Two different sialidases, KDN-sialidase and regular sialidase in the starfish *Asterina pectinifera*

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We have found the coexistence of two different sialidases in the entrails of the starfish *Asterina pectinifera*: a regular sialidase (RS), which cleaves sialic acid from sialoglycoconjugates, and a KDN-sialidase (KS) which releases the sialic acid analogue KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) from KDN-containing glycoconjugates that are resistant to RS. The 6700-fold purified KS and 1300-fold purified RS were prepared to study the properties of these two sialidases. KS and RS from *Asterina* starfish differ in several properties other than glycon specificity, including molecular mass, isoelectric point (pI) and susceptibility to competitive and non-competitive inhibitors. KS has a molecular mass of 31 kDa and a pI of 8.3 while RS has a molecular mass of 128 kDa and a pI of about 4.8. 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (NeuAc2en), but not 2,3-dehydro-2-deoxy-KDN (KDN2en), is a potent competitive inhibitor of RS ($K_i \sim 0.007$ mM); however, both NeuAc2en and

KDN2en are moderate inhibitors of KS ($K_i \sim 0.04$ mM). Hg^{2+} is a potent non-competitive inhibitor of RS but not of KS. KS and RS were examined for their ability to hydrolyse KDN- and NeuAc-containing glycoconjugates. KS hydrolyses 4-methylumbelliferyl- α -KDN (MU-KDN) 20 times faster than 4-methylumbelliferyl- α -NeuAc (MU-NeuAc), while RS hydrolyses MU-NeuAc 88 times faster than MU-KDN at the pH optimum of 4.0. KS effectively hydrolyses KDN- G_{M3} (where G_{M3} is NeuAc $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ Glc $\beta 1$ -1'Cer, and Cer is ceramide), KDN $\alpha 2 \rightarrow 3$ lactose, KDN $\alpha 2 \rightarrow 6$ lactose, KDN $\alpha 2 \rightarrow 6$ N-acetylgalactosaminitol, KDN $\alpha 2 \rightarrow 6$ (KDN $\alpha 2 \rightarrow 3$)N-acetylgalactosaminitol and KDN $\alpha 2 \rightarrow 6$ (GlcNAc $\beta 1 \rightarrow 3$)N-acetylgalactosaminitol. However, under the same conditions, these KDN-containing glycoconjugates are refractory to RS. Conversely, G_{M3} , NeuAc $\alpha 2 \rightarrow 3$ lactose and NeuAc $\alpha 2 \rightarrow 6$ lactose are effectively hydrolysed by RS but not by KS.

INTRODUCTION

2-Keto-3-deoxy-D-glycero-D-galacto-Nononic acid (KDN), a sialic acid analogue, was first isolated in 1986 from the cortical alveolar polysialoglycoproteins of rainbow trout (*Salmo gairdneri*) [1]. Since then, KDN-containing glycoconjugates have been found in a variety of organisms [2–6], yet very little is known about their catabolism. Structurally, KDN is very similar to NeuAc, however, KDN-containing glycoconjugates are resistant to bacterial sialidases [7,8]. Therefore, KDN-containing glycoconjugates must be catabolized by a specific enzyme other than the NeuAc-cleaving, regular sialidase (RS). To facilitate our understanding of the catabolism of KDN-containing glycoconjugates, we have undertaken studies of the specificity and distribution of the KDN-cleaving enzyme in nature. In 1994, we first found in the liver of the loach (*Misgurnus fossilis*), a sialidase which could also efficiently cleave KDN from KDN-containing glycoconjugates [8], and suggested the name KDN-sialidase (KS) to distinguish the enzyme from RS. Subsequently, Angata et al. found that the tissues of rainbow trout contained a sialidase with very weak KDN-cleaving activity combined with strong NeuAc-cleaving activity [9] and Kitajima et al. found that KDN-cleaving activity was inducible in the bacterium *Sphingobacterium multivorum* [10]. While surveying the distribution of KS in the animal kingdom, we found both KDN- and NeuAc-cleaving activities in

the entrails of several species of starfish, such as *Asterina pectinifera* and *Ceratonarcho semiregularis*, collected off the coast of southern Japan, and *Luidia clathrata*, a common sea star collected from the Gulf of Mexico. Prior to the discovery of KS activity, the *Asterias rubens* starfish was shown to contain NeuAc-cleaving activity [11]. We, therefore, critically examined the identities of the KDN- and NeuAc-cleaving activities in one of the starfish, *Asterina pectinifera*. This starfish was chosen because of its abundance in southern Japan and the relatively large amount of entrails that can be obtained per specimen. We found that the KDN- and NeuAc-cleaving activities of this organism could be resolved into two distinct enzymes, a KS and an RS. This paper presents evidence showing the coexistence of two different sialidases, KS and RS, in the entrails of *Asterina pectinifera* starfish.

EXPERIMENTAL PROCEDURES

Materials

Starfish (*Asterina pectinifera*) were collected from southern Japan. KDN was synthesized according to the method of Nakamura et al. [12] and 4-methylumbelliferyl- α -KDN (MU-KDN) according to the method of Myers et al. [13]. KDN $\alpha 2 \rightarrow$

Abbreviations used: KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid, which is synonymous with 3-deoxy-D-glycero-D-galacto-nonulosonic acid and deaminated neuraminic acid; KS, KDN-sialidase; RS, regular sialidase; MU-NeuAc, 4-methylumbelliferyl- α -NeuAc; MU-KDN, 4-methylumbelliferyl- α -KDN; Cer, ceramide; 3'-sialyllactose, NeuAc $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ Glc; 6'-sialyllactose, NeuAc $\alpha 2 \rightarrow 6$ Gal $\beta 1 \rightarrow 4$ Glc; 3'-KDN-lactose, KDN $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ Glc; 6'-KDN-lactose, KDN $\alpha 2 \rightarrow 6$ Gal $\beta 1 \rightarrow 4$ Glc; G_{M3} , NeuAc $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ Glc $\beta 1$ -1'Cer; 3'-KDN- G_{M3} , KDN $\alpha 2 \rightarrow 3$ Gal $\beta 1 \rightarrow 4$ Glc $\beta 1$ -1'Cer; 6'-KDN- G_{M3} , KDN $\alpha 2 \rightarrow 6$ Gal $\beta 1 \rightarrow 4$ Glc $\beta 1$ -1'Cer; 6'-KDN-GalNAc-ol, KDN $\alpha 2 \rightarrow 6$ N-acetylgalactosaminitol; 3'-KDN-GalNAc-ol, KDN $\alpha 2 \rightarrow 3$ N-acetylgalactosaminitol; 3',6'-di-KDN-GalNAc-ol, KDN $\alpha 2 \rightarrow 6$ (KDN $\alpha 2 \rightarrow 3$)N-acetylgalactosaminitol; 6'-KDN(3'-GlcNAc)GalNAc-ol, KDN $\alpha 2 \rightarrow 6$ (GlcNAc $\beta 1 \rightarrow 3$)N-acetylgalactosaminitol; NeuAc2en, 2,3-dehydro-2-deoxy-N-acetylneuraminic acid; KDN2en, 2,3-dehydro-2-deoxy-KDN; IEF, isoelectric focusing; pI, isoelectric point; SP, sulphydryl; NMML, nominal molecular mass limit.

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3Gal β 1 $\rightarrow$ 4Glc (3'-KDN-lactose), KDN α 2 $\rightarrow$ 6Gal β 1 $\rightarrow$ 4Glc (6'-KDN-lactose), KDN α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc β 1-1'Cer (3'-KDN-G_{M3}) (where G_{M3} is NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc β 1-1'Cer) and KDN α 2 $\rightarrow$ 6Gal β 1 $\rightarrow$ 4Glc β 1-1'Cer (6'-KDN-G_{M3}) were synthesized as described by Terada et al. [14]. KDN α 2 $\rightarrow$ 6N-acetylgalactosaminitol (6'-KDN-GalNAc-ol) and KDN α 2 $\rightarrow$ 6(KDN α 2 $\rightarrow$ 3)N-acetylgalactosaminitol (3',6'-di-KDN-GalNAc-ol) were prepared from loach skin mucus [15]. The following were gifts: KDN α 2 $\rightarrow$ 6(GlcNAc β 1 $\rightarrow$ 3)N-acetylgalactosaminitol [6'-KDN(3'-GlcNAc)GalNAc-ol] [5] from Dr. G. Strecker, Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille, France; 2,3-dehydro-2-deoxy-KDN (KDN2en) from Dr. M. 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 DEAE-650(M) and Fractogel EMD SP-650(S) (SP is sulphopropyl) (Merck, Darmstadt, Germany); Hypatite C (hydroxylapatite) (Clarkson Chemical, Williamsport, PA, U.S.A.); molecular mass and pI standard proteins, Superdex 75 HR and Mono P FPLC columns, Sephacryl S200-SF, Polybuffer 96 and 74, PhastGel isoelectric focusing (IEF) 3-9, and Ampholine pH 3-10 carrier ampholyte solution (40%) (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden); bicinchoninic acid assay reagents, 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (NeuAc2en) and PMSF (Pierce Chemical Co., Rockford, IL, U.S.A.); *Arthrobacter ureafaciens* sialidase and Azocoll (50-100 mesh) (Calbiochem), 4-methylumbelliferyl- α -NeuAc (MU-NeuAc), *Clostridium perfringens* sialidase, NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc (3'-sialyllactose), NeuAc α 2 $\rightarrow$ 6Gal β 1 $\rightarrow$ 4Glc (6'-sialyllactose), Trizma base and Bistris (Sigma, St. Louis, MO, U.S.A.); 300 kDa nominal molecular mass limit (NMML) cut-off PTMK and 10 kDa NMML cut-off PTGC polysulphone membrane plates (Millipore, Bedford, MA, U.S.A.); and Centricon-10 (10000 MM cut-off) microconcentrators (Amicon, Beverly, MA, U.S.A.).

Enzyme assays

Fluorimetric assay of MU-NeuAc- or MU-KDN-cleaving activity was carried out according to the procedure described by Potier et al. [16]. Briefly, the enzyme was incubated with 0.5 mM MU substrate in 50 mM sodium acetate buffer, pH 4.0, in a total volume of 100 μ l 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. MU released was determined using a Sequoia-Turner Model 450 fluorimeter. One unit of enzyme (MU-NeuAc- or MU-KDN-cleaving activity) is defined as the amount that liberates 1 nmol of MU per min at 37 °C. Time-course experiments were performed as previously described [8]. For detecting free NeuAc or KDN released from water-soluble sialoglycoconjugates by TLC, 30 nmol of substrate in 30 μ l of 50 mM sodium acetate buffer, pH 4.0, was incubated with an appropriate amount of enzyme at 37 °C. After incubation, 20 μ l of ethanol was added and 8 μ l aliquots of the reaction mixture were analysed by TLC as previously described [8]. For analysing the enzymic hydrolysis of the glycolipid substrates G_{M3} and KDN-G_{M3}, the reaction was carried out similarly, except the reaction mixture contained 10 nmol of substrate in 30 μ l of the same buffer. After incubation, the reaction was stopped by the addition of 20 μ l of ethanol and the tubes were dried under vacuum. The mixtures were redissolved in 10 μ l of methanol/water (1:1, v/v) and the entire mixture was spotted onto a TLC plate. The tubes were rinsed twice with 10 μ l of chloroform/methanol (2:1, v/v) to solubilize any remaining glycolipids. The

rinse was also applied onto the same positions on the TLC plate. The plates were developed by butan-1-ol/acetic acid/water (2:1:1, by volume), sprayed with diphenylamine reagent [17] and heated at 115 °C for 15 min to reveal glycoconjugates.

Kinetic analysis

Initial rate measurements for the enzyme-catalysed hydrolysis of MU-KDN and MU-NeuAc were conducted as described previously [8]. For determination of K_m values using sialyllactose and KDN-lactose, the amount of released sialic acid or KDN was determined quantitatively using a fluorimetric assay [18]. The kinetic parameters were determined from a direct fit of the v versus $[S]$ data to the Michaelis-Menten equation using the SigmaPlot curve-fitting program (Jandel Scientific, CA, U.S.A.). For determination of the K_i values for the two competitive inhibitors NeuAc2en and KDN2en against KS and RS, the conditions were the same as described above, with the reaction mixtures containing 5-50 μ M NeuAc2en or KDN2en. The effect of ionic strength was determined using NaCl and the effect of heavy metal ions was determined using Hg²⁺ (in the form of mercuric acetate) and Cu²⁺ (in the form of cupric chloride) over the concentration range 0.1 μ M-10 mM.

Assay for other enzymes

Exoglycosidases were assayed using *p*-nitrophenyl-glycosides in 50 mM sodium acetate buffer, pH 4.0, as previously described [19]. The following glycosidases were checked: α - and β -galactosidases, β -N-acetylhexosaminidase, α - and β -mannosidases, α - and β -glucosidases, α -N-acetylgalactosaminidase, α -N-acetylglucosaminidase, α -fucosidase and β -xylosidase. The presence of proteases was assayed using Azocoll as substrate [20].

Preparation of starfish crude sialidase

All operations were carried out at a temperature between 0 and 5 °C. Centrifugations were routinely carried out at 20000-30000 *g* for 20-30 min using a Sorvall RC5C refrigerated centrifuge. Unless otherwise indicated, ultrafiltration was carried out with an Amicon stirred cell using a PM10 membrane. Entrails derived from 46 *Asterina pectinifera* starfish (450 g wet weight) were homogenized with 50 vol. of cold acetone, filtered with a Buchner funnel and dried under vacuum to obtain 73 g of starfish acetone powder [21]. The acetone powder was homogenized with 20 vol. of water containing 1 mM EDTA and 1 mM PMSF as protease inhibitors using a Polytron (Brinkmann, Westbury, NY, U.S.A.), followed by centrifugation. The supernatant (water extract), which contained 30700 units of MU-NeuAc-cleaving activity and 16300 units of MU-KDN-cleaving activity, was filtered with a Minitan acrylic tangential-flow ultrafiltration system (Millipore) using four 300 kDa NMML cut-off PTMK polysulphone membrane plates. Under this condition, 78 % of the MU-KDN-cleaving activity and 36 % of the MU-NeuAc-cleaving activity in the water extract were recovered in the filtrate. The filtrate was concentrated by the Minitan system using 10 kDa NMML cut-off PTGC polysulphone membrane plates. The retentate (115 ml) was designated a crude sialidase preparation and used for further examination of the two sialidases. Protein concentration was determined using the bicinchoninic acid assay [22] with BSA as the standard.

Rotofor isoelectric focusing

IEF was performed in a 2% Ampholine pH 3–10 solution using a Rotofor preparative IEF cell (Bio-Rad, Hercules, CA, U.S.A.) according to the conditions described by the manufacturer.

RESULTS AND DISCUSSION

Presence of two different sialidases, KS and RS, in the starfish *Asterina pectinifera*

Separation of two sialidases by ultrafiltration, IEF and DEAE-Fractogel chromatography

Asterina starfish water extract was found to cleave both MU-NeuAc and MU-KDN. Using the Minitan tangential-flow ultrafiltration system with 300 kDa NMML cut-off membranes, 78% of the MU-KDN-cleaving activity was found to be preferentially expelled into the filtrate while the bulk of the MU-NeuAc-cleaving activity remained in the retentate, with only 36% of the original activity passing through the filters. The ratio of MU-KDN-cleaving activity to MU-NeuAc-cleaving activity changed from approximately 1:2 in the water extract to 1:1 in the filtrate. This suggested that the two activities were catalysed by two separate enzymes and the molecular mass of the MU-KDN-cleaving activity was smaller than that of the MU-NeuAc-cleaving activity. We took advantage of this property to enrich the MU-KDN-cleaving activity in the Minitan 300 kDa cut-off filtrate.

By Rotofor IEF of the crude sialidase preparation, MU-KDN-cleaving activity was broadly focused between pH 4.5 and 8.8, while the peak of MU-NeuAc-cleaving activity was focused sharply at pH 4.8, which again suggested that the MU-KDN-cleaving activity in *Asterina* starfish was distinct from the MU-NeuAc-cleaving activity. The pI of the RS purified from the *Asterias rubens* starfish was reported to be 4.9 [11].

The significant difference in the pIs of the two *Asterina* sialidase activities prompted us to use anion-exchange chromatography to resolve these two activities. The crude sialidase preparation derived from 450 g of starfish entrails was dialysed against 20 mM sodium phosphate buffer, pH 7.6, and applied to

a DEAE-Fractogel column (5 × 30 cm) equilibrated with the same buffer. After washing to remove the unadsorbed protein, the column was eluted with a linear NaCl gradient from 0 to 0.5 M in the same buffer (total volume 1500 ml), at 5 ml/min and 25-ml fractions were collected. Under this condition, RS but not KS was retained by the column (Figure 1). The unadsorbed fractions which contained the MU-KDN-cleaving activity (Figure 1, fractions 14–28) were pooled and concentrated to 30 ml by ultrafiltration to obtain DEAE-purified KS. The main MU-NeuAc-cleaving activity eluted from the column by the NaCl gradient was pooled (Figure 1, fractions 137–151) and concentrated to 30 ml by ultrafiltration to obtain DEAE-purified RS.

Hydroxylapatite chromatography

The DEAE-purified KS was dialysed against 5 mM sodium phosphate buffer, pH 7.0, and applied to a Hyapatite C (hydroxylapatite) column (2.5 × 10 cm) equilibrated with the same buffer. The column was washed with the same buffer at 3.5 ml/min and 15-ml fractions were collected. Under these conditions, the KS was not retained by the column. Subsequent elution with 1.0 M MgCl₂, followed by 200 mM sodium phosphate buffer, pH 7.0, eluted proteins without activity. Unadsorbed fractions containing KS activity were pooled and concentrated to 12.2 ml by ultrafiltration to obtain the Hyapatite C-purified KS.

Under the same conditions, the DEAE-purified RS was initially retained by the Hyapatite C column and was eluted by 1.0 M MgCl₂, followed by 200 mM sodium phosphate buffer, pH 7.0. The activity eluted by MgCl₂ was pooled and concentrated to 7 ml by ultrafiltration to obtain Hyapatite C-purified RS.

Sephacryl S200 gel filtration

The Hyapatite C-purified KS was applied to a Sephacryl S200 column (5 × 100 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The column was eluted with the same buffer at 0.5 ml/min and 17-ml fractions were collected. The MU-KDN-cleaving activity was pooled and concentrated to 4.2 ml by ultrafiltration to obtain Sephacryl S200-purified KS.

The Hyapatite C-purified RS was applied to the same Sephacryl S200 column under the same conditions. The main RS activity peak was eluted earlier than the MU-KDN-cleaving activity, and, using a molecular-mass calibration kit, the position of this peak was found to correspond to a molecular mass of about 128 kDa. The peak was pooled and concentrated to 2 ml by ultrafiltration to obtain Sephacryl S200-purified RS.

FPLC SP-Fractogel chromatography

The Sephacryl S200-purified KS was dialysed against 50 mM sodium acetate buffer, pH 4.5, and applied to an FPLC column (HR 10/10, 1 × 10 cm) packed with SP-Fractogel equilibrated with the same buffer. After washing to remove the unadsorbed protein, the column was eluted with a linear NaCl gradient from 0 to 0.4 M in the same buffer (total volume 160 ml) at 1 ml/min and 5-ml fractions were collected. No activity was found in the unadsorbed fractions, whereas the main MU-KDN-cleaving activity peak, which was separated from the major protein peak, was eluted from the column by the NaCl gradient and was pooled and concentrated to 1 ml by ultrafiltration to obtain SP-purified KS. Since RS had an acidic pI, SP-Fractogel chromatography was not suitable for the purification of this enzyme.

Figure 1 DEAE-Fractogel chromatography of *Asterina pectinifera* starfish crude sialidase preparation

The crude sialidase preparation obtained from 450 g of *Asterina* starfish entrails was fractionated by DEAE-Fractogel chromatography according to the conditions described in the text. Dotted line, absorbance at 280 nm; ●, MU-KDN-cleaving activity; ○, MU-NeuAc-cleaving activity; broken line, NaCl gradient.

Figure 2 FPLC gel filtration of the SP-purified KS and the Sephacryl S200-purified RS

Top: SP-purified KS (200 μ l aliquots) was applied to two FPLC Superdex 75 HR 10/30 columns connected in tandem and equilibrated with 50 mM sodium acetate buffer, pH 4.5, containing 0.1 M NaCl. The columns were eluted with the same buffer according to the conditions described in the text. The peak with MU-KDN-cleaving activity, as indicated by the horizontal bar, was concentrated by ultrafiltration using a Centricon-10 microconcentrator to obtain Superdex 75-purified KS. Bottom: Sephacryl S200-purified RS was also further purified by FPLC using the same conditions. The peak with MU-NeuAc-cleaving activity, as indicated by the horizontal bar, was concentrated by ultrafiltration to obtain Superdex 75-purified RS. Dotted line, absorbance at 280 nm; ●, MU-KDN-cleaving activity; ○, MU-NeuAc-cleaving activity.

FPLC gel filtration

The SP-purified KS (5 runs, 200 μ l each) was applied to two FPLC Superdex 75 HR 10/30 (1 $\times$ 30 cm) columns connected in tandem and equilibrated with 50 mM sodium acetate buffer, pH 4.5, containing 0.1 M NaCl. The columns were eluted with the same buffer at 0.3 ml/min and 0.5-ml fractions were collected. As shown in Figure 2 (top), this step effectively separated the KS from the bulk of the contaminating proteins. The MU-KDN-cleaving activity for all runs, as indicated by the horizontal bar (Figure 2, top), was pooled and concentrated to 0.1 ml by ultrafiltration using a Centricon-10 microconcentrator to obtain Superdex 75-purified KS. The position of this activity corresponded to a molecular mass of 31 kDa.

The Sephacryl S200-purified RS was also further purified by FPLC gel filtration using the same conditions. Unlike the MU-KDN-cleaving activity, the MU-NeuAc-cleaving activity appeared in the void volume, as shown in Figure 2 (bottom), and fractions indicated by the horizontal bar were pooled and concentrated to 0.75 ml by Centricon-10 microconcentrators to obtain the Superdex 75-purified RS. The results obtained by

Figure 3 Mono P chromatofocusing of Superdex 75-purified KS and RS

Top: Superdex 75-purified KS was applied to a Mono P HR 5/20 column and chromatofocused over the pH range 8.7–6.2 using 25 mM Tris/acetic acid, pH 8.7, as the starting buffer and Polybuffer 96 adjusted to pH 6.2 using acetic acid as the running buffer. Peak I was concentrated using a Centricon-10 microconcentrator to obtain Mono P-purified KS. Bottom: Superdex 75-purified RS was applied to the same Mono P column and chromatofocused over the pH range 6.3–3.8. The starting buffer was 25 mM Bistris/HCl, pH 6.5, and Polybuffer 74 adjusted to pH 3.8 using HCl was the running buffer. Dotted line, absorbance at 280 nm; ●, MU-KDN-cleaving activity; ○, MU-NeuAc-cleaving activity; ▲, pH. The fractions indicated by the solid bar in the top panel were pooled and concentrated. Detailed experimental conditions are described in the text.

FPLC gel filtration of KS and RS are in agreement with the results obtained by Sephacryl S200 gel filtration.

Chromatofocusing

The Superdex 75-purified KS and RS were examined by FPLC chromatofocusing using a Mono P HR 5/20 (0.5 $\times$ 20 cm) column. Based on the pIs of these two enzymes, eluents with pH ranges of 8.7–6.2 for KS and 6.3–3.8 for RS were chosen for chromatofocusing. The starting buffer used for KS was 25 mM Tris/acetic acid, pH 8.7, and the running buffer was Polybuffer 96 adjusted to pH 6.2 using acetic acid as described in the Pharmacia manual. After applying the sample onto the Mono P column, the column was eluted with the running buffer at 0.5 ml/min and 0.5-ml fractions were collected. As shown in Figure 3 (top), the KDN-cleaving activity was eluted over a wide pH range (8.5–6.9), with one major peak (peak I) being focused at pH 8.3 and several minor peaks focused over a lower pH range. Since peak I was the major peak and was well separated from other proteins, it was concentrated to 0.15 ml using a

Table 1 Purification of KS and RS from 450 g of the entrails of *Asterina pectinifera*

Step	Total protein (mg)		Total activity (units)		Specific activity (units/mg)		Purification (-fold)		Yield (%)	
	KS	RS	KS	RS	KS	RS	KS	RS	KS	RS
Crude sialidase	7000	7000	12650	11184	1.8	1.6	1	1	100	100
DEAE-Fractogel	3200	1900	10276	5800	3.2	3.1	1.8	1.9	81	52
Hypatite C	1700	576	7521	6604	4.4	11.5	2.4	7.2	59	59
Sephacryl S200	449	111	6665	3693	14.8	33.4	8.2	21	53	33
SP-Fractogel*	51	—	4555	—	89	—	49	—	36	—
Superdex 75	3.9	0.98	3825	2132	981	2183	545	1364	30	19
Mono P (I)†	0.08	—	961	—	12164	—	6758	—	8	—

* SP-Fractogel column was not used for the purification of the RS. See Results and discussion section.

† Due to low yield, Mono P was not suitable for the further purification of RS. See Results and discussion section.

Centricon-10 microconcentrator to obtain Mono P-purified KS. This highly purified preparation was used for studying the properties of KS from *Asterina* starfish. The elution profile shown in Figure 3 (top) is in agreement with the results of Rotofor IEF which indicated the presence of multiple isoelectric forms of KS with pIs ranging from 4.6 to 8.8. Owing to the complexities of the activities eluted after the main peak, no further attempt was made to study the properties of these activities.

For comparison, the Superdex 75-purified RS was also analysed by Mono P chromatofocusing using the starting buffer, 25 mM Bistris/HCl, pH 6.5, and the running buffer, Polybuffer 74 adjusted to pH 3.8 using HCl. As shown in Figure 3 (bottom), the MU-NeuAc-cleaving activity was eluted around pH 4.2. This again indicates that the MU-NeuAc-cleaving activity is distinct from the MU-KDN-cleaving activity. In contrast to KS, less than 10% of the RS activity applied to the Mono P column could be recovered. Therefore, this step was not suitable for further purification of RS and Superdex 75-purified RS and the Mono P-purified KS were used to compare the properties of the two sialidases.

Characterization of KS and RS

General properties of the two sialidases

Using the procedures described above, the KS obtained after the Mono P column was purified 6758-fold with 8% recovery. Table 1 summarizes the steps used to prepare KS and RS from the entrails of the *Asterina* starfish. By PhastSystem IEF, the Mono P-purified KS gave three major protein bands, as revealed by silver stain (Figure 4). Using an agarose overlay containing MU-KDN [23], the protein band focused at pH 8.3 was found to contain the MU-KDN-cleaving activity. This band also exhibited weak activity toward the hydrolysis of MU-NeuAc. Using a similar purification scheme, with the exception of the SP-Fractogel and Mono P chromatography, RS was purified 1364-fold from the crude sialidase preparation (Table 1). The RS preparation still contained several contaminating protein bands when examined by native PAGE and IEF. The molecular mass for KS using Superdex 75 gel filtration was estimated to be 31 kDa. However, under the same condition, RS was eluted in the void volume, confirming our observation made by Minitan ultrafiltration that the molecular mass of RS is larger than that of KS. By Sephacryl S200 gel filtration, the molecular mass of RS was estimated to be 128 kDa. Both Mono P-purified KS and Superdex 75-purified RS were completely free from other exoglyco-

Figure 4 PhastGel IEF of Mono P-purified KS

pI calibration standards (lane 1) and KS purified by the Mono P column (lane 2) were analysed by PhastGel IEF (pH 3–9) according to the conditions described by the manufacturer. Protein bands were visualized by silver staining. The arrow shows the position of MU-KDN-cleaving activity.

sidase activities using *p*-nitrophenyl-glycosides as substrates. They were also free from protease activity using Azocoll as the substrate.

Using MU-KDN or MU-NeuAc in 50 mM sodium acetate buffer, KS and the RS exhibited maximal activity at pH 4.0. This value is very similar to the optimal pH reported for RS from the *Asterias rubens* starfish (pH = 4.2) [11]. The KS of loach liver showed maximal activity at pH 4.5 [8], while the activity reported in the crude extract from rainbow trout ovaries exhibited maximal activity at pH 4.4 [9]. The KDNase activity partially purified from *S. multivorum* had maximal activity over the pH range 5.7–6.0 [10]. The K_m values of *Asterina pectinifera* starfish KS and RS, using MU-KDN and MU-NeuAc respectively as

Table 2 Properties of KS and RS

Detailed experimental procedures are described in the text.

Substrate	KS		RS	
	K_m (mM)	Relative rate* (%)	K_m (mM)	Relative rate* (%)
MU-KDN	0.05	100	0.42	1.1
MU-NeuAc	0.19	5	0.06	100
3'-KDN-lactose	2.8	11	—†	—
6'-KDN-lactose	11	5	—	—
3'-Sialyllactose	—	—	1.8	42
6'-Sialyllactose	—	—	1.8	28

* The relative rates of hydrolysis were determined by incubating 1 unit of KS or RS with 0.5 mM MU substrate or 5 mM oligosaccharide substrate at pH 4.0 and 37 °C. The amount of substrate hydrolysed was determined using the fluorimetric methods described in the text. The rates shown are the percentage of the rate of hydrolysis of MU-KDN by KS, or of MU-NeuAc by RS.

† Not determined due to the very low rate of hydrolysis.

substrates, were 0.05 mM and 0.06 mM (see Table 2). These values are similar to those of the KS from loach (0.07 mM and 0.12 mM, using MU-KDN and MU-NeuAc). The K_m of MU-NeuAc for RS isolated from the starfish *Asteria rubens* was reported to be 0.2 mM [11]. Using MU-KDN, the K_m for the KDN-cleaving activity in rainbow trout ovaries was 0.53 mM [9], while the K_m for *S. multivorum* KDNase was determined to be 0.019 mM [10]. It should be pointed out that the K_m values for the hydrolysis of MU-KDN were quite different for *Asterina* KS and RS (0.05 mM versus 0.42 mM), and the K_m for the hydrolysis of MU-NeuAc by KS and RS were also different (0.19 mM versus 0.06 mM) (Table 2). These results are consistent with the fact that KS and RS are distinct from each other in their recognition of these two substrates.

Mono P-purified KS was found to exhibit weak activity toward MU-NeuAc. Similarly, Superdex 75-purified RS also slowly hydrolysed MU-KDN. The ratio of MU-KDN- to MU-NeuAc-cleaving activities of Mono P-purified KS was found to be 20:1. Thus, the specificity of *Asterina* starfish KS is quite different from loach KS [8], which cleaved MU-KDN only slightly faster than MU-NeuAc, and the activities reported for trout tissues [9], which at best cleaved MU-KDN less than one tenth as fast as MU-NeuAc. The ratio of MU-NeuAc- to MU-KDN-cleaving activities of Superdex 75-purified RS was 88:1. For comparison, we found that the ratio of MU-NeuAc- to MU-KDN-cleaving activities of *Clostridium perfringens* and *Arthrobacter ureafaciens* sialidases were 350:1 and 60:1 respectively.

Asterina KS and RS were not greatly affected by Mg^{2+} , Ca^{2+} , Mn^{2+} or Co^{2+} at concentrations of up to 10 mM. EDTA at a concentration of 1 mM was found to inhibit both KS and the RS by 20%. Increasing NaCl concentrations were found to decrease the activity of both sialidases, with 1.0 M NaCl causing a reduction of 20 and 30% in the activity of KS and RS respectively. The effect of Cu^{2+} on KS and RS activities was also tested. Unlike some sialidases, KS was not inhibited by up to 10 mM Cu^{2+} , while RS was inhibited by 20% at this concentration. Similar to the report for RS of *Asterias rubens* starfish [11], Hg^{2+} was a potent non-competitive inhibitor of *Asterina* RS, reducing activity 50% at a concentration of 2 μ M. The MU-KDN-cleaving activity associated with this RS was also affected by Hg^{2+} to the same extent. This may indicate the presence of an essential SH group in RS. In contrast, KS from *Asterina* starfish

Figure 5 Hydrolysis of MU-KDN and MU-NeuAc by KS and RS

Top: The hydrolysis of MU-KDN and MU-NeuAc by KS. Middle: The hydrolysis of the same substrates by RS. ●, MU-KDN-cleaving activity; ○, MU-NeuAc-cleaving activity. Bottom: TLC showing the hydrolysis of MU-KDN (MK) and MU-NeuAc (MN) by *Asterina* starfish KS and RS. MK or MN (30 nmol in 30 μ l of reaction mixture) was incubated with 1 unit of KS or RS at 37 °C for 1 h. Lanes: 1, KS; 2, RS; 3, MK; 4, KDN; 5, MK + KS; 6, MK + RS; 7, MN + KS; 8, MN + RS; 9, NeuAc; 10, MN. Detailed experimental conditions are described in the text.

was found to be much less affected by Hg^{2+} , being inhibited only 15% at a concentration of 100 μ M. Similarly, the MU-NeuAc-cleaving activity associated with this KS was also not greatly affected by Hg^{2+} . These results indicate that the KDN-cleaving activity of RS and the NeuAc-cleaving activity of KS are the intrinsic activities of these two sialidases. Thus, the glycon specificities of *Asterina* starfish KS and RS are not absolute: KS can slowly hydrolyse the α -ketosides of NeuAc and the RS can also hydrolyse the α -ketosides of KDN at a very slow rate. The chemical structures of the two substrates differ only in the presence of an acetamido group at the C-5 position of NeuAc, which is substituted by a hydroxy group in KDN. This subtle difference must have a profound effect on the susceptibility of NeuAc- and KDN-containing glycoconjugates to RS and KS. In our survey of bacterial sialidases for KDN-cleaving activity [8], we noted that these sialidases contained a very weak activity towards MU-KDN. At high enzyme concentrations, the bacterial sialidases can also slowly hydrolyse the KDN-substrates [8].

Hydrolysis of MU-KDN and MU-NeuAc by KS and RS

Using time-course analysis, KS was found to hydrolyse MU-KDN at a much faster rate than MU-NeuAc (Figure 5, top). RS,

Figure 6 TLC showing the hydrolysis of 3'-KDN- G_{M3} , 6'-KDN- G_{M3} and G_{M3} (top), and 6'-KDN-GalNAc-ol, 3',6'-di-KDN-GalNAc-ol and 6'-KDN(3'-GlcNAc)GalNAc-ol (bottom) by KS and RS

Top: KDN-containing glycolipids (10 nmol) were incubated with 1 unit of KS or RS at 37 °C for 1 h. Lanes: 1, lactosylceramide; 2, 3'-KDN- G_{M3} ; 3, 6'-KDN- G_{M3} ; 4, KDN; 5, 3'-KDN- G_{M3} + KS; 6, 3'-KDN- G_{M3} + RS; 7, 6'-KDN- G_{M3} + KS; 8, 6'-KDN- G_{M3} + RS; 9, G_{M3} + KS; 10, G_{M3} + RS; 11, NeuAc; 12, G_{M3} ; 13, lactosylceramide; 14, KS; 15, RS. Bottom: KDN-containing acidic oligosaccharide alditols (30 nmol) were incubated with 1 unit of KS or RS at 37 °C for 1 h. Lanes: 1, KS; 2, RS; 3, 6'-KDN-GalNAc-ol; 4, 6'-KDN-GalNAc-ol + KS; 5, 6'-KDN-GalNAc-ol + RS; 6, KDN; 7, 3',6'-di-KDN-GalNAc-ol; 8, 3',6'-di-KDN-GalNAc-ol + KS; 9, 3',6'-di-KDN-GalNAc-ol + RS; 10, 6'-KDN(3'-GlcNAc)GalNAc-ol; 11, 6'-KDN(3'-GlcNAc)GalNAc-ol + KS; 12, 6'-KDN(3'-GlcNAc)GalNAc-ol + RS. The plates were developed with butan-1-ol/acetic acid/water (2:1:1, by vol.) and stained with diphenylamine reagent. The detailed assay conditions are described in the text.

on the other hand, hydrolysed MU-NeuAc at a much faster rate than MU-KDN (Figure 5, middle). The hydrolysis of MU-KDN and MU-NeuAc by the two sialidases was also analysed by TLC. As shown in Figure 5 (bottom), KS efficiently released KDN from MU-KDN but not NeuAc from MU-NeuAc. The reverse specificity was seen for the RS under the same conditions.

Hydrolysis of sialoglycoconjugates by KS and RS

As shown in Figure 6 (top), the two KDN-containing glycosphingolipids, 3'-KDN- G_{M3} and 6'-KDN- G_{M3} , were effectively hydrolysed by Mono P-purified KS, but were refractory to Superdex 75-purified RS under the same conditions. KS could also hydrolyse NeuAc-containing G_{M3} , but at a much slower rate. Equal units of RS released NeuAc from G_{M3} , but not KDN from either 3'-KDN- G_{M3} or 6'-KDN- G_{M3} . We found that the fluorimetric assay for free sialic acids [18] could also be used as a sensitive method to detect the released KDN. This assay was used to study the hydrolysis of oligosaccharides by KS and RS. The oligosaccharides 3'-KDN-lactose and 6'-KDN-lactose were found to be refractory to RS, but were readily hydrolysed by KS, while 3'-sialyllactose and 6'-sialyllactose were refractory to KS, but were hydrolysed by RS (results not shown). Table 2 shows the Michaelis-Menten constants obtained for these substrates with the two sialidases. Figure 6 (bottom) shows that Mono P-purified KS also hydrolysed three KDN-containing oligo-

Table 3 Effect of NeuAc2en and KDN2en on the hydrolysis of MU-KDN and MU-NeuAc by KS and RS

Experimental details are described in the text.

Inhibitor	K_i (mM)	
	KS	RS
NeuAc2en	0.04	0.0073
KDN2en	0.04	No effect

saccharide alditols, 6'-KDN-GalNAc-ol, 3',6'-di-KDN-GalNAc-ol and 6'-KDN(3'-GlcNAc)GalNAc-ol. However, these three KDN-glycoconjugates were refractory to Superdex 75-purified RS. The middle band in Figure 6 (bottom), lane 8, is the intermediate product [KDN α 2 $\rightarrow$ 3N-acetylgalactosaminitol (3'-KDN-GalNAc-ol) or 6'-KDN-GalNAc-ol], since prolonged incubation resulted in the disappearance of this band with a concomitant increase in KDN.

Effect of NeuAc2en and KDN2en on KS and RS

A well-known competitive inhibitor of regular sialidases, NeuAc2en, and its KDN analogue, KDN2en, were tested for their effects on the hydrolysis of MU-NeuAc and MU-KDN by KS and RS (Table 3). NeuAc2en was found to be an effective inhibitor ($K_i \sim 0.007$ mM) of RS, while it only had a moderate effect on KS ($K_i \sim 0.04$ mM). When KDN2en was tested, we found that it inhibited KS ($K_i \sim 0.04$ mM) equally well toward the hydrolysis of both substrates, but did not affect RS. These results provide another line of evidence showing that KS is distinct from RS.

Using synthetic and naturally derived KDN- and NeuAc-containing substrates, we have conclusively demonstrated the coexistence of two different sialidases in the starfish *Asterina pectinifera*. One is a regular NeuAc-cleaving sialidase, and the other, a KDN-cleaving sialidase. We were able to resolve these two enzymes based on their charge properties (Figure 1) and molecular sizes (Figure 2). Using ultrafiltration, KS activity was enriched in the filtrate while a large amount of protein and the majority of the RS remained in the retentate. As RS has been previously reported in *Asterias rubens* starfish [11], we directed our efforts to separate KS from RS in *Asterina pectinifera* starfish and characterize their properties.

The properties of *Asterina pectinifera* RS are similar to RS purified from another species of starfish, *Asterias rubens*, by Schauer and Wember [11]. These two sialidases have similar pIs, pH optima and susceptibilities to inhibition by Hg^{2+} . Unlike some sialidases [24–26], neither enzyme requires Ca^{2+} , Mg^{2+} or Mn^{2+} for full activity. On the other hand, KS from *Asterina pectinifera* differs greatly from RS isolated from both *Asterina pectinifera* and *Asterias rubens* with respect to molecular mass, pI, susceptibility to Hg^{2+} , inhibition by NeuAc2en and KDN2en and specificities toward sialoglycoconjugates.

The occurrence of KS was first reported in the liver of the loach, *Misgurnus fossilis* [8]. More recently, an extremely weak KDN-cleaving activity reported in the tissues of rainbow trout was suggested to be an intrinsic part of RS [9], as seen for bacterial sialidases [8]. A KDN-cleaving activity has also been induced in the bacterium *S. multivorum* using KDN-oligosaccharide alditols as the sole carbon source [10]. However, this activity was not completely separated from other exoglycosidases and has a low specific activity. The KS of *Asterina*

pectinifera starfish has a much higher specific activity and a marked preference toward the hydrolysis of KDN-containing glycoconjugates over NeuAc-containing glycoconjugates. Thus, this enzyme is apparently different from the 'KDNase' isolated from *S. multivorum* which cleaves exclusively KDN-glycoconjugates but not NeuAc-glycoconjugates [10].

An important question that remains to be answered is why starfish should contain KDN-sialidase. We have not detected KDN in the tissues of starfish, but we have preliminary evidence showing the presence of KDN in the plankton of the coastal waters of the Gulf of Mexico (J. A. Yuziuk, H. Nakagawa and Y.-T. Li, unpublished work). This suggests that KDN is present in the marine environment of starfish. The co-existence of KS and RS in *Asterina pectinifera* indicates the presence of a complex sialic acid catabolic system in the animal kingdom. Besides starfish, KS may coexist with RS elsewhere in nature and may have a distinct biological role.

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