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The *Pasteurella haemolytica O*-Sialoglycoprotein Endopeptidase Is Inhibited by Zinc Ions and Does Not Cleave Fetuin

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Culture supernatants of *Pasteurella haemolytica* A1 contain an *O*-sialoglycoprotein endopeptidase that cleaves human glycophorin A. This enzyme is inhibited by micromolar concentrations of Zn^{2+}. It can be separated from a neuraminidase activity in culture supernatants by ion-exchange chromatography. The neuraminidase activity can cause the de-sialation of the bovine soluble sialoglycoprotein, fetuin. However fetuin is not cleaved proteolytically either by culture supernatants from *P. haemolytica* A1 or by chromatographically purified *O*-sialoglycoprotein endopeptidase or neuraminidase.

An *O*-sialoglycoprotein endopeptidase (E.C. 3.4.24.57) (glycoprotease) has been identified in culture supernatants of most serotypes of the bovine lung pathogen *Pasteurella haemolytica* (1,2). The enzyme has a marked specificity for *O*-sialoglycoprotein, especially those with extensive clusters of sialoglycoprotein, such as human glycophorin A (3), human CD34, human CD43 and human CD44 (4). It has been shown to cleave other cell surface *O*-sialoglycoproteins such as human epitectin (5), and the ligands for P-selectin and L-selectin (6,7). The glycoprotease does not cleave exclusively *N*-linked sialoglycoproteins, and does not cleave de-sialated substrates (3). The gene for the *O*-sialoglycoprotein endopeptidase has been cloned, sequenced and expressed in *Escherichia coli* (8). The predicted amino-acid sequence includes a region resembling a metal ion binding site but differing from any of the 12 known classes of metalloprotease Zn^{2+}-binding sites (9). It has recently been reported that the *O*-sialoglycoprotein endopeptidase of *P. haemolytica A1* is Zn^{2+}-activated and can degrade fetuin, a soluble bovine sialoglycoprotein with potentially three *O*-linked and two *N*-linked glycans (10). We show here that the chromatographically purified *O*-sialoglycoprotein endopeptidase does not cleave fetuin and that its action on glycophorin A is inhibited by Zn^{2+}.

MATERIALS AND METHODS

Pasteurella haemolytica A1 cultures. P. haemolytica A1 (isolate 82, A.T.C.C. 43270) was cultured as described previously (11) except that 0.2 % (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was added to the RPMI 1640 culture medium instead of fetal calf serum. The culture supernatant was concentrated by diafiltration with 15 mM HEPES buffer, pH 7.4, through a Millipore 10 kD cut-off filter cartridge to remove lower molecular weight solutes.

Isolation of O-sialoglycoprotein endopeptidase. The concentrated culture supernatant (38 mL) was chromatographed on a DEAE-cellulose (Whatman DE52) ion-exchange column by elution with a concentration gradient of HEPES buffer pH 7.4 (15 mM to 500 mM) and 3 mL eluate fractions were collected.

O-sialoglycoprotein endopeptidase assay. An aliquot (2 μL) of each fraction was incubated with 3.5 μg [^{125}I]-glycophorin A in 50 mM HEPES buffer pH 7.4, total volume 25 μL, for 20 min at 37°C. The substrate and products were then separated by SDS-PAGE on 12% gels, which were dried and autoradiographed to locate the radio-labelled bands. The substrate bands for both dimeric and monomeric glycophorin A were excised from the gel, counted for radioactivity in a gamma counter, and the endopeptidase activity was calculated from the disappearance of substrate, after correction for variations in recovery of substrate and products (11).

N-Acetylneuraminidase assay. A fluorimetric method (12) was used to measure the hydrolysis of 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-NANA). The assay contained 2 nmol substrate in 20 μL 0.1M

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sodium acetate buffer, pH 5.0 with 5 μL enzyme fraction, incubated for 30 min at 37°C, and the fluorescent product was measured by excitation at 365 nm and emission at 450 nm in a Hitachi F-2000 spectrophotofluorimeter.

RESULTS

Ion-exchange chromatography of *P. haemolytica* A1 culture supernatant, concentrated by diafiltration, yields some *O*-sialoglycoprotein endopeptidase activity in the void volume (Figure 1, fractions 10–15) with most activity eluting in fractions 31–36, fraction 33 having a specific activity against human glycophorin A of 5 μg glycophorin A cleaved per hour per μg protein. Later fractions, 54–58, contain the N-acetylneuraminidase activity of the culture supernatant, as shown in Figure 2. It should be noted that while the N-acetylneuraminidase of *P. haemolytica* A1 has a high activity for the hydrolysis of the soluble fluorogenic substrate, MU-NANA. N-acetylneuraminidase activity against glycophorin A is undetectable (Figure 1, fractions 54–58). Thus the N-acetylneuraminidase fraction shown in Figure 2 does not de-sialate glycophorin A, under the conditions used to measure *O*-sialoglycoprotein endopeptidase activity. The *O*-sialoglycoprotein endopeptidase peak in Figure 2 was identified, not only by its enzyme activity against glycophorin A, but also by SDS-PAGE analysis and immunoblotting with a specific monoclonal antibody raised against the recombinant gene product, rGCP (13).

As shown in Figure 3A, when bovine glycoprotein fetuin (20 μg) was incubated for 2 h, at pH 7.4 in 50 mM HEPES buffer, with the DEAE-cellulose column eluant fractions (5 μL), containing the *O*-sialoglycoprotein endopeptidase (fractions 30-40) or N-acetylneuraminidase activities (fractions 54-60), no proteolysis of fetuin was seen by subsequent SDS-PAGE analysis and Coomassie blue staining of the gels. The N-acetylneuraminidase fractions (54-60) did however cause a slight increase in mobility of the fetuin 64 kD band during SDS-PAGE analysis, consistent with removal of sialate residues from fetuin. To confirm that the lack of cleavage of fetuin by *O*-sialoglycoprotein endopeptidase was not due to a lack of sensitivity in the assay, prolonged incubations of up to 21 hours were tested, with the same result. A more sensitive assay for fetuin degradation was performed, by incubating 3 μg [^{125}I]-fetuin with an *O*-sialoglycoprotein endopeptidase preparation (5 μg protein) for 2 or 4 hours at 37°C, that is, under conditions where an equivalent amount of glycophorin is totally degraded. This enzyme preparation was stabilized by fetal calf serum as previously described (11). It had a specific activity for glycophorin A of 8 μg per hour per μg protein and contained N-acetylneuraminidase activity of 0.7 nmol substrate cleaved

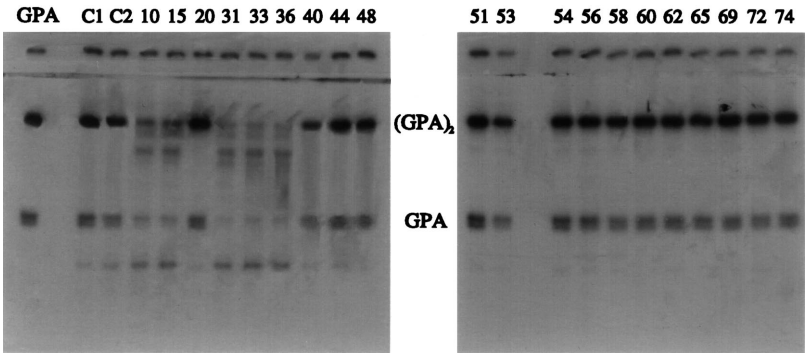

FIG. 1. The hydrolysis of glycophorin A by the chromatographically purified *O*-sialoglycoprotein endopeptidase. Aliquots (2 μL) of fractions of *P. haemolytica* culture supernatant proteins separated by DEAE-cellulose chromatography were incubated for 20 min at 37°C, with 3.5 μg [^{125}I]-glycophorin in 25 μL 50 mM HEPES buffer pH 7.4. Hydrolysis of glycophorin was measured by SDS–PAGE analysis on 12% gels and autoradiography, as described in the Methods. Controls (GPA lane) contain glycophorin A only, and show the dimer and monomer forms of glycophorin A. Lanes C1 and C2 show the activity of 0.1 μL and 0.2 μL concentrated culture supernatant enzyme activity, and the numbered lanes show the activities of column fractions, numbered as in Figure 2.

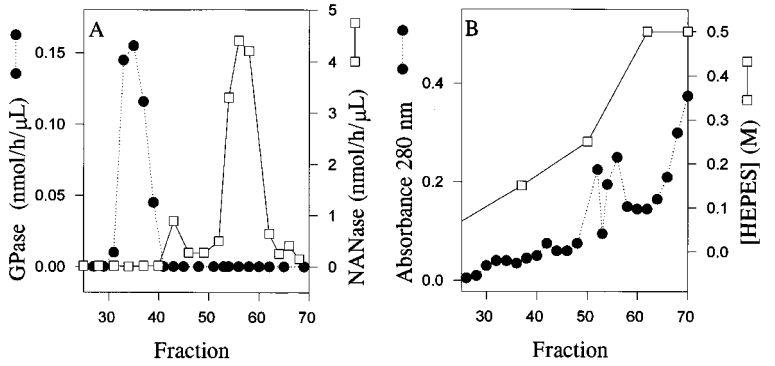

FIG. 2. The separation of *O*-sialoglycoprotein endopeptidase from neuraminidase by DEAE-cellulose chromatography. (A) The activities of the *O*-sialoglycoprotein endopeptidase (GPase) and the neuraminidase (NANase) measured in eluate fractions as described in the Methods. (B) The absorbance of eluates at 280 nm and the concentration of HEPES buffer pH 7.4 used to elute the protein fractions. Fraction numbers are identical with those in Figures 1 and 3.

per h per μg protein. Figure 3B confirms that no proteolysis of fetuin could be observed by SDS-PAGE analysis and autoradiography of the dried gels, but that there was some de-sialation as seen in Figure 3A shown by the slight increase in electrophoretic mobility.

Figure 4 shows that when human glycophorin A is degraded by the *O*-sialoglycoprotein endopeptidase, complete hydrolysis of the dimeric and the monomeric forms can be observed as shown by the disappearance of dimer and monomer bands at 60 min to 90 min. However, extensive

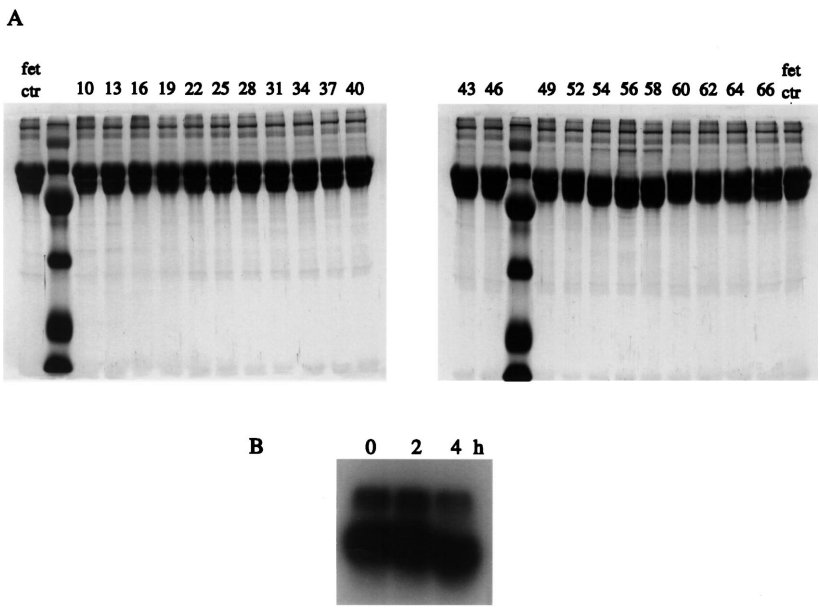

FIG. 3. The effect of fractions containing *O*-sialoglycoprotein endopeptidase or neuraminidase on bovine fetuin. (A) Aliquots (5 μL) of the DEAE-cellulose chromatographic fractions described in Figures 1 and 2 were incubated for 2 h with 20 μg bovine fetuin in 25 mL 50 mM HEPES buffer, pH 7.4, and then analyzed by SDS–PAGE on 12% gels. The fetuin was detected by Coomassie blue staining. The lanes show the untreated fetuin controls (fet ctr) and the eluate fractions numbered as in Figures 1 and 2. (B) An autoradiographic test for fetuin degradation shows the incubation of 3 μg[^{125}I]-fetuin with an *O*-sialoglycoprotein endopeptidase preparation (5 μg protein, specific activity against glycophorin A, 8 μg glycophorin degraded per h per μg protein) for 2 or 4 hours at 37°C. SDS–PAGE analysis and autoradiography of the dried gels was carried out to determine any proteolysis of fetuin. The three lanes show 0 h, 2 h and 4 h of enzyme treatment.

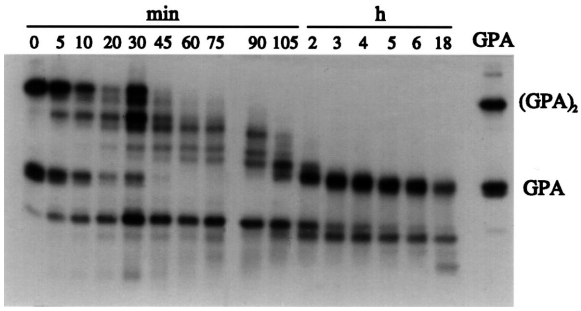

FIG. 4. The complete degradation of glycophorin A by the *O*-sialoglycoprotein endopeptidase results in the appearance of products with SDS–PAGE migration mobility similar to that seen for glycophorin A monomer. Aliquots (5μL) of fractions of *P. haemolytica* culture supernatant proteins stabilized in fetal calf serum were incubated for the times indicated at 37°C, with 3.5 μg [^{125}I]-glycophorin in 25 μL 50 mM HEPES buffer pH 7.4, in the presence of protease inhibitors, aprotinin, leupeptin, pepstatin A and antipain, all at 1 μg per mL. Hydrolysis of glycophorin was measured by SDS–PAGE analysis on 12% gels, and autoradiography, as described in the Methods. Control lanes show zero time incubation (O) or glycophorin A only (GPA), and show the dimer and monomer forms of undigested glycophorin A.

enzyme action gives rise to a product band on the gel with an apparent size equivalent to that of the undigested monomer. This band is relatively resistant to hydrolysis as seen by its presence after 18 h of enzyme treatment and it is likely due to association of two or more hydrophobic peptide products which contain the transmembrane region of the glycophorin A molecule. It is possible that the apparent disappearance of the monomer band, at 60 min to 90 min in Figure 4, is due in part to the association of monomer with hydrophobic peptide products to form complexes larger than the monomer.

It has been reported that the degradation of fetuin by *P. haemolytica* A1 culture supernatant extracts is stimulated by Zn^{2+} ions (10) However the chromatographically-purified *O*-sialoglycoprotein endopeptidase hydrolysis of glycophorin A was inhibited by Zn^{2+} at micromolar concentrations, as shown in Table 1. No cleavage of fetuin by this fraction was observed, in the presence or absence of Zn^{2+} ions (data not shown). The divalent transition metal chlorides, $NiCl_2$ and $CoCl_2$, also showed inhibition of the glycoprotease activity, though to a lesser degree. The divalent heavy metal ions Cu^{2+} and Hg^{2+} showed similar inhibition of the glycoprotease to that seen for Zn^{2+}. Magnesium and calcium ions showed apparent activation of the *O*-sialoglycoprotein endopeptidase activity. Other experiments, not shown, suggest that Ca^{2+} (1 mM) and Mg^{2+} (1 mM) stablize the *O*-sialoglycoprotein endopeptidase during isolation and assay. We have observed that the zwitterionic detergent CHAPS can substitute for serum proteins in *P. haemolytica* culture media, during the secretion and isolation of the *O*-sialoglycoprotein endopeptidase. The effect of this detergent on the hydrolysis of glycophorin A by the purified enzyme was tested, and as seen in Table 1, CHAPS gives an increased enzyme activity, similar to that seen for calcium and magnesium ions, suggesting that all three agents stabilize the enzyme during the assay, though any effects of these compounds on the physical state of the membrane protein substrate, glycophorin A, or its interaction with the enzyme, cannot be ruled out.

DISCUSSION

This study shows that the *O*-sialoglycoprotein endopeptidase from *P. haemolytica* A1 does not cleave the soluble glycoprotein, bovine fetuin,but that the neuraminidase secreted with the glycoprotease is capable of the desialation of bovine fetuin. The lack of proteolysis of fetuin was not due to a lack of sensitivity in the assay, since microgram quantities of [^{125}I]-fetuin were not degraded by the *O*-sialoglycoprotein endopeptidase, under conditions where glycophorin A hydrolysis is extensive. The fetuin-degrading proteolytic activity from *P. haemolytica* A1 was detected by Coomassie staining of milligram quantities of fetuin (10) and is clearly unrelated to the glycophorin

TABLE 1
The Effects of EDTA, Divalent Metal Ions, or the Zwitterionic Detergent CHAPS on the Cleavage of Glycophorin A by the Chromatographically Purified *O*-Sialoglycoprotein Endopeptidase

Control	EDTA		$ZnCl_2$	
mM:—	10	50	0.01	0.1
%: 100	30	0	50	2
CHAPS	$NiCl_2$		$CoCl_2$	
mM: 1.6	0.1	1	0.1	1
%: 127	27	5	99	58
	$MgCl_2$		$CaCl_2$	
mM:	0.1	1	0.1	1
%:	99	116	116	82
	$HgCl_2$		$CuCl_2$	
mM:	0.01	0.1	0.01	0.1
%:	51	0	14	0

Note. An aliquot (2 μL) of *O*-sialoglycoprotein endopeptidase from the DEAE-cellulose chromatography described in Figures 1 and 2 was incubated with the additives for one hour at 37°C, with 3.5 μg [^{125}I]-glycophorin in 25 μL 50 mM HEPES buffer pH 7.4. Hydrolysis of glycophorin was measured by SDS–PAGE analysis and gamma counting, as described in the Methods. The control assay activity was 14.5 μg glycophorin A degraded per h per μg protein and the assay contained 0.2 μg protein.

A-degrading activity of the *O*-sialoglycoprotein endopeptidase. The fetuin-degrading proteolytic activity was found to be stimulated by 1 mM Zn^{2+}, whereas the *O*-sialoglycoprotein endopeptidase activity against glycophorin A is 50% inhibited at one-hundredth of this Zn^{2+} concentration.

There are marked differences between the reported fetuin-degrading activity (10) and the *O*-sialoglycoprotein endopeptidase from *P. haemolytica* A1 described here. The fetuin-degrading activity was extracted from 24 h cultures of *P. haemolytica* A1, whereas the *O*-sialoglycoprotein endopeptidase activity is maximal in the supernatant at 4 h culture and then declines to zero activity by 20 h culture (data not shown). The fetuin-degrading activity was reported to be a major protein band in fractions retained on, and eluted from, carboxymethylcellulose ion-exchange columns, but the *O*-sialoglycoprotein endopeptidase activity is inactivated by cation-exchange chromatography under these conditions. The *O*-sialoglycoprotein endopeptidase only appears as a major protein band after the DEAE-cellulose purified fractions have been further resolved by gel-exclusion HPLC and anion-exchange HPLC (3).

The predicted amino acid sequence for the *O*-sialoglycoprotein endopeptidase was thought to contain a putative metal-binding site, namely His^{110}-His-Met-Glu-Gly-His. This site is conserved in the homologous open reading frames found in *E. coli* (13) and *Haemophilus influenzae* (14). This site was originally proposed to be a zinc ion binding domain (8), but subsequent analysis of 12 types of zinc ion binding motifs (9) has shown that the *O*-sialoglycoprotein endopeptidase putative metal-ion binding site does not fall into any of the known categories, since it shows a HHMEXH pattern rather than the general HEXXH sequence for Zn^{2+} binding. Though many metal ions have been tested for their ability to reactivate EDTA-inactivated and dialyzed *O*-sialoglycoprotein endopeptidase, no metal ion activator has yet been found. The glycoprotease may contain a tightly-bound metal ion of unknown nature, and the transition metals Zn^{2+}, Ni^{2+}, Co^{2+}, or the metal ions such as Hg^{2+} and Cu^{2+} which commonly inactivate enzymes, may displace an

essential metal ion activator from its binding site. The predicted amino acid sequence for the glycoprotease includes four cysteine residues, and the sensitivity of the glycoprotease to metal ions could be due to interactions with these cysteine thiols, though no effects of thiol-protecting reagents such as dithiothreitol has been detected (1,3). Studies in progress on the properties of the recombinant form of the *O*-sialoglycoprotein endopeptidase may throw more light on the specificity and metal-ion requirements for this enzyme.

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