2-Keto-3-deoxy-d-glycero-d-galacto-nononic Acid (KDN)-
and N-Acetylated neuraminic Acid-cleaving Sialidase (KDN-sialidase)
and KDN-cleaving Hydrolyse (KDNase) from the Hepatopancreas
of Oyster, Crassostrea virginica*

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KDN (2-keto-3-deoxy-d-glycero-d-galacto-nononic acid), a sialic acid analog, has been found to be widely distributed
in nature. Despite the structural similarity between KDN and Neu5Ac, α-ketosides of KDN are refractory to
conventional sialidases. We found that the hepatopancreas of the oyster, Crassostrea virginica, contains two
KDN-cleaving sialidases but is devoid of conventional sialidase. The major sialidase, KDN-sialidase, effectively
cleaves α-ketosidically linked KDN and also slowly cleaves the α-ketosides of Neu5Ac. The minor sialidase,
KDNase, is specific for α-ketosides of KDN. We were able to separate these two KDN-cleaving enzymes using hydrophobic
interaction and cation-exchange chromatography. The rate of hydrolysis of 4-methylumbelliferyl-α-KDN (MU-KDN)
by KDN-sialidase is 30 times faster than that of MU-Neu5Ac in the presence of 0.2 M NaCl, whereas
in the absence of NaCl this ratio is only 8. KDNase hydrolyzes MU-KDN over 500 times faster than MU-Neu5Ac and
is not affected by NaCl. KDN-sialidase purified to electrophoretically homogeneous form was found to have a molecular
mass of 25 kDa and an isoelectric point of 8.4. One of the three tryptic peptides derived from KDN-sialidase
contains the consensus motif, SXXDGGTW, that has been found in all conventional sialidases. Kinetic analysis of
the inhibition of the hydrolysis of MU-KDN and MU-Neu5Ac by 2,3-dehydro-2-deoxy-KDN (KDN2-en) and 2,3-
dehydro-2-deoxy-(Neu5Ac2-en) suggests that KDN-sialidase contains two separate active sites for the hydrolysis
of KDN and Neu5Ac. Both KDN-sialidase and KDNase effec-
tively hydrolyze KDN-GM3, KDN2a–3Gal β1–4Glc, KDN2a–6Galβ1–4Glc, KDN2a–6Galβ1–4Glc, KDN2a–6N-acetyl-
galactosaminitol, KDN2a–6(GalKN2a–3)N-acetyl-galactosaminitol, and KDN2a–6(GlcNacβ1–3)N-acetyl-
galactosaminitol. However, only KDN-sialidase also slowly hydrolyzes Gm3, Neu5Acα2–3Galβ1–4Glc, and Neu5Acα2–6Galβ1–4Glc. These two KDN-cleaving sialidases should be useful for
studying the structure and function of KDN-containing
glycoconjugates.

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After the initial detection of KDN in the cortical alveolar
polysialoglycoproteins of rainbow trout (Salmo gairdneri) in
1986 (1), KDN-containing glycoconjugates have been found to
be widely distributed in a variety of sources (2–7), including
mammalian tissues, human lung and ovarian cancer cells, and
human red blood cells (8, 9). It has been recently shown that
the expression of KDN-containing epitope in some mammalian
organs may be developmentally regulated (10–12). Despite the
wide occurrence of KDN-containing glycoconjugates, very little
is known about their catabolism. Structurally, KDN is very
similar to Neu5Ac; however, α-ketosides of KDN are refractory
to bacterial sialidases (1, 2, 4, 13). Based on the activity toward
the α-ketosides of Neu5Ac and KDN, sialidases can be divided into:
(a) conventional sialidase, which efficiently cleaves
Neu5Ac with very little or no activity toward the hydrolysis of
KDN; (b) KDN-sialidase, which hydrolyzes both KDN and
Neu5Ac; and (c) KDNase, which efficiently cleaves KDN with
very little activity toward Neu5Ac (13, 14).

In 1994, we reported the presence of a sialidase, which
cleaved the α-ketosides of both KDN and Neu5Ac in the liver
of the loach (Misgurnus fossilis) (13) and proposed the name
KDN-sialidase to distinguish the enzyme from conventional
sialidase and KDNase. The loach liver KDN-sialidase was able
to hydrolyze Mu-KDN and Mu-Neu5Ac in a ratio close to 1:1.
Kitajima et al. (15) and Nishino et al. (16) used KDN-oligosac-
charide alditols to induce in the bacterium, Sphingobacterium
multivorum, a KDNase that was reported to cleave KDN but
not Neu5Ac (15, 16). Previously, we reported the coexistence
of KDN-sialidase and conventional sialidase in the starfish, Aste-
rina pectinifera (14). While further surveying the distribution
of KDN-cleaving enzymes in the animal kingdom, we found
...
that the hepatopancreas of the oyster, *Crassostrea virginica*, was a convenient source rich in KDN-sialidase and devoid of conventional sialidase. The oyster hepatopancreas contains KDN-cleaving activities distributed throughout a wide range of isoelectric points (pI 4.5–8.8). This report describes the purification and characterization of the major KDN-cleaving enzyme, KDN-sialidase, from oyster hepatopancreas. During the course of purifying this enzyme, we also detected the presence of a minor KDN-cleaving enzyme, KDNase.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**

Oysters, *C. virginica*, collected from Barataria Bay in southern Louisiana, were purchased from P&J Oyster Company, New Orleans, LA. KDN was synthesized according to Nakamura et al. (17) and MU-KDN, according to Myers et al. (18). 3′-KDN-lactose, 6′-KDN-lactose, 3′-KDN-GalNAc, and 6′-KDN-GalNAc were synthesized as described by Terada et al. (19). 6′-KDN-GalNAc-ol and 3′,6′-di-KDN-GalNAc-ol were prepared from loach skin mucus (7). The following were generous gifts: 6′-KDN(3′-GlcNac)GalNAc-ol (5) from Dr. G. Strecker, Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille, France; KDN2-en from Dr. K. Furuhata, School of Pharmaceutical Science, Kitasato University, Shirokane, Tokyo, Japan, and Professor von Itzstein, Victorian College of Pharmacy, Parkville, Victoria, Australia. The following were purchased from commercial sources: pre-coated silica gel-60 TLC plates, Fractogel EMD SP-650(S), Merck; Macro-Prep ceramic hydroxyapatite type I, 40 μm, Bio-Rad; marker proteins for molecular weight and pl, Mono P FFPLC column, octyl-Sepharose SL-4B, Sephacryl S-200 HR and S-100 HR, Polybuffer 96, PhastGel IEP 3–9, PhastGel high density, Amersham Pharma Biotech; phenylmethylsulfonyl fluoride, and BCA protein assay kit, Pierce; Azocoll (50–100 mesh), Calbiochem; MU-Neu5Ac, Neu5Ac2-en, Clostridium perfringens sialidase, 3′-sialyllactose, 6′-sialyllactose, Tris, and bis-Tris, Sigma; Centricon-10 and Microcon (10,000 cut-off) micro-concentrators, Amicon; BioSep Sec S-200 HPLC column, Phenomenex, Inc. (Torrance, CA).

**Enzyme Assays—Fluorometric assay of MU-KDN- or MU-Neu5Acleaving activity was carried out essentially according to the procedure described by Potier et al. (20). The enzyme was incubated with 0.5 mm MU-KDN or MU-Neu5Ac in 50 mM sodium acetate buffer, pH 4.0, in a total volume of 100 μl at 37 °C. After a set 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 Sequioa-Turner Model 450 fluorometer. One unit of enzyme activity is defined as the amount that liberates 1 nmol of MU/min at 37 °C.

For detecting free Neu5Ac or KDN released from water-soluble sialylglycoconjugates by TLC, 30 nmol of substrate in 30 μl of 50 mM sodium acetate buffer, pH 4.0, were incubated with an appropriate amount of enzyme at 37 °C. After incubation, 30 μl of ethanol were added to the mixture, and the mixture was analyzed by TLC. For glycolipid substrates G α and KDN-G β, the reaction mixture contained 10 nmol of the substrate in 30 μl of the same buffer. The reaction was stopped by adding 30 μl of ethanol, dried under vacuum, redissolved in 10 μl methanol:water (1/1, v/v), and applied onto a TLC plate. The tube was rinsed twice with 10 μl of chloroform:methanol (2/1, v/v) to solubilize any remaining glycolipids, and the rinse was also applied onto the same positions on the TLC plate, as described previously (14). The plates were developed by 1-butanol:acetic acid:water (2/1/1, v/v/v), sprayed with diphenylamine reagent (21), and heated at 115 °C for 20 min to visualize glycoconjugates. The quantitative estimation of the stained bands on a TLC plate was accomplished by scanning the plate using a Scan Jet 2C/ADF scanner (Hewlett-Packard).

**Electrophoresis—** SDS-PAGE was performed according to Doucet and Trifaro (22) using Bio-Rad Mini-Protein cell. Native PAGE was carried out on Phast System (Amersham Pharmacia Biotech) using high density gel, reversed polarity electrode assembly (Amersham Pharmacia Biotech) and acidic buffer strips, pH 4.1, made according to Pharmacia Phast System application file number 300. IEF-PAGE was performed on Phast System using IEF 3–9 gels. Proteins were visualized with Coomassie Blue and silver staining.

**Kinetic Analysis—** The initial rates for the enzyme-catalyzed hydrolysis of MU-KDN and MU-Neu5Ac were evaluated from kinetic curves of product accumulation measured at least in duplicate experiments as described by Boeker (23). The reactions with 0.4 units of the enzyme were carried out at a fixed concentration of each substrate in the range between 0.025 and 0.85 mM. For inhibition assays, the solutions also contained a defined amount (0.02–0.1 mM) of the substrate analog NeuAc2-en or KDN2-en. The initial rates were analyzed by using multivariable nonlinear regression data analysis programs for determining the kinetic parameters of enzymatic hydrolysis and inhibition, kindly provided by Dr. A. R. Pavlov, Department of Ophthalmology, Tulane University School of Medicine.

**Amino Acid Sequencing of Tryptic Peptides Derived from KDN-sialidase—** Microsequencing was carried out by the W. M. Keck Foundation Biotechnology Resource Laboratory of Yale University. The gel slice containing the protein was digested with trypsin, and the eluate was subjected to microbore HPLC. Matrix-assisted laser desorption/ionization mass spectrometry was used to evaluate peak purity and identify candidate peaks suitable for sequencing. The sequences obtained were compared with GenBank™ and EST data bases.

**Assay for Other Enzymes—** Exoglycosidases were assayed using MU- and p-nitrophenyglucosides in 50 mM sodium acetate buffer, pH 4.0, as described previously (24). The proteolytic activity was assayed using Azocoll as a substrate (25). Protein concentrations were determined by the method of Lowry (26) or using BCA protein microassay method according to the user’s guide.

**Purification of Two KDN-cleaving Sialidases**

Unless otherwise indicated, all operations were performed at 0–5 °C. Chromatofocusing, octyl-Sepharose, and hydroxyapatite chromatographies were carried out at room temperature. Centrifugations were routinely carried out at 20,000–30,000 × g for 30 to 40 min using a Sorvall RC5C refrigerated centrifuge. Unless otherwise indicated, an Amicon stirred cell with a PM-10 membrane was used for concentration.

**Preparation of Crude Enzyme from Oyster Hepatopancreas—** Each hepatopancreas was manually removed from oyster with scissors. The hepatopancreas (500 g) derived from 1.5 gallon (about 210 oysters) of fresh oysters were homogenized with 3.5 liters of cold acetone, immediately filtered with a Buchner funnel, and dried under vacuum to obtain 135 g of oyster hepatopancreas acetone powder (27). This acetone powder can be kept in a freezer (-20 °C) for at least a year without losing the MU-KDN-cleaving activity. The acetone powder was homogenized with 3.4 liters of water containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors using a Polytron (Brinkmann), followed by centrifugation to obtain the acetone powder extract. The pH of the extract was adjusted to 4.0 with saturated citric acid, and the precipitate was removed by centrifugation. The supernatant was...
brought to 45% saturation (277 g/liter) with solid ammonium sulfate. After 2 h, the precipitate was removed by centrifugation, and the supernatant was further brought to 85% saturation (295 g/liter) with ammonium sulfate. After standing overnight, the precipitate was collected by centrifugation and dissolved in 40 ml of 50 mM sodium acetate buffer, pH 4.2. This crude enzyme preparation was used for further purification of the two KDN-cleaving enzymes.

**Sephacryl S-200 Gel Filtration**—The crude enzyme preparation was applied onto a Sephacryl S-200 column (5 × 100 cm) equilibrated with 50 mM sodium acetate buffer, pH 4.2, containing 0.15 M NaCl. The column was eluted with the same buffer at 1 ml/min, and 20-ml fractions were collected. The fractions containing both MU-KDN- and MU-Neu5Ac-cleaving activities, as shown in Fig. 1A, were pooled and concentrated by ultrafiltration.

**Octyl-Sepharose Chromatography**—The enzyme preparation obtained from Sephacryl S-200 filtration was equilibrated with 1.5 M ammonium sulfate using an Amicon stirred cell with PM-10 membrane, adjusted to 3 ml, and applied onto an octyl-Sepharose column (1.5 × 46 cm) equilibrated with 1.5 M of ammonium sulfate. The column was eluted with 1.5 M ammonium sulfate at 2 ml/min, followed by 50 mM sodium acetate buffer, pH 4.2, and 3-ml fractions were collected. The fractions containing KDN-cleaving activity, separated into two portions as shown in Fig. 1B, were concentrated and desalted using an Amicon stirred cell with PM-10 membrane. The leading edge of the ammonium sulfate-eluted peak, containing MU-KDN-cleaving activity with very low MU-Neu5Ac-cleaving activity (Fig. 1B, fractions 16 to 19), was used for the isolation of KDNase. The main fraction (Fig. 1B, fractions 20 to 29), containing both MU-KDN- and MU-Neu5Ac-cleaving activity, was used for the purification of KDN-sialidase.

**Purification of KDN-sialidase**—The enzyme preparation obtained after octyl-Sepharose chromatography (Fig. 1B, fractions 20 to 29) was applied onto a Sephacryl S-100 gel filtration column (1.5 × 90 cm) equilibrated with 40 mM sodium phosphate buffer, pH 6.7. The column was eluted with the same buffer at 0.3 ml/min, and 1-ml fractions were collected. The fractions containing both MU-KDN- and MU-Neu5Ac-cleaving activities were pooled and concentrated to 0.2 ml using a Centricon-10 microconcentrator. This preparation was further purified by FPLC chromatofocusing using the conditions described by the manufacturer. Both starting and running buffer contained 10% sucrose to protect the enzyme in Oyster Hepatopancreas and Isolation of Two KDN-cleaving Stialidases—By IEF-PAGE and agarose gel substrate overlay (28) using MU-KDN and MU-Neu5Ac as substrates, the enzyme preparation obtained by octyl-Sepharose chromatography was further purified by chromatofocusing using the conditions identical to that for the purification of KDN-sialidase to obtain KDNase. Table I summarizes the purification of KDN-sialidase and KDNase from 135 g of acetone powder.

**RESULTS AND DISCUSSION**

**Presence of Multiple Isoelectric Forms of KDN-cleaving Enzyme in Oyster Hepatopancreas and Isolation of Two KDN-cleaving Stialidases**—By IEF-PAGE and agarose gel substrate overlay (28) using MU-KDN and MU-Neu5Ac as substrates, the enzyme preparation obtained by octyl-Sepharose chromatography was further purified by chromatofocusing using the conditions identical to that for the purification of KDN-sialidase to obtain KDNase. Table I summarizes the purification of KDN-sialidase and KDNase from 135 g of acetone powder.

**TABLE I**

| Steps | Total protein | Total activity | Specific activity | Recovery | Purification fold |
|-------|--------------|----------------|------------------|----------|------------------|
|       | mg           | units          | units/mg         | %        | fold             |
| 1. Acetone powder extract | 15298 | 98213 | 6.4 | 100 | 1 |
| 2. (NH₄)₂SO₄, 45–85% | 3086 | 66500 | 21.5 | 68 | 3.4 |
| 3. Sephacryl S-200 | 688 | 58150 | 85 | 59 | 13.3 |
| 4. Octyl-Sepharose (KS) | 29 | 578 | 20.1 | 81 | 85 |
| 5. Sephacryl S-100 (KS) | 12 | 11105 | 925 | 11.3 | 145 |
| 6. Mono P (KS) | 0.12 | 1434 | 11950 | 1.5 | 1867 |
| 7. Octyl Sepharose (KDNase) | 91.5 | 3734 | 40.8 | 3.8 | 6.4 |
| 8. Fractogel SF (KDNase) | 3.3 | 878 | 286 | 0.9 | 42 |
| 9. Mono P (KDNase) | 0.07 | 216 | 3086 | 0.2 | 482 |

*Fig. 1B, fractions 20–29.*  
*Fig. 1B, fractions 16–19.*
tography (Fig. 1B, fractions 20 to 29) was found to contain multiple isoelectric forms of KDN-cleaving activity. The pI of the isoelectric forms ranged from 4.5 to 8.8 (Fig. 2, lane 7), with a major form focused at a pI in the vicinity of 8.4. Because of the heterogeneity of KDN-cleaving activity, the purification of KDN-cleaving enzymes from the hepatopancreas of oyster proved to be a challenge (Table I). Using Sephacryl S-200 chromatography, MU-KDN- and MU-Neu5Ac-cleaving activities were eluted as one peak (Fig. 1A). By subsequent octyl-Sepharose chromatography (Fig. 1B), the major MU-KDN- and MU-Neu5Ac-cleaving activities were eluted slightly behind the breakthrough protein peak. This step effectively removed the contaminating exo-glycosidases and the bulk of proteins that were retained by the column along with the rest of the MU-KDN- and MU-Neu5Ac-cleaving activities. Examination of the activity peak revealed that the leading edge of the peak (Fig. 1B, fractions 16 to 19) contained a high ratio of MU-KDN-to MU-Neu5Ac-cleaving activity. Therefore, the front portion of this peak was pooled separately and subjected to FPLC SP-Fractogel chromatography. As shown in Fig. 1D, two well separated peaks, I and II, with MU-KDN-cleaving activity were eluted by linear NaCl gradient. Peak I also exhibited some activity toward MU-Neu5Ac (the ratio of MU-KDN- to MU-Neu5Ac-cleaving activity was 10:1), whereas in peak II the MU-Neu5Ac-cleaving activity was extremely low. The ratio of MU-KDN- to MU-Neu5Ac-cleaving activity in this preparation was greater than 500:1. For comparison, the ratio of MU-KDN- to MU-Neu5Ac-cleaving activity of C. perfringens and Arthrobacter ureafaciens sialidases has been found to be 1 to 350 and 1 to 60, respectively (14). Thus, enzyme eluted in peak I represents a KDN-sialidase, whereas that from peak II, a KDNase.

**Purification of KDN-sialidase**—The main enzyme preparation obtained from the octyl-Sephacrose column (Fig. 1B, fractions 20 to 29) was found to contain multiple isoforms (Fig. 2, lane 7). This preparation was subjected to a second gel filtration followed by chromatofocusing. The second gel filtration resulted in a 2-fold increase in specific activity (see Table I). Based on the results obtained by IEF-PAGE, an eluent with a pH range of 8.6 to 7.0 was chosen for chromatofocusing. The elution profile from a Mono P column (Fig. 1C) was in agreement with that of isoelectric focusing (Fig. 2, lane 7). As shown in Fig. 1C, one major KDN-sialidase peak was focused at pH 8.4, whereas a number of other minor peaks were focused over a lower pH range. KDN-sialidase obtained from the major peak was found to be essentially free of other exo-glycosidases and proteases. Chromatofocusing enabled us to purify the major isoform of the enzyme, which contained about 15–20% total MU-KDN-cleaving activity as determined by IEF-PAGE and substrate overlay. To prevent the inactivation of the enzyme due to high pH, chromatofocusing was performed in the presence of 10% sucrose. Table I summarizes the results of a typical purification of KDN-sialidase from 135 g of the acetone powder. This purification scheme has been repeated more than 10 times and has been found to be quite reproducible. The preparation obtained by chromatofocusing could be further purified by ceramic hydroxyapatite chromatography. The main isoform of KDN-sialidase activity was eluted by 20 mM sodium phosphate buffer, pH 6.5, and was purified more than 12,000-fold (results not shown). When examined by IEF-PAGE and substrate overlay, this preparation was found to contain two isoforms focused around pH 8.4. No other proteins were detected in this preparation (Fig. 2, lanes 4 and 5). However, the recovery of this step was less than 5%. Therefore, the ceramic hydroxyapatite step was not practical for obtaining the pure enzyme.

By native PAGE at pH 4.1, the KDN-sialidase purified by chromatofocusing (Fig. 1C) moved as a single protein band (Fig. 3A, lane 1) coincident with the MU-KDN-cleaving activity, as revealed by the agarose-gel substrate overlay (Fig. 3A, lane 2). However, by IEF-PAGE (Phast system), it gave a closely spaced doublet of MU-KDN-cleaving activity together with several protein bands without enzyme activity (Fig. 2, lane 3 and 6). Using preparative SDS-PAGE, this enzyme preparation was resolved into one major protein band with a molecular mass of 25 kDa along with several minor protein bands. The protein in this 25-kDa band was extracted and subjected to a second SDS-PAGE (Fig. 3B) to obtain the electrophoretically homogeneous protein. This band was sent to the W. M. Keck Foundation Biotechnology Resource Laboratory of Yale University for tryptic digestion and subsequent sequence analysis.

**Presence of Asp Box Motif in KDN-sialidase**—Among 18 tryptic peptides separated by HPLC, three peptides were chosen for sequencing based on the purity and molecular mass. The amino acid sequences of the three well separated tryptic peptides were: (a) SGDSAGIWLSAR, (b) NTWLYPIYYAGGSSQEQTSNLK, and (c) GQPHLSAFFR. These sequences were used to screen GenBank™ and EST data bases, but no matches were found. Also, no matches were found for the tryptic peptides molecular mass search of OWL and EMBL data bases. Thus, KDN-sialidase represents a novel protein not reported previously. The peptide (a) was found to contain the “Asp box” Ser-Xaa-Asp-Xaa-Gly-Xaa-Thr-Trp, as shown in bold letters above. This sequence is conserved in many bacterial and viral sialidases (29). The Asp box was also found in sialidases isolated from Chinese hamster ovary cell (30) and rat skeletal muscle cytosol (31). The presence of the Asp box strongly indi-
cates that KDN-sialidase is a member of the sialidase family.

Further Purification of KDNase by Chromatofocusing—The KDNase preparation from peak II of SP-Fractogel chromatography (Fig. 1D) was further purified by chromatofocusing using conditions identical to those for purification of KDN-sialidase. The enzyme was eluted at pH 8.4. As shown in Table I, the yield of KDNase was only 0.2% that of the total KDN-cleaving activity in the crude extract. The final KDNase preparation was free of exo-glycosidase activities and was subsequently used for studying its properties.

General Properties of KDN-sialidase and KDNase—The final KDN-sialidase preparation showed two major closely spaced protein bands focused at pH near 8.4 on Phast system IEF-PAGE, as revealed by silver stain (Fig. 2, lane 4). Using the agarose-gel substrate overlay, both bands were found to contain the MU-KDN-cleaving activity (Fig. 2, lanes 1). Two bands also exhibited a weak activity toward the hydrolysis of MU-Neu5Ac (Fig. 4, lanes 3 and 4), indicating that the same protein possessed both the MU-KDN and MU-Neu5Ac-cleaving activities. In contrast, the KDNase preparation showed one major MU-KDN-cleaving activity band, which did not exhibit any activity toward MU-Neu5Ac when examined by the agarose-gel substrate overlay (Fig. 4, lanes 2 and 5). In the hepatopancreas of oyster, we did not detect the presence of conventional sialidase, which cleaves only MU-Neu5Ac. The sialidase from C. perfringens was used as a positive control for the hydrolysis of MU-Neu5Ac (Fig. 4, lanes 1 and 6). Both KDN-sialidase and KDNase were estimated to have an apparent molecular mass of about 30 kDa using Sephacryl S-100 gel filtration column at pH 6.5, 20 kDa using HPLC Phenomenex a-glucosidase activity in the KDN-sialidase preparation, both enzymes were free from other exo-glycosidase activities when analyzed using MU- and p-nitrophenyl glycosides as substrates. They were also free from protease activity using Azocoll as the substrate.

Using MU-KDN as substrate, KDN-sialidase and KDNase exhibited maximal activity between pH 3.6 and 4.0 in 50 mM sodium acetate buffer. The same pH optimum was also found for the hydrolysis of MU-Neu5Ac by KDN-sialidase. Both enzymes were found to be stable between pH 3.5 and 7.2. The optimal pH for oyster KDN-sialidase is similar to that reported for KDN-sialidase from starfish, pH 4.0 (14), loach liver, pH 4.5 (13), and rainbow trout, pH 4.4 (32). The optimal pH for oyster KDNase is lower than that for S. multivorum KDNase, pH 5.7–6.0 (15). As shown in Table II, the \( K_m \) (15.6 \( \mu \)M) for the hydrolysis of MU-KDN by oyster KDN-sialidase is slightly smaller than those reported for other KDN-cleaving enzymes: 50 \( \mu \)M for KDN-sialidase from starfish (14) and 70 \( \mu \)M for loach KDN-sialidase (13).

Similar to other sialidases (33–35), both KDN-sialidase and KDNase were not greatly affected by the divalent cations. At 10 mM concentration, Ca\(^{2+}\) and Mg\(^{2+}\) stimulated the hydrolysis of MU-KDN by KDNase by 15% but had no effect on the hydrolysis of MU-KDN by KDN-sialidase. At 10 mM concentration, Mg\(^{2+}\) inhibited the activities of both KDNase and KDN-sialidase by 40%. Ca\(^{2+}\) and Mn\(^{2+}\) had no effect on the hydrolysis of MU-KDN either by KDNase or KDN-sialidase. Hg\(^{2+}\) was found to be a more potent inhibitor of the hydrolysis of MU-KDN by KDNase than by KDN-sialidase (Fig. 5A, a and c). Between MU-KDN and MU-Neu5Ac, Hg\(^{2+}\) exerted more inhibitory effect on the hydrolysis of MU-Neu5Ac by KDN-sialidase (Fig. 5A, a and b).

### Table II

**Kinetic parameters for hydrolysis of MU-KDN and MU-Neu5Ac by KDN-sialidase**

| Substrate   | \( K_m \) ± S.E. | \( V_{max} \) ± S.E. | \( K_i \) ± S.E. |
|-------------|-----------------|-------------------|-------------|
| MU-KDN     | 15.6 ± 3.6      | 11.82 ± 0.41      | 7.24 ± 1.5  |
| MU-Neu5Ac  | 121 ± 10.2      | 1.56 ± 0.07       | 3.2 ± 0.3   |

**Fig. 5. Effect of Hg\(^{2+}\) (A) and ionic strength (B) on the hydrolysis of MU-KDN by KDN-sialidase (a) and KDNase (c) and hydrolysis of MU-Neu5Ac by KDN-sialidase (b).** In B, the ionic strength was altered by varying the NaCl concentration in 50 mM sodium acetate buffer. The detailed assay conditions are described under “Experimental Procedures.”

**Effect of Ionic Strength on KDN-sialidase and KDNase—**

While studying the ratio of the hydrolysis of MU-KDN and MU-Neu5Ac by KDN-sialidase, we initially noticed that at the optimal pH, this ratio was affected by the type of buffer used. Subsequently we found that this effect was due to the ionic strength of the buffer. After adjusting the buffer solutions to the same ionic strength, this ratio became constant. Therefore, we studied the effect of ionic strength by varying NaCl concentration. The effect of ionic strength on the hydrolysis of MU-KDN and MU-Neu5Ac by KDN-sialidase was profoundly different (Fig. 5B, a and b). Hydrolysis of MU-KDN by KDN-sialidase was stimulated 2.5-fold by the presence of 0.2 mM NaCl (Fig. 5B, a). However, the hydrolysis of MU-Neu5Ac by KDN-sialidase was inhibited by 35% under the same conditions (Fig. 5B, b). Although the rate of hydrolysis of MU-KDN by KDN-sialidase was 8 times faster than that of MU-Neu5Ac under the standard assay condition without NaCl, in the presence of 0.2 mM NaCl, the ratio of MU-KDN- to MU-Neu5Ac-cleaving activities became more than 30. In contrast, the ionic strength had no appreciable effect on the hydrolysis of MU-KDN by KDNase (Fig. 5B, c). These results suggest that KDN-sialidase and KDNase, although possessing similar biological activity, may be structurally dissimilar. Similar to MU-KDN, hydrolysis of
3'-KDN-lactose by KDN-sialidase was also stimulated by the presence of NaCl.

Effect of KDN2-en and Neu5Ac2-en on the Hydrolysis of MU-KDN and MU-Neu5Ac by KDN-sialidase—To further understand the hydrolysis of MU-KDN and MU-Neu5Ac by KDN-sialidase, we studied the inhibition of the hydrolysis of MU-KDN and MU-Neu5Ac by two substrate analogs, KDN2-en and Neu5Ac2-en. As shown in Fig. 6A, KDN2-en is a competitive inhibitor of KDN-sialidase for the hydrolysis of MU-KDN ($K_i = 7 \, \mu M$). In contrast, KDN2-en is a strong noncompetitive inhibitor ($K_i = 3.2 \, \mu M$) for the hydrolysis of MU-Neu5Ac by the same enzyme (Fig. 6B). Neu5Ac2-en competitively inhibited the hydrolysis of MU-Neu5Ac by KDN-sialidase ($K_i = 111 \, \mu M$) (Fig. 6C) but did not inhibit the hydrolysis of MU-KDN at concentrations up to 0.1 mM. These results clearly suggest that KDN-sialidase hydrolyzes MU-KDN and MU-Neu5Ac at two separate active sites. The presence of two active sites in KDN-sialidase is also supported by the differences in the effect of Hg$^{2+}$ and NaCl on the hydrolysis of MU-KDN and MU-Neu5Ac. The different effects of ionic strength on two active sites of angiotensin I-converting enzyme have been described (36).

It is interesting to compare the substrate specificity of oyster KDN-sialidase with that of another enzyme with two distinct active sites, lactose-phlorizin hydrolase (37). This enzyme uses one active site for the hydrolysis of glycosides with hydrophilic aglycon (lactase site) and another site for the hydrolysis of glycosides with hydrophobic aglycon (phlorizin hydrolase site). In contrast, the two active sites of oyster KDN-sialidase recognize a small structural difference between the sugar moiety of the glycosides (the C-5 is substituted by OH and acetamido group for KDN and Neu5Ac, respectively). KDN-sialidase from starfish, A. pectinifera (14), also hydrolyzes both MU-KDN and MU-Neu5Ac. However, the same active site was found to be responsible for the hydrolysis of MU-KDN and MU-Neu5Ac (14).

Kinetic Studies of KDNase—The hydrolysis of MU-KDN by oyster KDNase was also examined in the presence and absence of the substrate analogs, KDN2-en and Neu5Ac2-en. As in the case of KDN-sialidase, the hydrolysis of MU-KDN by KDNase followed Michaelis-Menten kinetics. The rate of hydrolysis of MU-Neu5Ac by the enzyme was at least 500 times slower than that of MU-KDN. The $K_m$ (57 $\mu M$) for the hydrolysis of MU-KDN by KDNase was larger than the $K_m$ values for hydrolysis of MU-KDN by KDN-sialidase (15 $\mu M$) and KDNase (19 $\mu M$) isolated from S. multivorum (15). Similar to that for KDN-sialidase, KDN2-en was also a potent competitive inhibitor ($K_i = 8 \, \mu M$) for the hydrolysis of MU-KDN by KDNase, whereas Neu5Ac2-en did not inhibit this reaction. This was also reported to be the case for S. multivorum KDNase with $K_i = 8.1 \, \mu M$ (38).

Hydrolysis of Sialoconjugates by KDN-sialidase and KDNase—As shown in Fig. 7, the two KDN-containing oligosaccharides, 3'-KDN-lactose and 6'-KDN-lactose, were effectively hydrolyzed by KDN-sialidase and KDNase. KDN-sialidase, but not KDNase, also hydrolyzed 3'-sialyllactose and 6'-sialyllactose at a much slower rate (longer incubation is needed).
needed to show the cleavage). Both KDN-sialidase and KDNase were able to hydrolyze 3'-KDN-G^3M^3 and 6'-KDN-G^3M^3, 6'-KDN-GalNAc-ol, 3',6'-di-KDN-GalNAc-ol, and 6'-KDN(3'-GlcNAc)-GalNAc-ol. Table III summarizes the substrate specificity of oyster KDN-sialidase and KDNase. Both KDN-sialidase and KDNase hydrolyzed KDN-GalNac-ol, 3'Gal and KDN linkage. Both enzymes hydrolyzed KDN-glycoconjugates much slower than KDNase (see Table III).

Among various organisms, tissues, and organs examined, the hepatopancreas of oyster was found to be the most convenient source for KDN-sialidase and KDNase. The existence of these two KDN-cleaving enzymes in the hepatopancreas of oyster is intriguing. It is possible that both enzymes originate from microorganisms inhabiting the digestive tract of oyster. However, attempts to culture microorganisms that contained KDN-cleaving activity from oyster hepatopancreas were not successful. The occurrence of two different active sites in KDN-sialidase in the absence of a regular sialidase is very intriguing. However, conclusive evidence must wait until the recombinant enzyme and the crystal structure of the enzyme become available. The finding of “Asp box” sequence motif in KDN-sialidase clearly places this enzyme in the sialidase family. KDNase from hepatopancreas of oyster is the first naturally occurring KDNase isolated from a higher organism. The only KDNase reported so far (15) is the one induced in S. mutovorum using KDN-oligosaccharide additts. Although KDN-containing glycoconjugates have been found to be widely distributed in nature, their biological functions are still not well understood. These two naturally occurring KDN-cleaving sialidases should become useful for studying the structures and functions of KDN-containing glycoconjugates.

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