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Original Article

Isolation of Highly Purified Rat Cerebral Lysosomes Using Percoll Gradients with a Variety of Calcium Concentrations

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Abstract

We were able to isolate a lysosomal fraction from rat brain which had a higher degree of purity than in previous studies with good recovery. This has been made possible by using percoll gradients following the swelling of mitochondria in the presence of calcium which would eliminate contamination from small amounts of mitochondria. By using percoll density gradient centrifugation after the swelling of mitochondria in the presence of calcium, cerebral lysosomes were purified 312-fold with the recovery of approximately 22 %, which is the highest reported for any cerebral lysosomal preparation. The most effective procedure for the separation was achieved by using 1.25 mM calcium incubation with post nuclear fraction. As brain lysosomes may play a major role not only in degrading macromolecules but also in their transport to the deposition site, obtaining purified rat cerebral lysosomes represents an important step in the study of the generation of macromolecules which accumulate in the brain.

Key words: isolation of lysosome - Percoll gradients - calcium concentration - rat brain

Introduction

The brain lysosomes may play a major role not only in degrading macromolecules but also in their transport to the deposition site. For example, Alzheimer's disease is characterized by extracellular deposition in the brain of insoluble aggregates of about 40 amino acids with the length of the amyloid B-protein (AB)[1]. The mechanism of extracellular accumulation of the AB in the brain is unknown and no simple model systems that produce extracellular AB have been reported.

Here we speculate that the AB is generated from the degradation of amyloid precursor protein in cerebral lysosomes or that lysosomal inhibitors block the secretion of the AB, or that a combination of the two processes occurs. To study the cellular mechanism of this secretion blockage or generation of the AB in the brain, a pure and well-characterized cerebral lysosomal fraction is needed. The useful methods for purification of brain lysosomes include : 1) a combination of differential and isopycnic centrifugation[2-4] ; 2) raising the density of mitochondria by deposition of formazan[5] ; 3) the use of a chemical field for displacing mitochondria from a crude mitochondrial-lysosomal preparation[6]; and 4) usage of a Percoll gradient[7]. But none of these procedures has been successful at producing a highly purified lysosomal fraction with good recovery from a crude preparation of brain. Therefore, we attempted to obtain a lysosomal fraction from rat brain which had a higher degree of purity in good recovery using percoll gradients following the swelling of mitochondria in the presence of calcium which would eliminate contamination from small amounts of mitochondria. Calcium accumulates within mitochondria but not within lysosomes; thus, after swelling, lysosomes can be successfully separated from contaminants by percoll density gradient centrifugation.

In this article, we report a new procedure based on the swelling of mitochondria in the presence of calcium for the isolation of lysosomes with high purity and in good recovery from rat brain using percoll density gradient centrifugation.

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Materials and Methods

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO. USA). Percoll and density marker were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Preparation of lysosomes

Four male Wistar rats from Japan SLC (Shizuoka,Japan) weight approximately 200 g each were killed by decapitation after being starved for a 12 hr period. Whole brains were excised and suspended in 3 volumes of ice cold 0.3 M sucrose solution. The brains were homogenized in sucrose solution in an ice-cooled Potter Elvehjem glass homogenizer with a Teflon pestle rotating at approximately 500 rpm with slow up-and-down strokes. The protein concentration of the homogenate was approximately 20 mg/ml in the sucrose solution, and this suspension was centrifuged at 750 x g for 10 min. The supernatant was collected and the pellet was resuspended in 10 ml of 0.3 M sucrose solution, rehomogenized and centrifuged under the same conditions. The final pellet was discarded and the two supernatant fractions were pooled. The pooled supernatant referred to as postnuclear supernatant, was further subfractionated on an isotonic percoll gradient. The percoll solution was prepared as follows: percoll was made isoosmotic by the addition of 9 volumes of percoll to 1 volume of 2.5 M sucrose containing 10 mM EDTA. This stock solution was diluted to 24 % Percoll (density approximately 1.08 g/ml) with 0.32 M sucrose containing 1 mM EDTA (SED solution). The postnuclear supernatant was incubated at 37 ℃ for 5 min in solutions containing calcium (0.4, 0.8 and 1.25 mM respectively). The individual incubated samples (0.5 ml) were layered onto 4 ml of the 24 % percoll solution and centrifuged at 20,000 x g in a Hitachi fixed-angle rotor at 4 ℃ for 20 min. The resulting gradients were aliquoted with 0.3 ml into tubes from the bottom of the rotor tube by aspiration. Total fraction numbers were 12. 1 to 6 fractions from the bottom were assayed the activity of the arylsulfatase (EC 3. 1. 6. 1) and INT reductase (EC 1. 3. 99. 1). The lysosomal layer corresponds to the fraction 1 (see Fig. 1).

For preparative preparation of brain lysosomes, four male Wistar rats were used. 0.3 ml of lysosomal fraction after percoll centrifugation was collected into 25 ml tubes from the bottom of the rotor tube by a Pasteur pipette. The fractions were diluted with 3 volumes of 0.3 M sucrose solution. The diluted suspension was centrifuged at 10,000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in the sucrose solution and recentrifuged at 10,000 x g for 10 min. The washed pellet was finally suspended in a small volume of 0.3 M sucrose solution and used as the lysosomal fraction.

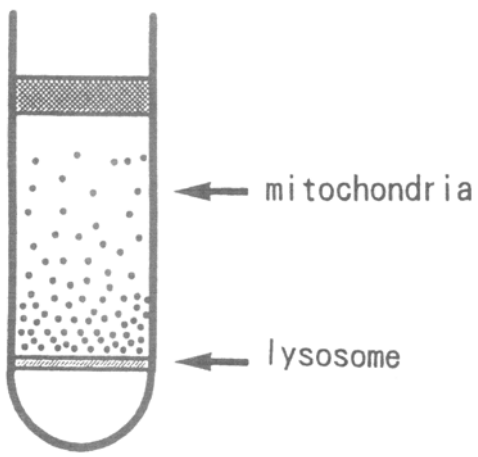

Fig. 1 Separation of mitochondria and lysosome on a percoll gradient.

Assays

The activities of arylsulfatase (for lysosome) and succinic-INT reductase (for mitochondria) were determined as markers. Arylsulfatase activity (combined activity of A and B) was determined by the method of Milsom et al.[8], while succinic-INT reductase was measured according to Morre[9]. Protein was determined by the method of Biurett[10] for the homogenate, and by the biocinchoninic acid (BCA)[11] for the lysosomal fraction.

Electron microscopy

The isolated lysosomal fraction was immediately fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 for 12 hr and postfixed with 1 % osmic acid in the cacodylate buffer for 1 hr. The fraction was routinely dehydrated in grade ethyl alcohol and propylene oxide and embedded in a mixture of epoxy resin (812) then cured overnight at 60 ℃. Seventy nm thick sections were cut with glass knives on an ultracut microtome (Reichert-Jung, Austria) after staining with uranyl acetate and lead citrate and examined with a Hitachi model H-1200 electron microscope.

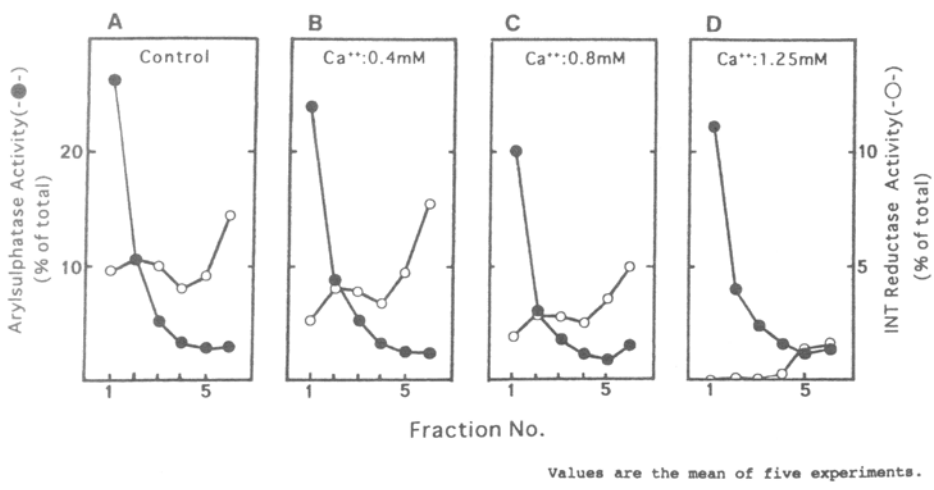

Fig. 2 Effect of Ca^{++} on the distribution profile of lysosomal and mitochondrial enzyme markers in Percoll density gradients.

Table 1 The relative specific activity of marker enzymes for lysosome (arylsulfatase).

Arylsulphatase Specific Activity (units/mg. protein)		Relative Specific Activity (Lysosome/homogenate)	Lysosomal Protein Content (mg/g tissue protein)
Homegenate	Lysosome	311.5 ± 7.8	3.0 ± 0.1
11.7 ± 0.3	3644.3 ± 83.4		

Values are mean ± SD of five exeperiments.

$$\text{Lysosomal protein content} = \frac{\text{Enzyme units/g total homogenate protein}}{\text{Enzyme units/mg lysosomal protein}}$$

Statistical analysis

The analysis of variance for multiple comparison proposed by H.Scheffe' was used for estimation of the most effective concentration of calcium for isolation of cerebral lysosomes using percoll gradients.

Results

Fig. 2 (A-D) shows the effect of calcium on the distribution profile of lysosomal and mitochondrial enzyme markers on the percoll gradient. Group A shows the control (absence of calcium), groups B, C and D show the Ca^{++} (0.4, 0.8 and 1.25 mM, respectively) incubation groups. Fraction 1 of each of the 4 groups contained the highest activity of the lysosomal marker, arylsulfatase, of any of the fractions. Although this lysosomal fraction of groups A, B and C contained a slight activity of the mitochondrial marker, succinic-INT reductase (4-10%), the same fraction of group D was almost entirely free of the activity of the mitochondrial marker (below 1%). Results of the analysis of variance on the activity of arylsulfatase of fraction 1 was not statistically significant in each group. On the other hand, results of the analysis of variance on the activity of succinic-INT reductase of fraction 1 was statistically significant in each group. Activity of succinic-INT reductase of fraction 1 of group D was the lowest level among the groups. Therefore, the most effective calcium concentration for the separation of cerebral lysosomes is 1.25 mM calcium incubation. Preparative preparation of brain lysosomes was done with 1.25 mM Ca^{++} concentration.

Table 1 shows the relative specific activity of marker enzymes for lysosomes (arylsulfatase). However, a marker enzyme of mitochondria (succinic-INT reductase) was not detected (data was not shown). Lysosomes were purified from the initial homogenate by approximately 312-fold, as determined by the specific activity of arylsulfatase. The recovery of arylsulfatase in the lysosomal fraction was 22 (21.6 ± 4.4) % that of the initial homogenate. On the other hand, the relative specific activity of succinic-INT reductase was very low as compared to the initial homogenate. These preparations showed only low levels of contamination by mitochondria. Assuming that arylsulfatase is exclusively localized in lysosomes and that the isolated lysosomes are 100% pure, we can estimate the lysosomal protein content from the specific activities of arylsulfatase in the total brain homogenate and in the isolated lysosomes. The lysosomal protein content in the brain is 3.0mg/g brain protein (Table 1).

Samples of intact lysosomes were analyzed by electron microscopy. Representative electron micrographs (x 5,000) are depicted in Fig. 3. Examination showed largely enriched vesicles with electron dense material along with a few mitochondrial particles. Therefore, this fraction is a lysosomal preparation of high purity with a little contamination by mitochondria.

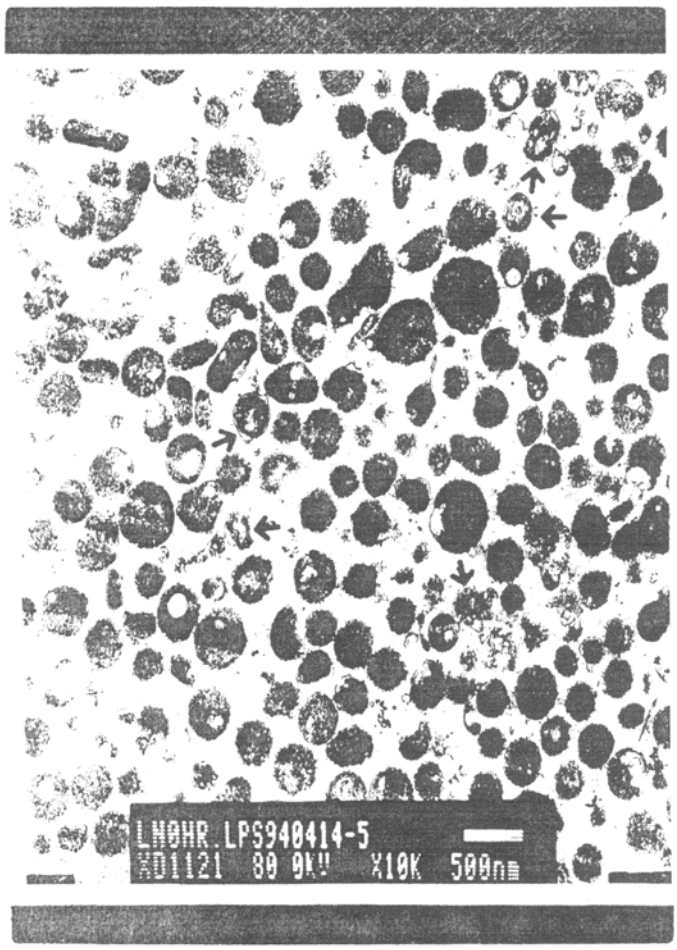

Fig. 3 Electron micrograph(x 5,000) of sample of intact lysosomes.
➡ - mitochondria

Discussion

The results reported in this paper indicate that significant quantities of rat cerebral lysosomes can be isolated with high purity. This has been made possible by introducing a new method which is based on the specific alteration of the isopycnic properties of mitochondria by calcium. The proposed mechanism is that calcium permeates the mitochondrial membrane and consequently the mitochondria swell. The swelling should cause acceleration of the sedimentation rate of mitochondria. Percoll appears to be a convenient centrifugation and helps to separate lysosomes from other particles. Calcium chloride incubation was done to change the density of mitochondria from the lysosomal fraction (Fig. 1). The most effective procedure for the separation was achieved by using 1.25 mM calcium incubation with postnuclear fraction. By using percoll density gradient centrifugation after the swelling of mitochondria in the presence of calcium, cerebral lysosomes were purified 312-fold with a recovery of approximately 22 %, which is the highest reported for any cerebral lysosomal preparation. This indicates that lysosomal protein content was approximately 3 mg protein/g brain protein. The brain lysosomal protein contents was less than the liver lysosomal protein contents (about 12 mg/g liver protein)[12]. The degree of purification previously reported for cerebral lysosomes (2-7) was markedly lower (10 to 100 fold) than the results reported here with the previously reported recovery being 4-8 %. These results may be compared with those obtained from liver using percoll gradients after swelling of mitochondria in the

presence of calcium where purification factors of 120-fold have been obtained for rat liver lysosomes with high recovery (25 %) and little contamination[13]. It appears that percoll gradient centrifugation after swelling of mitochondria by calcium can be used successfully to separate mitochondria from the lysosomal fraction in various cell types. Despite the efficiency of the separation between lysosomes and mitochondria by presently reported purification procedures, mitochondria seem to represent the major contamination of the cerebral lysosome preparation since a few mitochondria were observed on electron micrographs (Fig. 3). The disparity which exists between the degree of purity as analyzed by a biochemical marker and as determined by ultrastructural analysis is difficult to explain.

Burkart et al.[14] have reported that brain lysosomes may play a major role not only in degrading macromolecules but also in their transport to the deposition site. Therefore, obtaining purified rat cerebral lysosomes should represent an important step in the study of the generation of macromolecules which accumulate in the brain and their secretion and/or transport.

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