Cellular localization and substrate specificity of isoelectric forms of human liver neuraminidase activity

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The four major isoelectric forms of human liver neuraminidase (with pI values between 3.4 and 4.8) have been isolated by preparative isoelectric focusing and characterized with regard to their substrate specificity using glycoprotein, glycopeptide, oligosaccharide and ganglioside natural substrates. All forms exhibited a rather broad linkage specificity and were capable of hydrolyzing sialic acid glycosidically linked $\alpha 2-3$, $\alpha 2-6$ and $\alpha 2-8$, although differential rates of hydrolysis of the substrates were found for each form. The most acidic form 1 (pI 3.4) was most active on sialyl-lactose, whereas form 2 (pI 3.9) and 3 (pI 4.4) were most active on the more hydrophobic ganglioside substrates. Form 4 (pI 4.8) was most active on the low- M_r hydrophilic substrates (fetuin glycopeptide, sialyl-lactose). Each form was less active on the glycoprotein fetuin than on a glycopeptide derived from fetuin. Organelle-enriched fractions were prepared from fresh human liver tissue and neuraminidase activity on 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid was recovered in plasma membrane, microsomal, lysosomal and cytosolic preparations. Isoelectric focusing of the neuraminidase activity recovered in each of these preparations resulted in significantly different isoelectric profiles (number, relative amounts and pI values of forms) for each preparation. The differential substrate specificity of the isoelectric forms and the different isoelectric focusing profiles of neuraminidase activity recovered in subcellular-enriched fractions suggest that specific isoelectric forms with broad but defined substrate specificity are enriched at separate sites within the cell.

INTRODUCTION

Neuraminidase (sialidase, acylneuraminyl hydrolase, EC 3.2.1.18) catalyses the hydrolysis of sialic acid residues from the non-reducing termini of numerous sialoglycoconjugates. This enzyme has been studied in various mammalian organs including the brain (Tettamanti *et al.*, 1975; Venerando *et al.*, 1978; Cruz & Gurd, 1981), liver (Tulsiani & Carubelli, 1970; Schengrund *et al.*, 1979; Alhadeff & Wolfe, 1981; Zeigler & Bach, 1981; Verheijen *et al.*, 1983; Spaltro & Alhadeff, 1984; Van Dessel *et al.*, 1984; Schauer & Wember, 1984; Samollow *et al.*, 1985; Miyagi & Tsuiki, 1984), thyroid (Schengrund *et al.*, 1979; Van Dessel *et al.*, 1984), and spleen (Schengrund *et al.*, 1979). Soluble and particulate fractions of the enzyme have been demonstrated in rat liver (Tulsiani & Carubelli, 1970; Miyagi & Tsuiki, 1984), brain (Tettamanti *et al.*, 1975; Venerando *et al.*, 1978) and human liver (Alhadeff & Wolfe, 1981; Meyer *et al.*, 1981; Michalski *et al.*, 1982; Spaltro & Alhadeff, 1984; Den Tandt & Scharpe, 1984). Cellular localization studies of neuraminidase activity in rat liver (Tulsiani & Carubelli, 1970; Miyagi & Tsuiki, 1984) bovine thyroid (Schengrund *et al.*, 1979), human fibroblasts (Zeigler & Bach, 1981) and leucocytes (Verheijen *et al.*, 1983; Schauer & Wember, 1984) have revealed the occurrence of this enzyme in plasma membrane, microsomal, lysosomal and cytosolic fractions.

In a previous study, analytical isoelectric focusing of neuraminidase activity solubilized from human liver tissue revealed the presence of several isoelectric forms (Spaltro & Alhadeff, 1984). In the present investigation

preparative isoelectric focusing has been performed on neuraminidase activity solubilized from human liver tissue and the four major isoelectric forms have been characterized with regard to their substrate specificity using sialoglycoconjugate natural substrates. In addition, human liver subcellular fractions have been prepared and isoelectric focusing has been performed on neuraminidase activity recovered in these organelle-enriched preparations. The results of our studies indicate that the isoelectric forms of neuraminidase display broad but differential substrate specificity and that the isoelectric forms appear to be differentially distributed in the enriched subcellular fractions.

MATERIALS AND METHODS

General

All procedures were carried out at 2–4 °C unless otherwise stated. N-Acetylneuraminic acid was purchased from Kantoishi Biochemicals, Japan. Dowex AG-1 X8 (acetate form, 200–400 mesh) was purchased from Bio-Rad Laboratories. 4-MU-NANA and fetuin (type III) were purchased from Sigma Chemical Co. Ampholines (pH 4–6) were purchased from LKB Produktur and Servalytes (pH 4.5–5.0) were purchased from Serva. Glycopeptides from fetuin were prepared by Pronase digestion of the glycoprotein and fractionation was performed on DEAE-cellulose according to Spiro (Spiro, 1962; Spiro & Bhoyroo, 1974). Ovine submaxillary glands were purchased from Pel-Freez Biologicals and OSM was prepared according to the method of

Abbreviations used: 4-MU-NANA, 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid; OSM, ovine submaxillary mucin.

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Tettamanti & Pigman (1968). Bovine brain was obtained from a slaughterhouse and gangliosides were prepared by extraction according to Suzuki (1965). Ganglioside G_{M1} was purified by reverse-phase h.p.l.c. according to Williams & McCluer (1980). Bovine colostrum was obtained at a local dairy and sialyl-lactose (mixed isomers) was prepared according to the method of Ohman & Hygstedt (1968).

Neuraminidase assay using 4-MU-NANA

The neuraminidase assay was performed essentially as previously described (Spaltro & Alhadeff, 1984) using 0.26 mM-4-MU-NANA in 0.25 M-acetic acid/sodium acetate buffer (pH 4.6) under linear conditions. Most assays were run in triplicate for 45 min at 37 °C. A unit of activity is defined as 1 nmol of 4-MU-NANA hydrolysed/h at 37 °C.

Natural substrate assays

All substrates for the natural substrate assays were prepared as aqueous solutions containing 50 µg of bound sialic acid/20 µl of solution. Solutions containing 100 µl of neuraminidase preparation, 50 µl of 0.25 M-acetic acid/sodium acetate buffer (pH 4.6) and 20 µl of substrate were incubated at 37 °C for 1–6 h. Tissue and substrate blanks were run in parallel with all determinations. Enzyme reactions were stopped by immersion in an ice bath and all reaction products were applied directly to mini-columns containing 0.3 ml of Dowex AG-1 X8 (acetate form, 200–400 mesh). These columns were washed with 3 ml of distilled water and free sialic acid was subsequently eluted with 2 ml of 1 M-NaCl. The sialic acid eluted from the mini-columns was determined fluorimetrically according to the method of Hess & Rolde (1964). Briefly, 0.5 ml aliquots of sialic acid eluants were combined with an equal volume of 0.005 M-diaminobenzoic acid, sealed into ampoules and heated for 16 h at 100 °C. Fluorescence was measured with a Turner fluorimeter equipped with Turner Blue Lamp no. 110–853, Corning CS-1-64 and Turner 2A primary filters (excitation wavelength, 440 nm) and a Baird 520–3C-GRT secondary filter (emission wavelength, 520 nm). All rates of natural substrate hydrolysis are presented as nmol of free sialic acid released/6 h at 37 °C.

Subcellular fractionation

Fresh human liver tissue which appeared normal upon gross pathological examination was obtained at autopsy and kept on ice until used. Enriched plasma membrane and microsomal fractions were prepared according to the method of Fleischer & Kervina (1974). Lysosomal fractions were prepared according to the method of Sawant *et al.* (1964) and cytosolic fractions were prepared by centrifugation of post-nuclear supernatant fluids at 125000 g for 1 h. Marker enzyme assays were performed to follow the preparation of organelle-enriched fractions. 5'-Nucleotidase activity (EC 3.1.3.5), a plasma-membrane-associated enzyme, was assayed essentially as described by Michell & Hawthorne (1965) and released inorganic phosphate was determined according to Bernhart & Wreath (1955). Glucose-6-phosphatase activity (EC 3.3.1.9), used as a measure of microsomal content, was assayed according to Rip & Cherry (1976) with inorganic phosphate determined as for 5'-nucleotidase. Monoamine oxidase activity (EC

1.4.3.4), associated with mitochondrial membranes, was estimated by following the formation of benzaldehyde from benzylamine according to Tabor *et al.* (1954). α -Mannosidase (EC 3.2.1.6), a lysosomal hydrolase, was determined by fluorimetric assay using 4-methylumbelliferyl α -D-mannoside as substrate according to Alhadeff *et al.* (1982). Lactate dehydrogenase activity (EC 1.1.1.27), a cytoplasmic marker, was assayed according to Hochella & Weinhouse (1965). Protein was determined by the Coomassie dye-binding method (Peterson, 1983) using human serum albumin as a standard.

Isoelectric focusing of neuraminidase activity

Isoelectric focusing was carried out essentially as previously described (Spaltro & Alhadeff, 1984) on Triton X-100-solubilized human liver preparations (approx. 300–600 units of neuraminidase activity) using a 140 ml preparative column, sucrose gradients (0–67%, w/v) and ampholytes with a pH range of 4.0–6.0. For some experiments, restricted range ampholytes (pH range 4.5–5.0) were employed for better resolution of isoelectric forms. Focused preparations were eluted, the pH of each fraction was determined and each fraction was assayed for 4-MU-NANA neuraminidase activity. Defined peaks of activity were pooled and aliquots were assayed in triplicate in buffer solutions ranging in pH from 3.2 to 7.2 (pH values 3.2–5.3, 0.25 M-citric acid/sodium citrate; pH values 3.8–5.6, 0.25 M-acetic acid/sodium acetate; pH values 5.8–7.2, 0.25 M-KH₂PO₄/K₂HPO₄) to determine their respective pH optima curves and any buffer effects.

Neuraminidase activity in organelle-enriched preparations was solubilized using Triton X-100 (0.85%, w/v) as previously described (Spaltro & Alhadeff, 1984) and analytical isoelectric focusing was performed on solubilized preparations using 40 ml columns and restricted range pH (4.5–5.0) ampholytes. Focusing was performed at constant voltage (800 V) for 15–18 h essentially as previously described (Spaltro & Alhadeff, 1984).

The distribution of activity in the isoelectric focusing profiles was determined by integrating (by triangulation) designated pI ranges and calculating the percentage of recovered activity found in each of these pI ranges.

RESULTS

Typical results of preparative isoelectric focusing of 4-MU-NANA neuraminidase activity derived from a Triton X-100-solubilized human liver supernatant fluid preparation are depicted in Fig. 1. At least four major isoelectric forms are present (numbered 1–4 from most to least acidic) with approximate pI values of 3.4, 3.9, 4.4 and 4.8. For a given liver, the pI values of the forms immediately after autopsy (prior to freezing) were comparable to the pI values after short term (approx. 1 week) or long term (approx. 1 year) storage at –20 °C even though the overall amount of neuraminidase activity decreased substantially ($\geq 50\%$) (Alhadeff & Wolfe, 1981). Each of these four major forms was separately combined and used for the subsequent kinetic and substrate specificity studies.

The pH optima curves for the four major forms of solubilized 4-MU-NANA neuraminidase activity typical of three separate experiments are shown in Fig. 2. Each form has a major pH optimum at or near pH 4.6 and each form exhibits significant buffer effects (increased

Fig. 1. Isoelectric focusing profile of Triton X-100-solubilized human liver 4-MU-NANA neuraminidase activity with pH 4.0-6.0 ampholytes

See the Materials and methods section for details.

activity in acetate buffer). All four forms demonstrate peaks of activity at or near pH 4.2 and 5.0 with the percentage of maximum activity at pH 4.2 increasing to 100% in the least acidic form (Fig. 2*d*). Since it was impractical to run pH optimum curves for each of the four major forms for each of the six natural substrates studied, it was assumed that the natural substrates would have sufficient activity for analysis at or near the pH optimum for 4-MU-NANA. Therefore, all natural substrate assays were run at pH 4.6 in 0.25 M-acetate buffer.

The results of substrate specificity studies on crude solubilized human liver neuraminidase activity and on the four major forms of neuraminidase activity representative of six separate experiments are summarized in Table 1. Preliminary experiments indicated that the rates of hydrolysis for the natural substrates were approximately linear up to 6 h incubation time and the data in Table 1 are given as nmol of sialic acid release/6 h at 37 °C. In addition, the relative rates of hydrolysis for a given substrate (expressed as percentage maximum hydrolysis by the four forms) are given in parentheses. The approximate linearity over 6 h may appear somewhat surprising considering the well-known lability of neuraminidase activity. However, the presence of natural substrates (sialoglycoconjugates) and/or NANA (released from the natural substrates) in the assay solutions probably stabilized enzymatic activity since we have shown previously that NANA can preserve almost all (86%) of solubilized neuraminidase activity even after storage for 29 days at 2-4 °C (Spaltro & Alhadeff, 1984). Inspection of the data in Table 1 indicates that, although the crude solubilized neuraminidase activity and the four major forms have similarly high activity towards 4-MU-NANA, the preparations (particularly the forms) exhibit different patterns of natural substrate hydrolysis. The crude solubilized neuraminidase has highest activity on sialyl-lactose but quite similar activity on all the other natural substrates with little specificity exhibited. Forms 1 and 4 showed a wider range of activity and were most active on sialyl-lactose, least active on fetuin and exhibited intermediate activity on the glycolipid (ganglio-

Fig. 2. pH-activity curves of the four major isoelectric forms of Triton X-100-solubilized 4-MU-NANA neuraminidase activity with citric acid/sodium citrate (●), acetic acid/sodium acetate (○) and $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (△) buffers

(a), Form 1 (pI 3.4); (b), form 2 (pI 3.9); (c), form 3 (pI 4.4); (d) form 4 (pI 4.8). See the Materials and methods section for details.

Table 1. Rate of hydrolysis of natural substrates and 4-MU-NANA by four major isoelectric forms of neuraminidase and by crude solubilized human liver neuraminidase

Values in parentheses show the relative rate of hydrolysis for a given substrate by the four major forms expressed as a percentage of maximum hydrolysis.

Substrate	Crude solubilized neuraminidase activity	Activity (nmol of sialic acid released/6 h at 37 °C)			
		Isoelectric form			
		1 (pI 3.4)	2 (pI 3.9)	3 (pI 4.4)	4 (pI 4.8)
Fetuin	3.0	1.6 (34)	4.6 (100)	1.6 (34)	1.6 (34)
Fetuin glycopeptide 8	5.7	4.1 (52)	5.8 (74)	5.1 (65)	7.8 (100)
OSM	4.3	4.4 (69)	6.4 (100)	4.5 (70)	5.9 (92)
Mixed bovine brain gangliosides	5.1	6.4 (59)	8.0 (74)	10.8 (100)	7.6 (70)
Ganglioside G _{M1}	4.1	4.5 (47)	9.5 (100)	8.2 (85)	3.2 (34)
Sialyl-lactose	14.3	9.8 (94)	7.4 (70)	6.9 (66)	10.4 (100)
4-MU-NANA	10.9	9.6 (100)	8.6 (89)	9.1 (95)	8.2 (85)

Table 2. Subcellular fractionation of 4-MU-NANA neuraminidase activity in human liver

Fraction (marker enzyme)	Marker enzyme purification factor	4-MU-NANA neuraminidase activity		
		Specific activity (nmol/h per mg of protein)	Purification factor	Relative recovery*
Homogenate	1.0	0.92	1.0	—
Plasma membrane (5'-nucleotidase)	2.2	2.4	2.6	3
Microsomal (glucose-6-phosphatase)	8.5	7.9	8.6	43
Lysosomal (α -mannosidase)	11.0	13.3	14.5	36
Cytosolic (lactate dehydrogenase)	19.2	3.3	3.6	18

* Percentage of recovered activity associated with subcellular fraction.

side) substrates. In contrast, forms 2 and 3 were most active on the more hydrophobic ganglioside substrates. Form 2 was most active on the macromolecular substrates (fetuin, OSM) whereas form 4 was most active on the low- M_r hydrophilic substrates (fetuin glycopeptide, sialyl-lactose). Each form was less active on the glycoprotein fetuin than on a glycopeptide derived from fetuin, suggesting preference by all forms for low- M_r substrates. All forms exhibited a rather broad linkage specificity and were capable of hydrolyzing sialic acid glycosidically linked α 2-3, α 2-6 and α 2-8.

Subcellular fractionation studies on fresh human liver resulted in plasma membrane, microsomal, lysosomal and cytosolic preparations which were enriched 2-19-fold

as determined by marker enzyme specific activities in the various preparations (Table 2). 4-MU-NANA neuraminidase activity was recovered in all four subcellular-enriched preparations with purification factors (as determined by increases in specific activity) ranging from approx. 3- to 15-fold (Table 2). Approx. 79% of the recovered neuraminidase activity was found associated with the microsomal and lysosomal preparations with only about 3% associated with the plasma membrane preparation. The remainder of the recovered activity (18%) was found in the cytosolic preparation.

Isoelectric focusing profiles representative of three separate experiments on each subcellular-enriched fraction using restricted range (pH 4.5-5.0) ampholytes are

Fig. 3. Isoelectric focusing profiles of 4-MU-NANA neuraminidase activity from subcellular-enriched fractions of human liver tissue with restricted range (pH 4.5–5.0) ampholytes

(a), Plasma membrane fraction; (b), microsomal fraction; (c), lysosomal fraction; (d) cytosolic fraction. See the Materials and methods section for details.

depicted in Fig. 3. Plasma membrane-associated neuraminidase activity (Fig. 3a) demonstrates the presence of one isoelectric form (with an approximate pI value of 3.3) whereas microsomal-associated neuraminidase activity (Fig. 3b) contains at least two forms (with approximate pI values of 3.5 and 3.7). Lysosomal-associated (Fig. 3c) and cytosolic-associated (Fig. 3d) neuraminidase activities both contain multiple (four to six) isoelectric forms (with approximate pI values of 3.3–4.4 and 3.9–4.6, respectively). The pI distributions of crude solubilized liver 4-MU-NANA neuraminidase activity and of 4-MU-NANA neuraminidase activity from the four subcellular-enriched fractions (determined by integrating the designated pI ranges in the respective profiles) are summarized in Table 3. The quantitative distribution of activity is strikingly different in the subcellular fractions with increased amounts of acidic activity (pI 3.2–3.9) found for the microsomal and, particularly, the plasma membrane fractions. Increased amounts of the more neutral activity (pI 3.9–4.7) were found for the lysosomal and, particularly, the cytosolic fractions. The small amount (7%) of activity in the pI range 4.7–5.1 for the crude solubilized activity was not detected in any of the subcellular fractions.

DISCUSSION

Sialic acids are widespread in nature and occur glycosidically bound to other sugars primarily by α 2–3, α 2–6 and α 2–8 linkages in both low- and high- M_r glycoconjugates, including oligosaccharides, glycoproteins, glycopeptides and glycolipids (Corfield & Schauer, 1982). This great structural diversity of molecules containing sialic acids suggests the probable existence of several neuraminidases which could differ in specificity regarding linkage type, M_r and class of compound.

Numerous previous investigators have given evidence for the existence of more than one neuraminidase in various mammalian tissues (see the Introduction). Evidence has been obtained in subcellular fractionation studies (Tulsiani & Carubelli, 1970; Schengrund *et al.*, 1979; Zeigler & Bach, 1981; Verheijen *et al.*, 1983; Van Dessel *et al.*, 1984; Miyagi & Tsuike, 1984; Schauer & Wember, 1984) which have localized neuraminidase activity in plasma membrane, microsomal, lysosomal and cytosolic fractions, with membrane fractions having mostly ganglioside-specific activity and lysosomal fractions having mostly glycoprotein-specific activity. In addition to the subcellular localization studies, substrate specificity studies on various crude and partially purified neuraminidase preparations using oligosaccharide, glycoprotein and glycolipid substrates have provided further evidence for the existence of different neuraminidases (Schengrund *et al.*, 1979; Zeigler & Bach, 1981; Van Dessel *et al.*, 1984; Miyagi & Tsuike, 1984). A more recent study has provided genetic evidence (independent variation in breeding) for two neuraminidases controlled by different genes in both rat and mouse liver (Samollow *et al.*, 1985). In addition, at least two genetically distinct neuraminidases appear to be present in humans: a glycoprotein-specific enzyme which is deficient in the lysosomal storage disease sialidosis (Warner & O'Brien, 1979) and a ganglioside-specific enzyme which is deficient in mucopolipidosis IV (Kuriyama *et al.*, 1982).

Table 3. Distribution of 4-MU-NANA neuraminidase activity after isoelectric focusing of crude solubilized liver activity and enriched subcellular fractions of human liver tissue

Preparation	Neuraminidase activity recovered (%) in pI ranges:				
	3.2-3.6	3.6-3.9	3.9-4.2	4.2-4.7	4.7-5.1
Crude solubilized activity	9	18	35	31	7
Plasma membrane	100	0	0	0	0
Microsomal	46	54	0	0	0
Lysosomal	17	43	28	12	0
Cytosolic	0	6	5	89	0

In a previous study, several isoelectric forms of solubilized human liver neuraminidase activity were demonstrated (Spaltro & Alhadeff, 1984). In the present investigation preparative isoelectric focusing has been employed to isolate the four major forms of human liver neuraminidase. The forms had pI values somewhat more acidic than in our previous study (Spaltro & Alhadeff, 1984) which may be due to the use of different batches of ampholytes, the use of a different (preparative) focusing column and/or microheterogeneity differences between individual livers. Studies on these major forms have indicated that all exhibit a relatively broad substrate specificity and are capable of hydrolyzing all linkage types on structurally diverse natural substrates, but that differential rates of substrate hydrolysis are found for each form. Forms 1 and 4 were most active on hydrophilic low- M_r substrates whereas forms 2 and 3 were most active on the hydrophobic ganglioside substrates.

Subcellular fractionation studies indicated that 4-MU-NANA neuraminidase activity is present in plasma membrane, cytosolic, microsomal and lysosomal fractions, with approx. 80% of the recovered activity found in the latter two fractions. These results confirm and extend the work of other investigators on other tissues and demonstrate neuraminidase activity in enriched subcellular fractions derived from human liver. Although insufficient amounts of neuraminidase activity were recovered (because of the lability of enzymic activity) in the subcellular fractions to run substrate specificity studies, enough 4-MU-NANA activity was available to perform analytical isoelectric focusing on each fraction. Each fraction gave a different profile of isoelectric forms with the more acidic forms predominating in the membrane fractions and the more neutral forms predominating in the lysosomal and cytosolic fractions. These new results indicate that the isoelectric forms of neuraminidase are not similarly distributed in the subcellular compartments and organelles of human liver cells. Furthermore, this asymmetric distribution of forms may reflect functional differences at different subcellular sites within liver cells since these forms demonstrate differential substrate specificity on sialoglycoconjugates. At present it is not known whether the major forms of neuraminidase investigated in this study represent post-translationally modified forms of the same gene product or genetically distinct enzymes.

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