biotechnology. Re-LPS from Escherichia coli K12, D31 m4, lot 5 (KDO content 11.7%); Re-LPS from Salmonella minnesota R595, lot 30457B (KDO content 11.3%) and Rd-LPS from S. minnesota R7, lot 3 (KDO content 16.5%) were from List Biological Laboratories (Campbell, CA). DeOa-LPS prepared from Re-LPS R595 by mild hydrazinolysis as described previously (8) was a gift from Dr. S. C. Szu (National Institutes of Health, Bethesda, MD).

Preparation of the Glycosyl Donor (2) from the Per-O-acetylated Methylester of KDO (1)

Compound 1, methyl 2,4,5,7,8-penta-O-acetyl-3-deoxy-D-manno-2-octulosopyranosonate, was prepared by the acetylation of 20 g of KDO (ammonium salt) with acetic anhydride-pyridine (1:1; 160 ml). After 20 h at 25 °C, the reaction mixture was evaporated and co-evaporated with toluene, and the residue was partitioned between water (50 ml) and CHCl3 (150 ml). The organic layer was separated and washed with 5% HCl and water, dried (Na2SO4), and filtered. The filtrate was evaporated to give a pale yellow foam. TLC (ethyl acetate:AcOH:H2O, 3:2:1) revealed that it contained the 2,4,5,7,8-penta-O-acetyl-3-deoxy-α-D-manno-2-octulosopyranosonic acid (Rf 0.5) as the major component, together with several unknown, fast-moving components. Treatment of the mixture with excess diazomethane in CH2Cl2 (200 ml), followed by silica gel column chromatography using toluene:ethyl acetate (2:1) as
the eluent, gave compound 1 (18.8 g, 52% from KDO) as a white solid: 

\[
R_p 0.30 \text{ (toluene:ether, 1:1); m.p. 154–156 °C; m.p. (literature) 155–158 °C (9); MS (m/z, chemical ionization-positive mode) 480 (M + NH_4^+, 100%), 420 (M + AcOH + NH_4^+, 8%), and 403 (M + AcOH + H^+, 22%; 1)} \]

\[
^1H \text{ NMR (CDCl}_3) \gamma 3.818 \text{ (OCH}_3\text{), 5.151, 2.120, 2.057, 2.010, and 2.006 (each s, 3 H, CH}_3\text{). The data for sugar protons are listed in Tables I and II. To prepare compound 2, dry hydrogen chloride gas was bubbled into a solution of 1 (3.85 g, 7.96 mmol) in acetyl chloride (40 ml) in a 100-ml flask at 0 °C for 30 min, then the flask was sealed and kept at 4 °C. After 24 h the solution was evaporated and the residual solvent was co-evaporated with toluene to give compound 2 (3.6 g, quantitative) as a colorless oil.} \]

**Preparation of Methyl (4-Methylumbelliferyl) 4,5,7,8-Tetra-O-acetyl-3-deoxy-β-d-mannopyranosyl(1-3)glucose (3) and Its α-Anomer (4)**

The conjugation of 2 with 4-methyl-umbelliferyl was accomplished by S_2,2 coupling in DMF and catalyzed phase-transfer reaction as described below.

\[
S_2,2 \text{ coupling in DMF—To a solution of 2 (2.5 g, 5.7 mmol) in DMF (20 ml), 4-methylumbelliferyl (sodium salt) (1.70 g, 8.5 mmol) was added. The mixture was stirred at 25 °C for 4 h, and TLC (toluene-ethyl acetate, 1:1) indicated the disappearance of 2. DMF was evaporated in vacuo, and the residue was partitioned between water (20 ml) and CHCl_3 (50 ml). The organic layer was separated and washed with aqueous NaHCO_3 and water, dried (Na_2SO_4), and filtered. The filtrate was evaporated, and the residue was subject to silica gel column chromatography using toluene-ethyl acetate (3:1) as the eluent to give a mixture of two UV-absorbing and carbohydrate-containing compounds that migrated closely on TLC (toluene-ethyl acetate, 2:1 or toluene:ether, 1:1). The mixture was further fractionated by silica gel column chromatography using toluene-ether (1:1) as the eluent to obtain the β-anomer 3 (2.5 g, 76%) and the α-anomer 4 (224 mg, 6.8%). Alternatively, the two anomers could be simply separated by agitation of the mixture in toluene. The β-anomer 3 is quite soluble in toluene but the α-anomer 4 is not. Filtration of the suspension gave pure 3 in toluene and pure 4 as a solid.**

**Catalyzed Phase-Transfer Reaction—**

A mixture of 2 (440 mg, 1.0 mmol), 4-methylumbelliferyl (sodium salt) (324 mg, 1.5 mmol), and tetrabutylammonium bisulfate (399 mg, 1.0 mmol) in CH_2Cl_2 (10 ml) and aqueous NaHCO_3 (0.5 M, 10 ml) was shaken vigorously at 25 °C for 12 h. Then, the organic layer was separated and washed with brine and water, dried (Na_2SO_4), and filtered. The filtrate was evaporated and the products were purified by the procedure described above to give the β-anomer 3 (463 mg, 80%) and α-anomer 4 (44 mg, 7.6%).

**Preparation of 4-Methylumbelliferyl 3-Deoxy-β-d-mannopyranoside Acid (5, β-KDO-MU)**

MeONa/MeOH (0.5 M, 2 ml) was added to a solution of 3 (1.0 g, 1.73 mmol) in MeOH (40 ml), and the mixture was stirred at 25 °C. After 1.5 h, the reaction mixture was concentrated to 10 ml and diluted with water (50 ml). The solution was adjusted to pH 11 and maintained at this pH by adding 2 M NaOH. TLC (CHCl_3/MeOH:H_2O:AcOH, 65:25:4:1) indicated the complete saponification of the methyl ester after 2 h. The solution was neutralized by Dowex 50W-X8 (H+ form) to pH 4 and filtered. The filtrate was then adjusted to pH 8 with 0.1 M NH_4OH and evaporated. The residue was applied onto a Sephadex G-10 column (2.5 × 95 cm), which was equilibrated and eluted with 50 mM NH_4OH, to obtain β-KDO-MU (5) as its ammonium salt (601 mg, 84%; R_p 0.26 (CHCl_3/MeOH:H_2O:AcOH, 65:25:4:1); m.p. 112–116 °C; MS (m/z, chemical ionization mode) 596 (M + NH_4^+, 100%) and 579 (M + H^+, 11%); 13C NMR (CDCl_3, set CDCl_3 at 77.16 ppm) 170.22, 169.92, 169.37, 160.74, 156.90, 154.81, and 152.02 (C-2, C-3, C-4), 77.23): 1H NMR (CDCl_3) δ 7.494 (d, 1 H, J = 8.7 Hz, H-5), 6.994 (d, 1 H, J = 2.4 Hz, H-4), 6.956 (dd, 1 H, J = 2.4 and 8.7 Hz, H-6), 6.210 (q, 1 H, J = 1.2 Hz, H-3), 3.788 (s, 3 H, OCH_3), 2.407 (d, 3 H, CH-α at C-3), 2.143, 2.035, 1.973, and 1.545 (each s, each 3 H, COCH_3). The data for sugar protons are listed in Tables I and II.

**Preparation of 4-Methylumbelliferyl 3-Deoxy-β-d-mannopyranoside Acid (5, β-KDO-MU)**

\[
^1H \text{ NMR data for the sugar protons of KDO derivatives} \]

The NMR spectra were measured at 300 MHz in CDCl_3 (for 1, 3, and 4) or D_2O (for 5 and 6).

| Hydrogen | Chemical shift (δ) | Multiplicity \( ^a \) |
|----------|-------------------|---------------------|
| H-3a     | 2.209 t           | s                   |
| H-3e     | 2.261 dd          | s                   |
| H-4      | 5.335 ddd         | s                   |
| H-5      | 5.407 m           | d                   |
| H-6      | 4.184 ddd         | t                   |
| H-7      | 5.232 ddd         | t                   |
| H-8a     | 4.488 ddd         | m                   |
| H-8b     | 4.125 ddd         | m                   |

\( ^a \text{Chemical shift (δ) was expressed in ppm.}

\( ^b \text{Multiplicity: s, singlet; d, doublet; t, triplet; m, multiplet.} \)
Preparation of 4-Methylumbelliferyl-3-Deoxy-α-D-manno-2-octulopyranosidonic Acid (6, α-KDO-MU)

The de-O-acetylation and alkaline saponification of 4 (200 mg, 0.346 mmol) followed by Sephadex G-10 chromatography were performed according to the procedures described for the preparation of β-KDO-MU to give α-KDO-MU (6) as its ammonium salt (130 mg, 91%). Rf 0.21 (CHCl₃:MeOH:H₂O:AcOH, 65:25:4:1); m.p. 129–131 °C; MS (m/z, fast atom bombardment-positive mode) 419 (M + Na⁺), 2%), 397 (M + H⁺, 4%), 154 (100%); ¹³C NMR (D₂O, set external dioxane at 66.67): δ 174.18 (C-1), 164.63, 157.73, 156.37, and 153.73 (C-2’, 4’, 7’, 9’), 126.28, 114.57, 114.51, 110.88, 104.39, and 101.18 (C-2, 3, 5, 6, 8, 10), 72.86, 69.10, 66.23, 65.76, and 63.34 (C-4, 5, 6, 7, 8), 34.83 (C-3), 17.94 (CH₃). ¹H NMR (D₂O, set the internal HDO at 4.778): δ 7.640 (d, 1 H, J = 8.8 Hz, H-5’), 7.001 (dd, 1 H, J = 2.2 and 8.8 Hz, H-6’), 6.958 (d, 1 H, J = 8.8 Hz, H-8’), 6.147 (s, 1 H, H-3) 2.356 (s, 3 H, CH₃). The data for sugar protons are listed in Tables I and II.

Enzyme Assays

When the KDO-cleaving activity of α-KDOase was assayed using Re-LPS or DeOA-LPS as substrate, 40 µg of LPS was incubated with an appropriate amount of enzyme in 70 µl of 50 mM sodium formate buffer pH 4.5 or 3.0 and an appropriate amount of the enzyme. After incubation at 37 °C for a preset time, the reaction was stopped by adding 40 µl of ethanol followed by brief centrifugation. The supernatant was evaporated to dryness using a SpeedVac, dissolved in 12 µl of methanol:water (1:2) and analyzed by TLC using chloroform:methanol:12 mM MgCl₂ (45/40/12) as the developing solvent. The plate was sprayed with diphenylamine reagent (10) and heated at 120 °C for 20 min to reveal glycoconjugates. The fluorometric assay of α-KDOase activity using α-KDO-MU as substrate was carried out according to the procedure described by Potier et al. (11). The enzyme was incubated with 0.5 mM α-KDO-MU in 50 mM sodium formate buffer, pH 3.0, in a total volume of 100 µl at 37 °C. After a preset time, 1.5 ml of 0.2 M sodium borate buffer, pH 9.8, was added to the reaction mixture to stop the reaction. The released MU was determined using a Sequoia-Turner Model 450 fluorometer. One unit of α-KDOase is defined as the amount of the enzyme that liberates 1 nmol of KDO per min at 37 °C.

Purification of α-KDOase

All operations were carried out at a temperature between 0 and 5 °C. Centrifugations were routinely carried out at 20,000 × g for 30–40 min using a Sorvall RC5C refrigerated centrifuge. Ultrafiltration was carried out with an Amicon stirred cell using a PM10 membrane.

The hepatopancreas (450 g) dissected from 1 gallon of fresh oysters was homogenized with 3.2 liters of cold acetone using a Polytron homogenizer, quickly filtered through a Buchner funnel, and immediately dried under vacuum to obtain 90 g of the acetone powder of oyster hepatopancreas. The acetone powder was extracted with 2.25 liters of cold acetone using a Polytron homogenizer, followed by centrifugation. The pH of the water extract was adjusted to 4.3 with a saturated solution of citric acid, and the precipitate was removed by centrifugation. The supernatant was brought to 45% saturation with solid ammonium sulfate (277 g/liter). After standing for 2 h, the precipitate was removed by centrifugation, and the supernatant was quickly filtered through a Buchner funnel, and immediately homogenized with 3.2 liters of cold acetone using a Polytron homogenizer, followed by centrifugation. The supernatant was brought to 45% saturation with solid ammonium sulfate (277 g/liter). After standing for 2 h, the precipitate was removed by centrifugation, and the supernatant was evaporated to dryness using a SpeedVac, dissolved in 12 ml of methanol:water (1:2) and analyzed by TLC using chloroform:methanol:12 mM MgCl₂ (45/40/12) as the developing solvent. The plate was sprayed with diphenylamine reagent (10) and heated at 120 °C for 20 min to reveal glycoconjugates. The fluorometric assay of α-KDOase activity using α-KDO-MU as substrate was carried out according to the procedure described by Potier et al. (11). The enzyme was incubated with 0.5 mM α-KDO-MU in 50 mM sodium formate buffer, pH 3.0, in a total volume of 100 µl at 37 °C. After a preset time, 1.5 ml of 0.2 M sodium borate buffer, pH 9.8, was added to the reaction mixture to stop the reaction. The released MU was determined using a Sequoia-Turner Model 450 fluorometer. One unit of α-KDOase is defined as the amount of the enzyme that liberates 1 nmol of KDO per min at 37 °C.

**TABLE II**

| Coupled H | J value | 1 | 2 | 3 | 4 | 5 | 6 |
|-----------|---------|---|---|---|---|---|---|
| 2a, 3b    | 12.0    | 12.0 | 12.5 | 12.5 | 12.8 |
| 3a, 4     | 11.2    | 11.5 | 12.2 | 12.5 | 12.0 |
| 3e, 4     | 5.7     | 5.8  | 5.6  | 4.6  | 5.4  |
| 4, 5, 6   | 3.0     | 2.8  | 3.0  | —    | 2.5  |
| 5, 6      | 1.3     | <1.0 | 1.2  | <1.0 | <1.0 |
| 7, 8a     | 9.8     | 8.5  | 9.5  | 9.3  | 10.0 |
| 7, 8b     | 2.2     | 2.6  | 2.2  | —    | —    |
| 8a, 8b    | 3.8     | 3.4  | 3.2  | —    | —    |
| 8a, 8b    | 12.4    | 12.5 | 12.5 | —    | —    |

*Not analyzed because of the overlapping of signals.*

*Fig. 2. Purification of α-KDOase from oyster hepatopancreas.*

A, purification by sephacryl S-200 chromatography; B, purification by SP-Fractogel chromatography. Dotted line, absorbance at 280 nm; O, α-KDOase-cleaving activity. Detailed conditions are described under "Experimental Procedures."
RESULTS AND DISCUSSION

Preparation of \( \alpha \)- and \( \beta \)-KDO-MU—The glycosyl chloride of the per-O-acetylated methylester of KDO (2) was chosen as the glycosyl donor for the synthesis of \( \alpha \)- and \( \beta \)-KDO-MU. Two procedures, which have been used for the preparation of 4-methylumbelliferyl ketoside of NeuAc, were applied for the coupling of 2 with the sodium salt of 4-methylumbelliferone, namely, the direct coupling in a dipolar aprotic solvent such as DMF (12) and the catalyzed phase transfer reaction (13). In both cases, the methyl (4-methylumbelliferyl 4,5,7,8-tetra-O-acetyl-3-deoxy-\( \beta \)-D-manno-2-octulopyranoside)onate (3) was obtained as the major product (80%), together with the \( \alpha \)-anomer (4) as the minor product (7%). Compounds 3 and 4 could be separated either by repeated column chromatography or by taking advantage of their differential solubility in toluene. The \( \beta \)-anomer 3 was more soluble in toluene than the \( \alpha \)-anomer 4. The structures of 3 and 4 were determined by mass spectrometry and by \( ^1 \)H and \( ^{13} \)C NMR spectroscopy. Although it is difficult to deduce the anomeric configuration by \( ^1 \)H NMR analysis, we assigned the major product as the \( \beta \)-anomer 3 based on the following considerations. First, it is reasonable to assume that due to the anemic effect, compound 2 should exist predominantly in the \( \alpha \)-anomeric configuration. Second, the coupling reaction under a given condition should occur by the usual SN2 mechanism to give the product an amemic inversion. This assignment was confirmed by the proton-coupled \( ^{13} \)C NMR signals for C-1 in the final products.

Several attempts to improve the yield of the \( \alpha \)-anomer 4 were unsuccessful. For example, silver triflate-catalyzed reaction of 2 with anhydrous 4-methylumbelliferone in CH\(_2\)Cl\(_2\) in the presence of molecular sieves (MS4A) led to the formation of methyl 4,5,7,8-tetra-O-acetyl-2,6-anhydro-3-deoxy-D-manno-oct-2-enonate (14) (the glycal derivative of KDO) in 82% yield instead of the coupling product. It is noteworthy that the reaction of 2 with the sodium salt of 4-methylumbelliferone in DMF in the presence of tetrabutylammonium chloride (2 molecular equivalents) did not improve the yield of 4. Therefore, the formation of the \( \alpha \)-anomer 4 did not result from in situ anemicization of the glycosyl chloride by chloride ion that was generated during the coupling reaction, but instead, the \( \alpha \)-anomer must have come from the contaminated \( \beta \)-glycosyl chloride. It should be pointed out that the formation of isomeric products was not reported in the similar preparation of MU-ketoside of sialic acid (12, 13).

De-O-acetylation of 3 and 4, followed by alkaline hydrolysis of the methyl ester and purification by Sephadex G-10 chromatography, gave the \( \beta \)-anomer 5 (84%) and the \( \alpha \)-anomer 6 (91%), respectively, as their ammonium salts. The \( ^1 \)H NMR spectra (Fig. 3) of 5 and 6 revealed that the sugar protons of the two anomers had distinctly different chemical surroundings. In 5, the signals for H-4, 5, 6, 7, and 8 were overlapping in a narrow area ranging from \( \delta \) 4.04 to 3.82; whereas in 6, the signals for each proton of 4, 5, 6, 7, 8 were completely separated in a wide range (\( \delta \) 4.26–3.12).

**Table III**

| Step                      | Protein Total | Specific Activity | Purification | Recovery |
|---------------------------|---------------|------------------|--------------|----------|
| Crude extract             | 22,000        | 134,200          | 6.1          | 1        | 100    |
| 40–85% (NH\(_4\))\_SO\(_4\) | 1,955         | 100,080          | 51           | 8.4      | 75     |
| Sephacryl S200            | 435           | 69,403           | 160          | 26       | 52     |
| SP-Fractogel              | 59            | 37,990           | 644          | 106      | 28     |
| Con A-Sepharose           | 16.3          | 13,300           | 816          | 134      | 10     |
| Octyl-Sepharose           | 5.8           | 8,312            | 1433         | 235      | 6.2    |

**Fig. 3.** Proton NMR spectra of \( \beta \)-KDO-MU (top) and \( \alpha \)-KDO-MU (bottom).

**Fig. 4.** Proton-coupled \( ^{13} \)C NMR signals for C-1 of \( \alpha \) and \( \beta \)-KDO-MU.

**Fig. 5.** Comparative acidic hydrolysis of \( \alpha \) and \( \beta \)-KDO-MU. Each KDO-MU anomer (20 nmol) was dissolved in 200 \( \mu \)l of 0.2 M sodium acetate buffer (pH 4.5) and heated at 70 °C for a predetermined time. The hydrolysate was diluted to 1 ml and 50 \( \mu \)l-samples were taken for high performance anion exchange chromatography analysis.
**Determination of the Anomeric Configuration by Proton-coupled $^{13}$C NMR**—Some empirical $^1$H NMR rules have been used for deducing the anomeric configurations of KDO derivatives. One such rule is that the difference in chemical shift between C-3a and H-3e in $\beta$-anomer is usually bigger than that in $\alpha$-anomer (9). However, these empirical rules often lead to ambiguous assignments. For example, in the present case, the difference between $\delta$ (H-3e) and $\delta$ (H-3a) in $\beta$-anomer 5 is bigger than that in $\alpha$-anomer 6, whereas the difference between $\delta$ (H-3e) and $\delta$ (H-3a) in $\beta$-anomer 3 is actually smaller than that in $\alpha$-anomer 4. This is due to the fact that the substituents could greatly influence the chemical shifts of neighboring protons. A definitive determination of the anomeric configurations of KDO derivatives could be achieved by comparison of the proton-coupled $^{13}$C NMR signals of the C-1 in $\alpha$- and $\beta$-anomers (9). In a typical $^3$C$_2$ chair conformation of KDO derivatives, the dihedral angles of (C-1)-(C-2)-(C-3)-(H-3a) in $\alpha$- and $\beta$-anomers are nearly 60° and 180°, respectively. Therefore, the $\alpha$-anomer would give a small value for the coupling constant between C-1 and H-3a ($J_{C-1, H-3a} < 1$ Hz), and the $\beta$-anomer would give a relatively large coupling constant ($J_{C-1, H-3a} = 5$–6 Hz), according to the Karplus relationship (15). In the proton-coupled $^{13}$C NMR spectra (Fig. 4), the C-1 signal of 5 appeared at $\delta$ 173.73 as a doublet ($J_{C-1, H-3a} = 5.5$ Hz), whereas the C-1 signal of 6 appeared at $\delta$ 174.18 as a broad singlet ($J_{C-1, H-3a} < 1$ Hz). Accordingly, 5 should be in the $\beta$-configuration and 6 in the $\alpha$-configuration. This method was previously used for the determination of the anomeric configurations of sialic acid derivatives (16). The present study shows that this assignment is equally applicable for KDO derivatives.

**Acid Stability of $\beta$-KDO-MU (5)**—Under mildly acidic conditions (0.2 M acetate buffer, pH 4.5, 70°C), the $\beta$-anomer was found to be much more labile than the $\alpha$-anomer (Fig. 5). A similar observation was reported for the anomeric pairs of the ketosides of sialic acid, in which the equatorial aglycon was hydrolyzed much faster than the axial aglycon (17, 18).

KDO-containing glycoconjugates have been found to contain both $\alpha$- and $\beta$-linked KDO (7). Therefore, $\alpha$- and $\beta$-KDO-MU should be useful for studying $\alpha$- and $\beta$-KDOases, and the availability of these two enzymes will facilitate the studies of the structure and function of KDO-containing glycoconjugates.

**KDOase from Oyster Hepatopancreas**—Despite the medical importance of LPSs, virtually nothing is known about their degradation. The enzyme (KDOase) that cleaves $\alpha$-KDO from LPSs has never been reported. The hepatopancreas of the oyster, _C. virginica_, was found to be rich in various glycoconjugate-cleaving enzymes. Using the periodate-thiobarbituric acid reaction (7) and TLC, the crude extract of oyster hepatopancreas was found to liberate KDO from Re-LPS prepared from _E. coli_ and _S. minnesota_. Since KDO residues in LPSs have been shown to be $\alpha$-ketosidically linked (19, 20), we used the synthesized $\alpha$-KDO-MU as substrate to follow the enzyme activity to purify $\alpha$-KDOase from the crude extract of oyster hepatopancreas. Before a $\alpha$-KDO-MU was synthesized, we used Re-LPS as substrate, and the liberated KDO was determined by the periodate-thiobarbituric acid reaction. Initially, this method was used to monitor the purification of $\alpha$-KDOase during Sephacryl-S-200 gel filtration. We subsequently found that in the column fractions, the enzyme activity detected by the Re-LPS/periodate-thiobarbituric acid reaction coincided well with that detected using $\alpha$-KDO-MU as substrate. The availability of $\alpha$-KDO-MU greatly facilitated the purification of $\alpha$-KDOase.

**Oyster $\alpha$-KDOase was found to be stable in the pH range**
Interestingly, the pH optima for this enzyme using the synthetic and the natural substrates were found to be significantly different. The pH optimum of this enzyme was determined to be 4.5 for releasing KDO from Re-LPS, whereas that for the hydrolysis of α-KDO-MU was 3.0 (Fig. 6). Thus, the optimal pH for the hydrolysis of KDO from KDO-containing glycoconjugates cannot be inferred from the hydrolysis of α-KDO-MU.

The time courses of the liberation of KDO from DeOA-LPS by α-KDOase and by acid hydrolysis are compared in Fig. 7. The oyster α-KDOase was able to completely detach the KDO from DeOA-LPS. The amount of KDO released by the enzyme was found to be comparable to that liberated by acid hydrolysis. Fig. 8 shows the TLC analysis of the release of KDO from DeOA-LPS and α-KDOase isolated from oyster hepatopancreas was able to cleave KDO efficiently from all LPS substrates tested, including Re-LPS from E. coli, Re-LPS from S. minnesota R595, Rd-LPS from S. minnesota R7, and DeOA-LPS. However, β-KDO-MU was found to be refractory to this enzyme. Thus, the specificity of α-KDOase also supports the assignment of the anomeric configurations of the two KDO-MU anomers. The oyster α-KDOase represents the first α-KDO-cleaving enzyme, and the presence of such an enzyme in the hepatopancreas of oyster may suggest the wide occurrence of KDO in marine organisms. α-KDOase capable of liberating KDO from LPSs should be important and useful for studying the structure and function of LPSs and other α-KDO-containing glycoconjugates.

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