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Identification and Characterization of Cathepsin D in a Highly Purified Sialidase from Starfish *A. pectinifera*

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A sialidase [EC 3.2.1.18] from the ovary of starfish *Asterina pectinifera* was isolated and highly purified by preparative PAGE. The SDS–PAGE separation of the purified enzyme revealed two natures of protein bands, upper (50 kDa) and a lower (47 kDa). To identify the protein, N-terminal amino acid sequence of the upper band was done. The sequence matched with the N-terminal amino acid sequence of human lysosomal mature cathepsin D and cathepsin D activity was also found in all the preparation steps. Protease inhibitor pepstatin A inhibited the proteolysis activity of cathepsin D against a synthetic substrate. The two enzymes sialidase and cathepsin D were separated from each other by using high-performance gel-filtration chromatography. The Western blot analysis and isoelectric focusing showed the co-purified cathepsin D is a 50 kDa protein with a PI value of 4.2.

Key words: Asterina pectinifera, cathepsin D, enzymatic properties, sialidase, ovary of starfish.

Abbreviations: 4MU-NeuAc, 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid; HPGFC, high-performance gel-filtration chromatography; IPG, immobilized pH gradient; Neu I, neuraminidase I; PPCA, protective protein/cathepsin A; PVDF, polyvinylidine difluoride; β-Gal, beta-galactosidase; TBS, tris-buffered saline.

Sialidase (neuraminidase) [EC 3.2.1.18] which catalyses the hydrolysis of terminal sialic acid residues of oligosaccharides, glycoproteins and glycolipids, takes part in the metabolism of sialoglycoconjugates (*1*). Sialidases are widely distributed in viruses, bacteria, fungi, mycoplasma and protozoa as well as in avian and mammalian species (*2*). In microorganisms, sialidases believed to be involved in pathogenesis (*3*) and nutrition (*4*) and they were purified to homogeneity and characterized for their properties and structures. In mammals sialidases are of four types namely neuraminidase I (Neu I), neuraminidase II (Neu II), neuraminidase III (Neu III) and neuraminidase IV (Neu IV) existing in lysosomal membrane, cytoplasm, plasma membrane and lumen of lysosome, respectively (*5–8*). Mammalian sialidases are involved in modulating cellular events such as activation, differentiation, maturation and growth (*9–11*).

The deficiency diseases of Neu I are of two forms; sialidosis and galactosialidosis. The former is due to the mutation of *NEU I* gene (*12*) and the later is due to the deficiency of the protective protein/cathepsin A (PPCA) (*13*). Neu I exist as multi-enzyme complex with beta-galactosidase (β-Gal) and PPCA. The enzymatic activity of Neu I and β-Gal is stabilized and protected against other proteases due to the interaction with PPCA (*14*, *15*). So when there is a mutation in PPCA gene or deficiency of PPCA, the complex formation is hindered and thus the undefended enzymes lose their activities to other proteases resulting in galactosialidosis disease.

Since Neu I forms complex with β-Gal and PPCA, in our earlier studies we used a *p*-aminophenyl thio-β-D-galactoside-CH-sepharose (PATG-sepharose), an affinity column for β-Gal, for purification of the enzyme complex and characterized it from human placenta (*16*). The purified sample gave five protein bands 78, 64, 46, 32 and 20 kDa in 10% SDS–PAGE. Although the protein band of sialidase was not confirmed, others were identified as immunoglobulin M heavy chain (78 kDa) (*12*), β-Gal (64 kDa) (*11*), α-*N*-acetylgalactoseaminidase (46 kDa) (*14*) and subunits of PPCA (32 and 20 kDa) (*14*, *17*, *18*). Other than PPCA, there are no literatures available stating the co-purification of a protease enzyme with sialidase. Previously, we purified a sialidase from starfish, *Asterina pectinifera*, and compared the enzymatic properties with those of human placental sialidase (*19*). On SDS–PAGE, the purified preparation generously gave two protein bands; an upper (50 kDa) and a lower (47 kDa) bands. Here we report the identification and characterization of the upper band.

MATERIALS AND METHODS

Materials—Pepstatin A, antipain, chymostatin, leupeptin, E-64 and MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 were purchased from Peptide Institute, Inc. (Osaka, Japan). Goat anti-mouse

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recombinant cathepsin D antibody was obtained from Neuromics Antibodies Inc. (Northfield, MN, USA) and rabbit anti-goat IgG-HRP from Acris Antibodies GmbH (Himmelreich, Germany). Chemiluminescence Western blotting detection reagent (ECL Plus) was purchased from GE Healthcare Bio-sciences Corp. (Piscataway, NJ, USA). Pepstatin A-Agarose, bicinchoninic acid and bovine spleen cathepsin D were from Sigma-Aldrich Corp. (St Louis MO, USA) and IPG strip from Bio-Rad laboratories (Hercules CA, USA). 4-Methylumbelliferyl-α-D-*N*-acetylneuraminic acid (4MU-NeuAc) was synthesized in our laboratory. All other chemicals used in the experiments were of analytical grade.

Purification of Sialidase—Sialidase from the homogenate of starfish ovary was purified by ammonium sulphate precipitation and octyl sepharose, Con-A sepharose, CM-sephadex columns sequentially. Finally it was purified by preparative gel electrophoresis and procedure for all the steps were previously described (*19*).

Gel Electrophoresis—Fifty nanograms of proteins were separated on 10% normal PAGE and 12.5% SDS–PAGE by procedure described by Laemmli (*20*) (for normal PAGE, SDS was withheld in every step). One hundred nanograms of protein was separated according to their net charge on a 7 cm immobilized pH gradient (IPG) strip pH 3 to 10 by using *PROTEAN IEF Cell* (Bio-Rad) as stated by the manufacturer. Then the strip was equilibrated with 3 ml each of buffer I and II for 10 min successively [urea (6 M), SDS (2%), Tris/HCl pH 8.8 (0.375 M), glycerol (20%) containing DTT (130 mM) (buffer I) or iodoacetamide (135 mM) and bromophenol blue (0.001%) (buffer II)]. Carefully the strip was placed over a 12.5% SDS–PAGE, sealed by 1% agarose and separated by the above method. The proteins separated on the gels were visualized by Daiichi silver staining kit (Daiichi Pure Chemicals Co., Ltd Tokyo, Japan).

N-terminal Amino Acid Sequencing—Five micrograms of proteins were separated by the above method on a 12.5% SDS–PAGE and transferred to the polyvinylidine difluoride (PVDF) membrane. Proteins were identified by Amido black staining [amido black (0.1%), methanol (40%), acetic acid (10%)]. The 50 kDa band was excised, destained [methanol (50%) and acetic acid (5%)] and the N-terminal amino acid sequence was analysed by PSQ-1 protein sequencer (Shimadzu).

High-Performance Gel-Filtration Chromatography—High-performance gel-filtration chromatography (HPGFC) column Shodex KW403-4F (Showa Denko K.K. Chemicals Division, Kanagawa, Japan) was equilibrated with 200 mM sodium acetate buffer pH 4.2 at a flow rate of 0.3 ml/min and the same condition was maintained throughout the separation. Twenty-five micrograms of CM-sephadex purified sample was injected and the protein separation pattern was monitored at 280 nm. Fractions of 70 μl (every 15 s) from the 5th minute were collected and tested for sialidase and cathepsin D activities as described later.

Western Blot Detection—Five hundred nanograms protein of CM-sephadex purified fraction, each 75 ng protein of HPGFC purified cathepsin D and sialidase fractions were separated on a 12.5% SDS–PAGE by the previously described method. Then the proteins were blotted on PVDF membrane using an electroblot apparatus (ATTO, Model AE-6677). The membrane was exposed to goat anti-mouse recombinant cathepsin D antibody (Neuromics Antibodies) (1:10,000) in Tween tris-buffered saline (TBS) after blocking with 5% non-fat milk, and then further exposed to the secondary antibody, rabbit anti-Goat IgG-HRP (Acris Antibodies) (1:20,000), in Tween TBS. Immunodetection was performed with an ECL Plus Western blotting detection reagents (Amersham Biosciences).

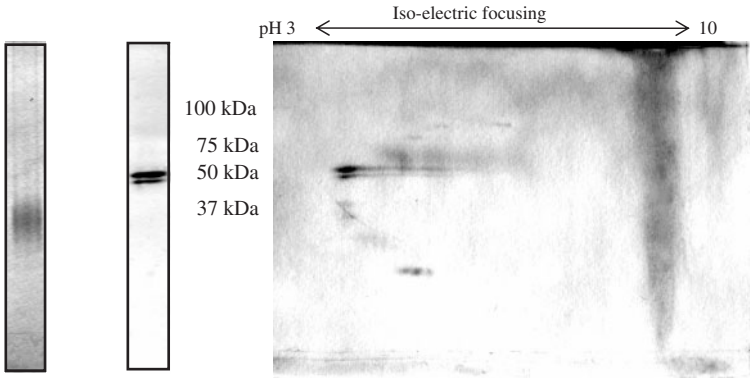
Pepstatinyl Column Chromatography—The CM-sephadex purified sample (30 μg) was loaded to pepstatinyl agarose column ($0.6 \times 5.8\,cm^2$) pre-equilibrated with 200 ml of 50 mM sodium acetate (pH 3.5) buffer containing 200 mM sodium chloride. The column was left over night in 4°C for the enzymes to be adsorbed. The column was washed with 200 ml of same buffer and eluted with 50 ml of 50 mM Tris–HCl (pH 8.5) buffer containing 200 mM sodium chloride. Two millilitres fractions were collected and tested for cathepsin D and sialidase activities by the below said procedures.

Enzyme Assays—Sialidase activity towards the synthetic substrate 4MU-NeuAc was determined according to the literature (*21*). One unit of enzyme is defined as the amount of enzyme which catalysed the release of 1 nmol of sialic acid per minute. Cathepsin D activity was determined towards the synthetic substrate MOCAc-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 as stated in the literature (*22*). For inhibitory studies stock solutions of pepstatin A, antipain, chymostatin, leupeptin and E-64 were added to the enzymes to get a final concentration of 1, 100, 200, 10 and 10 μM, respectively. The inhibitors were allowed to bind to the proteins for 5 min at 4°C prior adding the substrates. The residual sialidase and cathepsin D activities were determined by the previous procedures.

Protein Analysis—Protein concentrations were determined by the bicinchoninic acid assay (*23*) using BSA as a standard.

RESULTS AND DISCUSSION

The sialidase purified by preparative PAGE gave a broad band in the 10% normal PAGE (Fig. 1a) but in 12.5% SDS–PAGE two bands, upper (50 kDa) and a lower (47 kDa), were observed (Fig. 1b). To further investigate the nature of the proteins, the same preparation was analysed by two-dimensional gel electrophoresis (2D-PAGE). For the first dimension, isoelectric focusing, IPG strip pH 3–10 and for the second dimension 12.5% SDS–PAGE were used. Proteins were visualized by silver staining. The 2D-PAGE (Fig. 1c) revealed both the protein has same PI value of about 4.2. In order to identify the proteins, the upper band was subjected to N-terminal amino acid sequencing as stated in the materials and methods. The N-terminal amino acid sequence (Fig. 1d) matched with human lysosomal matured cathepsin D [EC 3.4.23.5] and also a lesser extent to human cathepsin E. We repeated the sequencing experiments several times and found, sometimes, some of the amino acids (underlined) are replaced by



d

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Starfish	-	G*	P*	V	P*	E*	V	L*	H	Y	Y*	Y	D*	A*	E	Y*	
	-	A			A	K	P		P	N*	I	A			Q	L	
Aspartic protease	-	G	P	V	P	E	P	L	S	N	Y	L	D	A	Q	Y	
Mature cathepsin D	-	65 G	P	I	P	E	V	L	K	N	Y	M	D	A	Q	Y	79
Cathepsin E	-	Q	S	A	K	E	P	L	I	N	Y	L	D	M	E	Y	

Fig. 1. **Protein-separation pattern on normal PAGE, SDS–PAGE and 2D SDS–PAGE and N-terminal sequence alignment.** Fifty nanograms protein of preparative PAGE-purified sample separated on a 10% normal PAGE (a) and 12.5% SDS–PAGE (b). One hundred nanograms protein of preparative PAGE-purified sample separated by isoelectric focusing on IPG strip pH 3–10 and 12.5% SDS–PAGE for the second dimension (c). Proteins were visualized by silver staining. The N-terminal sequence of the upper band (SDS–PAGE) was aligned with human aspartyl protease, human cathepsin D and human cathepsin E (d). The amino acids matched with cathepsin D are identified by asterisk (*). Upon repetition of the sequencing experiments, sometimes some of the amino acids (_) are replaced by other amino acids (2nd row).

Table 1. **Cathepsin D activity in the different purification steps of sialidase from the ovary of starfish, *A.pectinifera*.**

	Sialidase		Cathepsin D		Sialidase activity
Steps	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Cathepsin D activity
$(NH_4)_2SO_4$ precipitation	1350	1.5	1400	1.55	0.97
Octyl-Sepharose	950	16.8	350	6.2	2.7
Con-A Sepharose	190	42	43.4	9.6	4.4
CM-Sephadex	94.5	210	9.72	21.6	9.7
Preparative PAGE[a]	31.5	15750	2.3	1150	13.7
Preparative PAGE[b]	15	7500	1.25	625	12.0

[a]A gel concentration of 7% and 4 cm height was used.
[b]A gel concentration of 10% and 7 cm height was used. Equivalent amount of different CM-sephadex samples were used for both of the preparative PAGE (a & b) purification.

other amino acids (2nd row). It seems to be a possible contamination by another same molecular weight protein.

The enzyme preparations were tested for sialidase and cathepsin D activities against synthetic substrates. All the fractions, $(NH_4)_2SO_4$ precipitate, octyl-sepharose, Con-A sepharose, CM-sephadex including the final preparative PAGE purified one, have the cathepsin D activity (Table 1). It should be noted that both the enzymes are purified in all the purification steps with the significant increase in their specific activities. It implies that all the purification steps enhance the purification of both the enzymes. So the protease cathepsin D might not have co-purified as an impurity but intentionally in sialidase preparation.

We attempt to separate these two enzymes from each other in the final preparative PAGE method. Previously we used a gel concentration of 7.5% and height of 4 cm which is good for purifying 50 kDa proteins according to the manufacturer. Since this condition did not separate these two enzymes completely, we increased the concentration of acrylamide to 10% and height of the gel to 7 cm. However the two enzyme activities could not be separated completely (Table 1). The ratio of sialidase and cathepsin D activities of the preparations from 7.5 and 10% gel did not change significantly. Preparative

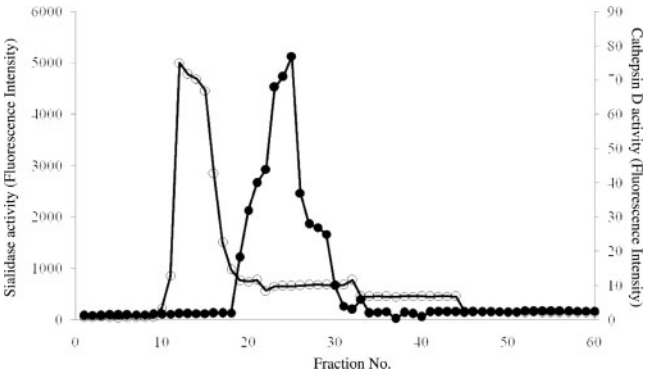


Fig. 2. **HPGFC separation of starfish sialidase and cathepsin D.** Twenty-five micrograms protein of CM-sephadex-purified preparation was separated by HPGFC using Shodex KW403-4F gel-filtration column previously equilibrated with 200 mM sodium acetate buffer (pH 4.2) at a flow rate of 0.3 ml/min. Fractions of 70 µl were collected and tested for enzyme activities. Open circle-sialidase activity, filled circle—cathepsin D activity.

PAGE has been used successfully to separate proteins with few kilo Dalton differences (*24*) and hence the difficulty in separation may be due to very similar molecular sizes and net charges (PI values) of these two proteins. So we employed a HPGFC method for the purification. CM-sephadex purified preparation was injected to Shodex KW403-4F column previously equilibrated with 200 mM sodium acetate buffer pH 4.2 and eluted with the same buffer. Sialidase and cathepsin D activities could be separated by HPGFC as shown in the Fig. 2.

The HPLC fractions showing sialidase and cathepsin D activities were pooled and concentrated by Microcon Ultracel YM-10 centrifugal filter devices (Millipore). Then the fractions were analysed by Western blot by using anti mouse cathepsin D antibody as primary antibody and HRP conjugated anti goat IgG as the secondary antibody. The antibody bound proteins were visualized by chemiluminescence (Fig. 3). The anti mouse cathepsin D antibody bound mainly to a 50 kDa protein, and also slightly to proteins over 75 kDa which seems to be non-specific binding of CM-sephadex purified sample (lane 1). The antibody bound to the same 50 kDa protein of HPGFC purified cathepsin D fraction (lane 2) and not to any of the protein bands of sialidase fraction (lane 3). This implies that the 50 kDa protein has specific affinity to cathepsin D antibody and sialidase fraction is free from cathepsin D.

The effect of various protease inhibitors upon starfish cathepsin D, starfish sialidase and bovine spleen cathepsin D were conducted. The protease inhibitors pepstatin A (1 µM), antipain (100 µM), chymostatin (200 µM), leupeptin (10 µM) and E-64 (10 µM) were added to the enzyme preparations and the residual activities were assayed. Among the inhibitors tested pepstatin A, a well-known cathepsin D inhibitor, showed highest inhibitory effect of about 99% in a very low concentration against starfish and bovine cathepsin D (Fig. 4). The sialidase activity was not disturbed upon inhibiting cathepsin D activity.

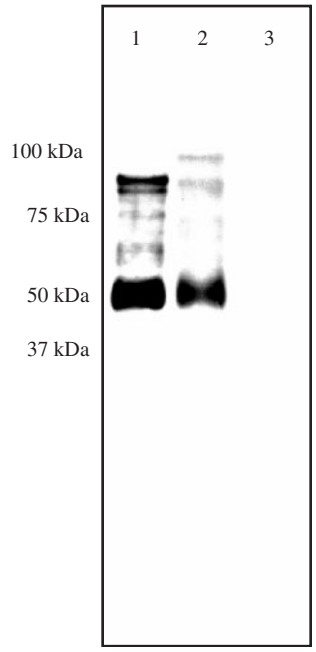


Fig. 3. **Western blot detection of cathepsin D.** Five hundred nanograms protein of CM-sephadex purified fraction (lane 1), each 75 ng protein of HPGFC-purified cathepsin D (lane 2) and sialidase (lane 3) fractions separated on 12.5% SDS–PAGE and transferred to PVDF membrane were probed with anti mouse cathepsin D antibody and detected by chemiluminescence.

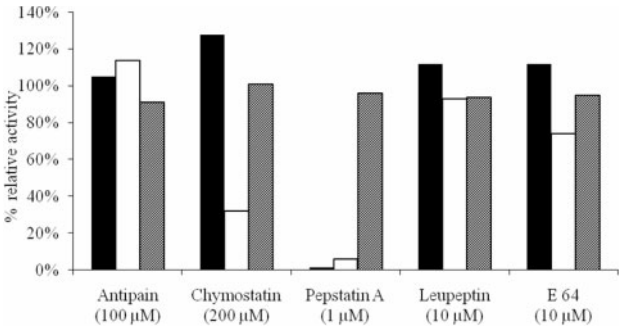


Fig. 4. **Comparative profile of the inhibitory effects of protease inhibitors upon bovine spleen cathepsin D (filled square), starfish cathepsin D (open square) and starfish sialidase (line-filled square).** CM-sephadex-purified starfish enzymes and commercial bovine spleen cathepsin D were used.

From the amino acid sequence homology, Western blot and inhibitory studies, we understood that starfish cathepsin D is similar to mammalian cathepsin D in means of structure and catalytic activity. D'Agrosa, R. M., and Callahan, J. W. reported that human lysosomal sialidase lost its activity immediately when exposed to cathepsin D, a well-known largest lysosomal protease which actively involves in catabolism of waste proteins (*25*). But the 12,287-fold purified starfish sialidase preparation was not bothered by cathepsin D. This shows that starfish sialidase has some unique character when compared with that of human source.

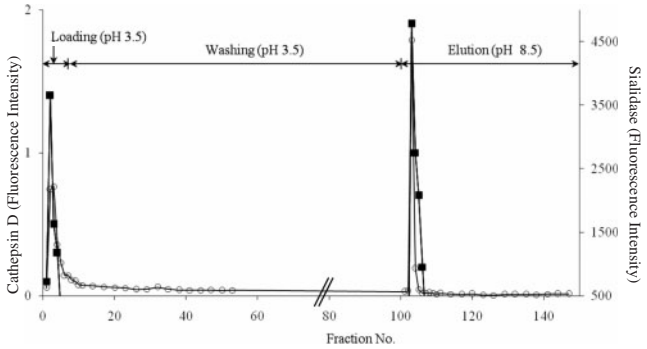


Fig. 5. **Cathepsin D and sialidase activity in pepstatinyl column chromatography.** The CM-sephadex purified sample (30 μg) was loaded to pepstatinyl agarose column ($0.6 \times 5.8\,cm^2$) pre-equilibrated with 50 mM sodium acetate (pH 3.5), 200 mM sodium chloride buffer. The column was washed with same buffer and 2 ml fractions were eluted with 50 mM Tris–HCl (pH 8.5), 200 mM sodium chloride buffer. Open circle—sialidase activity, filled square—cathepsin D activity.

To elucidate the interaction of sialidase and cathepsin D, we tried to separate sialidase from cathepsin D by using cathepsin D affinity column (pepstatinyl column). After loading the column with CM-sephadex purified fraction it was left overnight at 4°C for the enzymes to be adsorbed. The column was washed with 30 volumes of starting buffer to make sure all unbound are washed away from the column. Then the column was eluted and the fractions were tested for enzyme activities. As shown in the Fig. 5 fractions with cathepsin D activity also contained sialidase activity. Pepstatinyl column has been used to purify cathepsin D from various sources (*26–28*). But the pepstatinyl column purified starfish cathepsin D preparation also contained sialidase activity. This result suggests that there could be a possible interaction between these two enzymes as like that of mammalian Neu I and PPCA. And this interaction may be weaker since the enzymes are separated completely by HPGFC where as the mammalian enzymes can be separated partially (*29*). With further evidences for this interaction and domains of the starfish sialidase which are impervious to aspartic proteases will help constructing new recombinants as enzyme replacement therapy for the human galactosialidosis and sialidosis diseases.

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