Volume 282, number 2, 235–238 FEBS 09661 May 1991

ADONIS 001457939100368S

Occurrence of sialidase activity in two distinct and highly homogeneous populations of lysosomes prepared from the brain of developing mouse

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Received 25 February 1991

Light and heavy lysosomes of mouse forebrain were separated from each other by centrifugation on a Percoll gradient. Light lysosomes were then freed from mitochondria and membranes by sucrose density gradient centrifugation and further purified by flotation-centrifugation on a sucrose gradient. The final preparations of light and heavy lysosomes, fairly homogenous, carried sialidase activity, assayed on MU-NeuAc. The optimal pH was 4.0 and 4.2, the apparent K_m value 2.8×10^{-3} M and 4.2×10^{-3} M and the apparent V_{max} value 0.11 and 0.47 mU·mg^{-1} protein, for the light and heavy lysosome sialidase, respectively. From 4 days to adulthood the specific activity of the light and heavy lysosome sialidase increased 3-fold and 1.7-fold, respectively.

Brain development; Sialidase; Lysosome; Subcellular fractionation

1. INTRODUCTION

Sialidase occurs in mammalian cells, including neural cells, at different subcellular levels, the lysosomes, the cytosol and the plasma membrane [1–9]. The presence of sialidase in brain lysosomes was recently assessed [2] using a highly purified preparation of lysosomes obtained from the brain of 17-day-old mice. These lysosomes must be considered heavy lysosomes, on the basis of the procedure used for their preparation [10]. However, it is known that in neural cells, as well as in other cells, light lysosomes also occur [11,12]. The attempts so far made to separate these two populations of lysosomes led to preparations having a relatively low level of homogeneity [11,12]. Whether light lysosomes carry sialidase activity is not known, the question remaining open of possible differential roles played by lysosomal sialidases in the degradation and turnover of sialosylglycoconjugates, compounds that are known to be highly concentrated in the central nervous tissue [13].

In the present work, performed on developing mouse forebrain, we perfected a procedure capable to separate light and heavy lysosomes into distinct preparations of very high homogeneity. We observed that both light and heavy lysosomes carry sialidase activity and that the two lysosomal sialidases appear to be expressed throughout post-natal development.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

All chemicals (Merck, Darmstadt, Germany) were of analytical grade. Crystalline bovine serum albumin, substrates for marker enzyme assays and pepstatin A were obtained from Sigma (St. Louis, MO, USA); Percoll from Pharmacia (Uppsala, Sweden); 4-methylumbelliferone, 4-methylumbelliferyl-β-D-galactoside (MU-Gal) and 4-methylumbelliferyl-α-D-*N*-acetylneuraminic (MU-NeuAc) acid from Koch-Light Laboratories (Colnbrook, UK).

2.2. *Separation of the two populations of 'light' and 'heavy' lysosomes. All the operations were performed at 0–4 °C.*

The procedure was worked out using 17-day-old mice and then extended to animals of other ages.

Brown mice of the C57Bl strain originating from Jackson Laboratories (Bar Harbor, ME, USA) and supplied by Charles River (Milan, Italy) were used. The age of the animals was 3, 10, 17 and 49 post-natal days. Groups of 30–70 animals for each age were killed by decapitation and the brains, without cerebellum, stem and medulla oblungata, and freed from meninges, were pooled, weighed, washed with cold homogenizing solution (0.32 M sucrose containing 1 mM potassium phosphate buffer, pH 7.0, and 0.1 mM EDTA) and homogenized with 3 vols of the same solution. The mild homogenization conditions specified by Caimi et al. [10] were used. The P_2 fraction (precipitating between $1000 \times g$ and $17500 \times g$) was prepared from the homogenate as described [10].

2.2.1. *Percoll gradient centrifugation*

The P_2 fraction was resuspended with 3 vols. of homogenizing solution and submitted to centrifugation on a self-generating density gradient of Percoll following the conditions specified by Caimi et al. [10]. The gradient was collected into 1.0 ml aliquots from top to bottom by pumping into the tube (punctured at the bottom) a 2.2 M sucrose solution in a Jasco gradient fractionator apparatus (mod. 640). Three fractions were obtained; F_1, F_2 and F_3 (from the top), in accordance with Caimi et al. [10]. Fractions F_1 and F_3, which carried almost all of the lysosomal enzyme activities, were diluted (3 times) with the homogeneizing solution and pelletted by centrifugation ($105\,000 \times g$, 15 min, fixed angle rotor, Beckman TL100 centrifuge).

2.2.2. *Sucrose gradient centrifugation (all the solutions used were added with 0.2 μg/ml Pepstatin A in order to prevent sialidase degradation by proteases)*

The pellet of fraction F_1 (15–20 mg, as protein) was dissolved with 4–5 ml of homogenizing solution and laid on the top of a discontinuous sucrose gradient (0.8 M; 1.0 M; 1.2 M; 1.4 M; 1.6 M and 1.8 M, 5 ml each) [14] and centrifuged (50 000 × g, 90 min, swinging buckets rotor SW28, Beckman L70 ultracentrifuge). The gradient was collected into 1.0 ml aliquots as specified above. The material laid above 1.4 M sucrose (1.4 M sub-fraction), that carried almost all of the lysosomal enzyme activities (see Fig. 1), was diluted (4 times) with the homogenizing solution and pelletted as described above.

2.2.3. *Floatation centrifugation (all solutions used were added with 0.2 μg/ml Pepstatin A as above)*

The pellets of the F_3 fraction and 1.4 M sub-fraction (1.5–2 mg, as protein) were separately dispersed with 9 ml 1.6 M sucrose, overlaid with a solution of 1.4 M (9 ml) and 1.0 M (9 ml) and 0.8 M sucrose (9 ml), and centrifuged at 50 000 × g for 90 min (as above). The gradient was collected into 1.0 ml aliquots. The materials laid above 1.4 M and 1.6 M sucrose (see Fig. 2) were withdrawn, diluted and pelletted. They constituted the purified 'light' and 'heavy' lysosomal populations, respectively.

2.3. *Preparation of the microsomal and cytosolic fractions*

The microsomal fraction and the cytosolic fraction (from which proteins were precipitated by addition of saturating amounts of ammonium sulphate and salts removed by dialysis) obtained from 4 g of forebrain homogenate were prepared as previously described [2,4]. The two fractions were used as crude preparations of the plasma membrane and cytosolic sialidase, respectively.

2.4. *Determination of marker enzymes*

The lysosome marker β-D-galactosidase (E.C. 32.1.23) was determined by the fluorimetric procedure of Kint [15]; the plasma membrane marker 5'-nucleotidase (E.C. 3.1.3.5) by the method of Ipata [16], as perfected by McIntosh and Plummer [17]; the mitochondrial marker succinate dehydrogenase (E.C. 1.1.1.49) by the method of Pennington [18]; the cytosol markers lactate dehydrogenase (E.C. 1.1.1.27) and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) by the methods of McIntosh and Plummer [17] and of Bourre et al. [19], respectively. Protein content was determined by the method of Lowry et al. [20]; when Percoll was present, the fluorescamine method [21] was used.

2.5. *Assay of sialidases*

Sialidases were fluorimetrically assayed using MUB-NeuAc as substrate following the indications previously given [2] and under the optimal conditions that were established in preliminary experiments. The assay mixtures contained, in a final volume of 0.1 ml, 0.2–0.5 mM of MUB-NeuAc, proper amounts of enzyme protein (2–20 μg for the lysosomal sialidases; 70–400 μg for the microsomal sialidase; 50–200 μg for the cytosolic sialidase), 20 mM sodium acetate-acetic acid buffer at the optimal pH, that resulted to be 4.0 for the light lysosome sialidase, 4.2 for the heavy lysosome sialidase, 5.1 for the microsomal (plasma membrane-bound) sialidase, and 6.2 for the cytosolic sialidase. The incubation times at 37°C were 10–30 min. The specific activity of sialidases and marker enzymes was expressed as nmol of hydrolyzed substrate, or released product, $\cdot min^{-1}$ (mU), $\cdot mg^{-1}$ of protein.

2.6. *Ultrastructural analysis*

The final preparations of light and heavy lysosomes were submitted to submicroscopic examination using the experimental conditions described in [10] and a Philips model 301 electron microscope.

3. RESULTS AND DISCUSSION

Application of the sub-fractionation method on self-generating Percoll gradient to the P_2 fraction from mouse forebrain, provided three well defined fractions, F_1, F_2 and F_3, in accordance with Calmi et al [10], at all the animal ages. The distribution of protein was 88–92% in fraction F_1, less than 1% in F_3 and the remainder in F_2, and the distribution of β-D-galactosidase 45–55% in F_1 fraction, 15–10% in F_2 and 35–50% in F_3. Sialidase activity at pH 4.0–4.2 (lysosomal sialidase) distributed about 50% in fraction F_1 and 45–50% in F_3, and almost all of the sialidase at pH 5.1 (plasma membrane sialidase), together with 5'-nucleotidase, was contained in fraction F_1. As shown in Fig. 1, fraction F_1 was divided, upon sucrose density gradient centrifugation, into 5 subfractions laid on 0.8 M, 1.0 M, 1.2 M, 1.4 M and 1.6 M sucrose, respectively. At all ages the subfraction over 0.8 M sucrose carried the highest amount of protein (50–65%), and of 5'-nucleotidase and sialidase pH 5.1 (65–70%). The subfraction over 1.4 M sucrose, that contained only a minor portion of protein (2–4%), carried almost all (75–85%) of β-D-galactosidase and sialidase pH 4.0 activities with virtually no contamination of the plasma membrane marker and a slight contamination of the mitochondrial marker.

When the subfraction over 1.4 M sucrose was submitted to floatation-centrifugation on a sucrose gradient (Fig. 2a), practically all the protein, β-D-galactosidase and sialidase pH 4.0 content was carried by the material

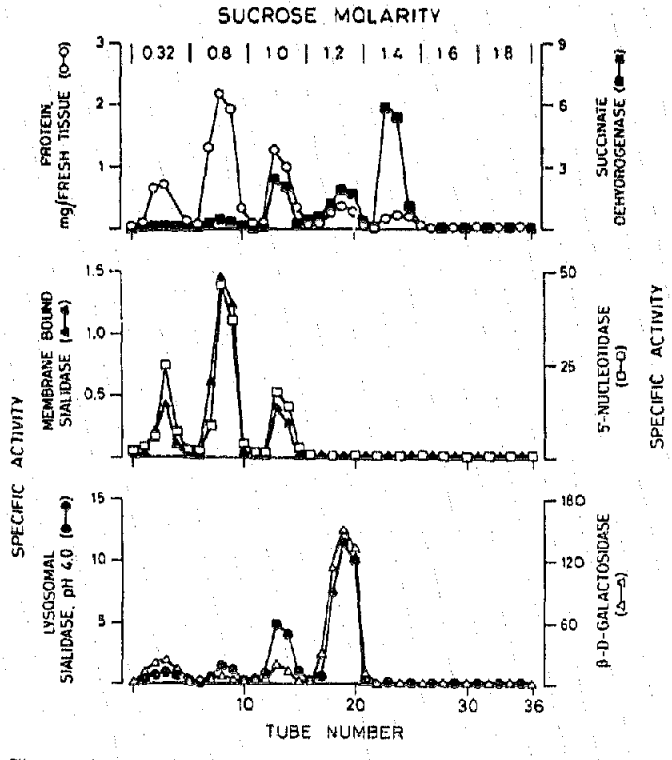

Fig. 1. Subfractionation of the 'F_1 fraction' on a discontinuous sucrose gradient. The protein content and the specific activity of sialidases and different marker enzymes are reported. For details see section 2.

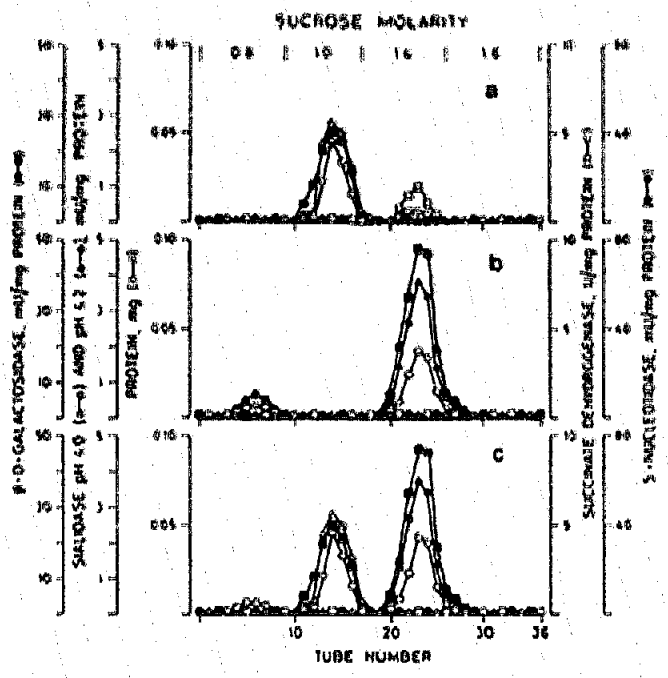

Fig. 2. Purification and separation by floatation centrifugation on a discontinuous sucrose gradient of the '1.4 M subfraction' and 'F_3 fraction' obtained from mouse forebrain homegenate. The protein content and the specific activity of sialidases and some marker enzymes are reported. For details see section 2a, '1.4 M subfraction'; b, 'F_3 fraction'; c, '1.4 M subfraction' and 'F_3 fraction', mixed together prior to floatation centrifugation.

laid over 1.4 M sucrose ('light lysosome fraction'). This material was devoid of any measurable amounts of the plasma membrane and mitochondrial markers (Table I). The Percoll fraction F_3, submitted to the same procedure, gave rise (Fig. 2b) to a major band, overlaid on 1.6 M sucrose ('heavy lysosome fraction') that carried practically all of the protein, β-D-galactosidase and sialidase pH 4.2, contained in fraction F_3, with no detectable amounts of the non-lysosomal markers (Table I). In a control experiment the 1.4 M subfraction and fraction F_3 were mixed together and submitted to the same floatation centrifugation step (Fig. 2c). Two major bands were obtained, laying on 1.4 M and 1.6 M sucrose, respectively; the former carried almost all of the protein and sialidase pH 4.0, contained in the starting 1.4 M subfraction, and the latter one the protein and sialidase pH 4.2, contained in the starting F_3 fraction. Examination of the purified preparations of light and heavy lysosomes showed a large preponderance of vesicles of various size, loaded with electron-dense material with occasional contamination (particularly the light lysosome fraction) with mitochondria or mitochondriaal particles. All this indicates that the light and heavy lysosome fractions are lysosomal preparations of high purity and that the lysosomes herein contained, behave as distinct organelles. The pH optimum for the sialidase contained in the light lysosome fraction was 4.0, and that of the enzyme carried by the heavy lysosome fraction 4.2; the values of apparent K_m and V_{max} (referring to MU-NeuAc) were 2.8×10^{-5} and 4.2×10^{-5} M, and 0.11 and 0.47 mU·mg^{-1} protein, for the two enzymes, respectively.

As reported in Table I, at all investigated ages the enrichment (RSA, referring to the starting homogenate) of β-D-galactosidase was about 100-fold in the heavy lysosome fraction, in accordance with Caimi et al. [10], and about 50-fold in the light lysosome fraction. On this basis the homogeneity of the separated lysosome populations can be judged more than 10 times higher than that reported previously [11,12]. The enrichment

Table I

Specific activity and RSA values of sialidase and several marker enzymes in the purified heavy and light lysosome preparations from the forebrain of developing mice. The lysosome preparations were those obtained after floatation centrifugation on a sucrose gradient. The relative specific activity (RSA) values are referred to the starting homogenate

Animal age	Sialidase (mU/mg protein)	RSA	β-Galactosidase (mU/mg protein)	RSA	5'Nucleotidase (mU/mg protein)	RSA	Succinate dehydrogenase (mU/mg protein)	RSA	Lactate dehydrogenase (mU/mg protein)	RSA
3 days										
Heavy lysosomes	9.75	65.0	81.0	90.0	n.d.	–	n.d.	–	n.d.	–
Light lysosomes	2.80	18.5	40.7	45.2	n.d.	–	n.d.	–	n.d.	–
10 days										
Heavy lysosomes	13.94	58.0	155.0	96.9	n.d.	–	n.d.	–	n.d.	–
Light lysosomes	6.44	21.0	77.0	48.1	n.d.	–	n.d.	–	n.d.	–
17 days										
Heavy lysosomes	16.38	68.0	180.2	106.0	n.d.	–	n.d.	–	n.d.	–
Light lysosomes	7.90	19.5	84.1	49.5	n.d.	–	n.d.	–	n.d.	–
49 days										
Heavy lysosomes	17.55	59.0	178.0	98.9	n.d.	–	n.d.	–	n.d.	–
Light lysosomes	8.10	20.0	93.0	51.7	n.d.	–	n.d.	–	n.d.	–

n.d. = not detectable

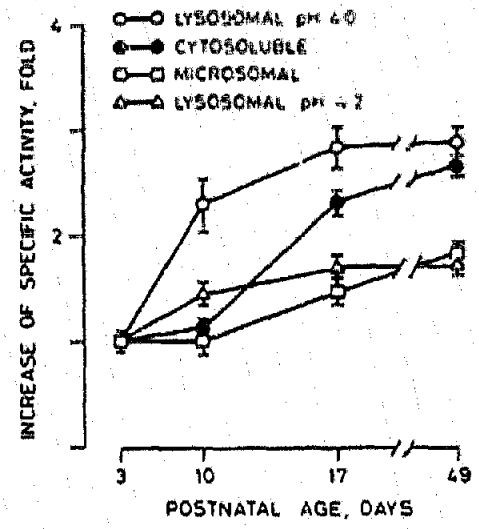

Fig. 3. Changes with post-natal age of the specific activities of different sialidases in mouse forebrain. For this study the starting forebrain material was about 4 g regardless of age, the light and heavy lysosome preparations were those obtained after floatation centrifugation on a discontinuous sucrose gradient, and the amounts of materials loaded on the various gradients and numbers of the fractions collected after centrifugation on the gradients were rigorously the same at all ages. The data given are the mean values of 4 experiments ± SD values.

of sialidase pH 4.2 in the heavy lysosome fraction was about 60-fold at all ages and that of the sialidase pH 4.0 in the light lysosome fraction about 20-fold. The reason why the RSA values for the assayed lysosomal enzymes were markedly higher in the heavy than in the light lysosomes is not known. One possible explanation is a lesser degree of homogeneity of the light lysosome preparation, as compared to the heavy one; alternatively the stability of the light lysosomes may be lower than that of the heavy ones, leading to partial inactivation of the lysosomal enzymes during the subfractionation procedure.

The changes with age of the specific activity of the light and heavy lysosomal sialidases are reported in Fig. 3, in comparison with those of the cytosoluble and plasma membrane bound sialidases. All sialidases show an increase of specific activity with age, as already reported previously for some of them [3,8]. However, differences are present. The two lysosomal sialidases undergo the most marked increase of specific activity in the first 10 days of age, whereas the cytosoluble and plasma membrane-bound sialidases after 10 days of age. From 4 days to adulthood the light lysosomal sialidase displays the highest increase (about 3-fold), followed by the cytosoluble and plasma membrane enzyme, and the heavy lysosomal sialidase the lowest (about 1.7-fold).

These developmental differences, together with property differences (for example the optimal pH) may be in favour of the concept [22,23] that the various sialidases are different proteins, not different post-translational products of the same protein.

In conclusion, this work provides clear evidence that two distinct populations of lysosomes, light and heavy lysosomes, obtained from mouse forebrain, carry sialidase activity, and that an enrichment of sialidase occurs in post-natal life in both lysosomal populations. The availability of the present procedure, able to separate light and heavy lysosomes, can enable one to study the kinetics and properties of the sialidases herein contained and to inspect the specific role played by these enzymes in sialosylglycoconjugates' metabolism in the brain.

Acknowledgements: The present work was supported in part by a grant from the Consiglio Nazionale delle Ricerche (CNR), Rome, Italy (Progetto finalizzato Ingegneria Genetica Grant no. 91.00052. PF99).

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