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Study of Lysosomal Enzymes in Fish Muscle Tissues− V Partial Purification of β−N−Acetylglucosaminidase in Carp Red Muscle and their General Property

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The present study was undertaken to partially purify β-N-acetylglucosaminidase from carp red muscle and to examine their general property. β-N-Acetylglucosaminidase is separated by DEAE-cellulose chromatography into two components, lysosomal and soluble β-N-acetylglucosaminidase. The former consists of three isoenzymes, which have the optimum pH at 4.5 with citrate buffer and at 5.5 with acetate buffer. These enzymes are inhibited by acetate, N-acetylglucosamine and N-acetylgalactosamine. The latter has the optimum pH at 6−7 with both buffers and is inhibited by N-acetylgalactosamine only. The enzyme is easily denatured by incubation at 50°C for 30 min. It is suggested that lysosomal β-N-acetylglucosaminidase is the type B enzyme of human serum and kidney reported by ROBINSON and STIRLING (1968) and that soluble β-N-acetylglucosaminidase might be the type C enzyme of human and bovine brain reported by BRAIDMAN *et al.* (1974) and OVERDIJK *et al.* (1975).

Key words : β-N-acetylglucosaminidase, lysosomal enzymes, carp muscle, purification

β-N-Acetylglucosaminidase (NAG), one of the typical lysosomal enzymes, is widely distributed in animal tissues. The enzyme participates in hydrolyzing the terminal β-N-acetylglucosaminyl links on oligosaccharides derived from glycolipids, mucopolysaccharides, mucoproteins and so on (SELLINGER *et al.* 1960, CONCHIE and HAY 1963, ROBINSON and STIRLING 1968).

In the previous paper (UENO *et al.* 1979), it has been reported that two types of NAG were found in the white and red muscles of carp, separating by differential centrifugation into two components, lysosomal and soluble NAGs. The former which had the optimum pH at 4.5 with citrate buffer was present in the lysosomal fraction, and the latter which had the optimum pH at 6.0 with acetate buffer was in the final supernatant.

The present works are undertaken to purify partially both NAGs from carp red muscle by means of DEAE-cellulose chromatography and to examine their general property.

Materials and Methods

Carp, *Cyprinus carpio,* average weight 500 g, obtained from the Tado Carp Farm (Mie). 4-Methylumbellifery-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch-Light) was used as the substrate of NAG. The other reagents used were obtained from the Wako Pure Chemical Industries, Ltd.

Preparation of enzyme: The red muscle was separated from the white, rinsed and held in 0.25 M sucrose containing 1 mM EDTA and 0.2 M KCl, pH 7.4. The muscle was cut into small pieces with scissors, a portion (usually 50 g) was suspended in 250 ml of the sucrose solution and disrupted in a Waring Blender (Sakuma Seisakusho Co. Ltd) at speed scale 2 for 30 seconds. Then, the homogenate was filtered through four layers of gauze which had been wet with the sucrose solution. The filtrate (about 240 ml) was divided into two portions. The first portion (100 ml) was centrifuged at 78,480 x g for 45 min. The resulting supernatant, the final supernatant, was kept in a freezer at −80°C and used as the soluble NAG rich source. The second portion (140 ml) was subjected to tissue fractionation, using the differential centrifugation descrided in the previous paper (UENO *et al.* 1981). The lysosomal fraction was obtained by centrifuging the mitochondrial fraction at 22, 500 x g for 10 min. The sediment was washed three times with 0.25 M sucrose solution and resuspended with 30 ml of the solution. The suspension was made to 0.1% Triton X 100 and stood at 4°C for 30 min. After centrifugation, the resulting supernatant, the lysosomal fraction, was kept in a freezer at −80°C and used as the lysosomal NAG rich source.

Enzyme assay: 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (39.0 mg, 10 mM) was dissolved in 10 ml of ethylene glycol monomethyl ether. The solution was kept in a refrigerator as the stock solution of substrate. The solution was diluted with acetate or citrate buffer to 0.2 mM when used. β-N-Acetylglucosaminidase was measured by the fluorometric method described in the previous paper (UENO *et al.* 1979). The reaction mixture consisting of 0.1 ml of the enzyme solution and 0.1 ml of the substrate solution was incubated at 37°C for 60 min. After incubation, 3.3 ml of 50 mM glycine buffer containing 5 mM EDTA, pH 10.4, was added, and the released 4-methylumbelliferone was measured at Em 460 nm and Ex 365 nm by a spectrofluorometer (Japan Spectroscopic Co. Ltd., Type FP−4). For the assay of lysosomal NAG, 0.2 M citrate buffer, pH 4.5, was used, and 0.2 M acetate buffer, pH 6.0, was used for that of soluble NAG.

DEAE−Cellulose chromatography: The enzyme solution was concentrated by ultrafiltration to a small volume (about 5-fold concentration). Then, an aliquot (5 ml) was applied to the column (1.0 × 12.0 cm), which had been equilibrated with 0.01 M phosphate buffer containing 1 mM EDTA, pH 7.0. The enzyme was eluted with a gradient consisting of 0.01 M phosphate buffer containing 1 mM EDTA and 0.3 M NaCl.

Determination of protein: Protein was determined by the method of LOWRY *et al.*

(1951). The amount of protein was expressed as bovine serum albmine.

Results

DEAE—Cellulose chromatography of NAG in carp red muscle.

Figure 1 shows the elution patterns of carp red muscle NAG on DEAE-cellulose chromatography. Three peaks I, II and III showing high activity were observed in the lysosomal fraction and the final supernatant. In addition to these peaks, a peak IV

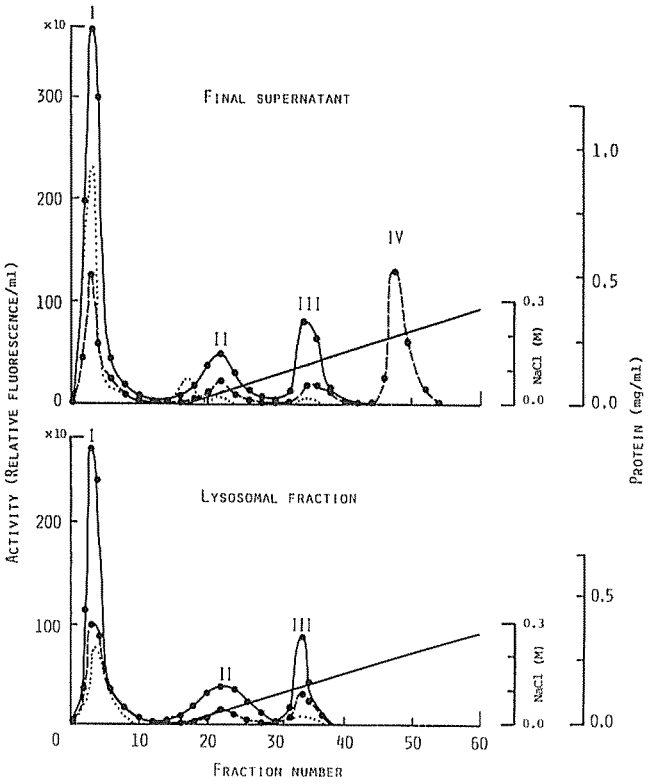

Fig. 1. DEAE-Cellulose chromatography of β-N-acetylglucosaminidase in carp red muscle.
Fraction volume was 5 ml. Activity was determined at pH 4.5 (citrate buffer) for lysosomal β-N-acetylglucosaminidase ●—● and at pH 6.0 (acetate buffer) for soluble β-N-acetylglucosaminidase ●···●. The solid and dotted lines represent the concentration of NaCl and the amount of protein, respectively. The experimental conditions are described in the text.

showing high activity was only in the final supernatant. The fractions were pooled and subjected to the following experiments.

pH—dependence of NAG activity

The activity was measured by incubating the reaction mixture of the enzyme and

the substrate solution at various pH, and the result is shown in Fig. 2. The optimum activity of peaks I－III was obtained at pH 4.5 with citrate buffer and at pH 5.5 with

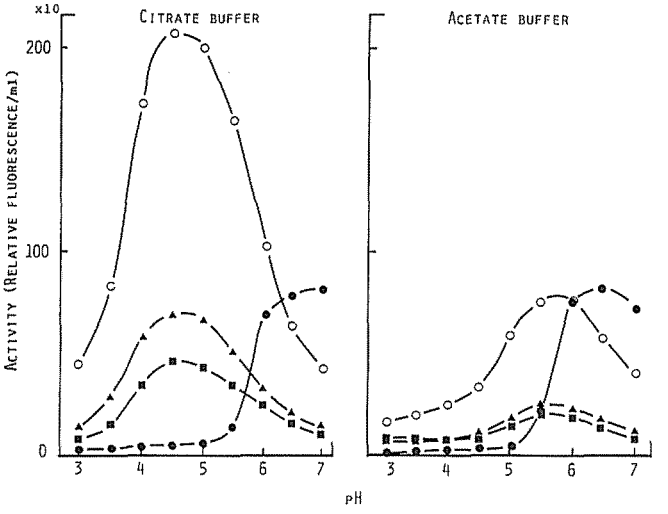

Fig. 2. Effect of pH on the activity of β-N-acetylglucosaminidase in carp red muscle. Incubation in 0.1 M citrate or acetate buffer was at 37 °C for 60 min. The β-N-acetylglucosaminidase assay are described in the text. ○ : peak I, ■ : peak II, ▲ : peak III, ● : peak IV.

acetate buffer, while that of peak IV was at pH 6－7 with both buffers. It was also observed that acetate buffer inhibited the NAG activity in peaks I－III more strongly than citrate buffer.

pH stability

As shown in Fig. 3, the activity of peaks I－III was considerably stable in the pH ranged from 3.5 to 7.0, whereas that of peak IV was lost rapidly when the pH varied from 7.0 to less than 6.0.

Heat stability

Figure 4 shows the effect of heat on the stability of NAG activity. The NAG in peak IV was denatured more easily by heat than that of peaks I－III. The residual activity of peaks I, II, III, and IV was 88, 88, 83, and 5%, respectively when exposed to incubation at 50 °C for 30 min.

Effect of citrate and acetate

It is well known that NAG is inhibited by acetate but not citrate (ROBINSON and STIRLING 1968). Figure 5 shows the effect of citrate and acetate on the activity of NAG. The activity in peak I was inhibited strongly by acetate (about 83 % inhibition by 0.1 M at the final concentration), and that in peak IV showed about 13 and 21 % inhibition with citrate and acetate, respectively.

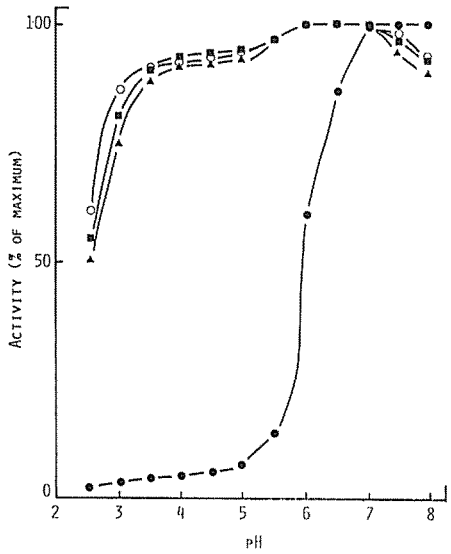

Fig. 3. Effect of pH on the stability of β-N-acetylglucosaminidase activity in carp red muscle.
The enzyme solution was buffered to the desired pH with 0.1 M citrate—0.2 M phosphate and stood at 4 °C for 30 min. Then, the residual activity was measured with the β-N-acetylglucosaminidase assay described in the text. Incubation was at 37°C for 60 min. For the assay in peaks I – III, 0.2 M citrate buffer, pH 4.5, was used and 0.2 M acetate buffer, pH 6.0, was for that in peak IV. ○: peak I, ■: peak II, ▲: peak III, ●: peak IV.

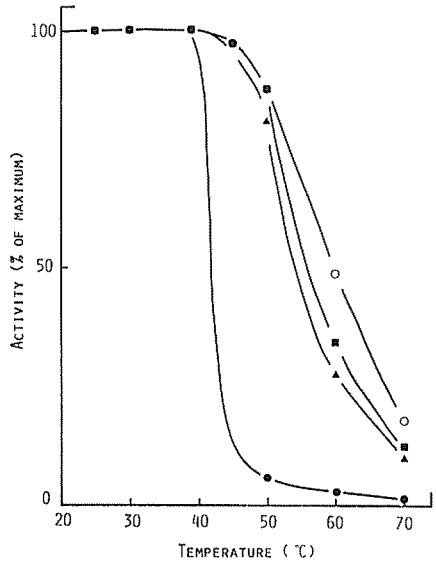

Fig. 4. Effect of heat on the stability of β-N-acetylglucosaminidase activity in carp red muscle.
The enzyme solution was heated to the desired temperature for 30 min. Then, the residual activity was measured with the β-N-acetylglucosaminidase assay described in the text. Incubation was at 37 °C for 60 min. For the assay in peaks I – III, 0.2 M citrate buffer, pH 4.5, was used and 0.2 M acetate buffer, pH 6.0, for that in peak IV. ○: peak I, ■: peak II, ▲: peak III. ●: peak IV.

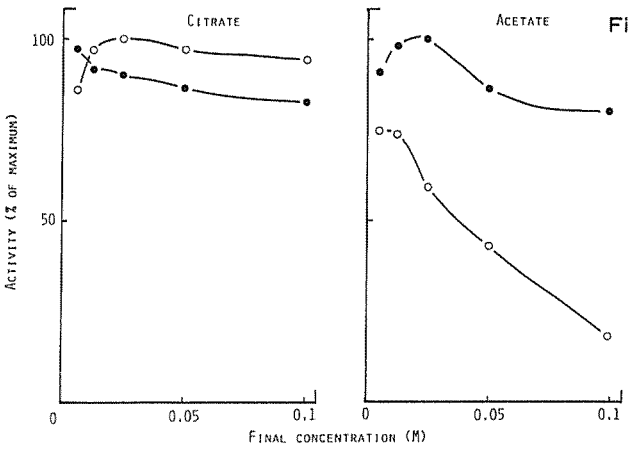

Fig. 5. Effect of citrate and acetate on the activity of β-N-acetylglucosaminidase in carp red muscle.
The enzyme solution containing various amounts of citrate or acetate was adjusted to pH 7.0 and stood at 37 °C for 30 min. Then, the residual activity was measured with the β-N-acetylglucosaminidase assay described in the text. Incubation was at 37 °C for 60 min. For the assay in peak I ○—○, 0.2 M citrate buffer, pH 4.5, was used and 0.2 M acetate buffer, pH 6.0, for that in peak IV ●—●.

Effect of N−acetylglucosamine and N−acetylgalactosamine

Figure 6 shows the effect of N-acetylglucosamine and N-acetylgalactosamine on the activity of carp red muscle NAG. There was strong inhibition of NAG activity in peak

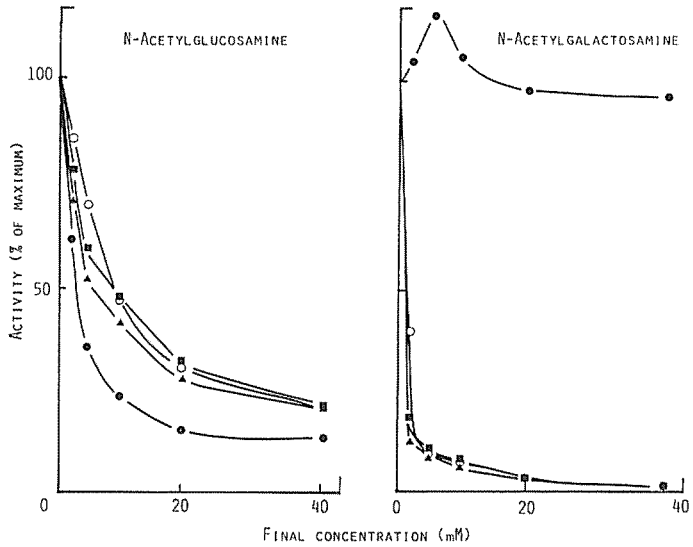

Fig. 6. Effect of N-acetylglucosamine and N-acetylgalactosamine on the activity of β-N-acetylglucosaminidase in carp red muscle.
Incubation was at 37 °C for 60 min. The β-N-acetylglucosaminidase assay are described in the text. ○ : peak I, ■ : peak II, ▲ : peak III, ● : peak IV.

I − IV by N-acetylglucosamine. N-Acetylgalactosamine also inhibited NAG activity in peaks I − III but not that in peak IV.

Effect of dialysis on the activity of NAG

Table 1. Effect of dialysis on the activity of soluble β-N-acetylglucosaminidase

Outer liquid	Soluble NAG*2 activity (RF*3 × 10/ml)	
	Before dialysis	After dialysis
1 % NaCl	153.0	3.6
1 % NaCl + 2 mM ME*1, pH 7.2	153.0	76.2
1 % NaCl + 2 mM ME + 25 mM EDTA, pH 7.2	170.4	131.7
1 % NaCl + 2 mM ME + 50 mM EDTA, pH 7.2	180.0	153.9
1 % NaCl + 2 mM ME + 100 mM EDTA, pH 7.2	195.3	162.9
1 % NaCl + 2 mM ME + 200 mM EDTA, pH 7.2	201.0	154.2

*1 2-Mercaptoethanol, *2 β-N-Acetylglucosaminidase, *3 Relative fluorescence

The enzyme solution of peak IV was dialyzed in a cellophane bag against various solution at 4 °C overnight. Then, the residual activity was measured with the β-N-acetylglucosaminidase assay described in the text. For the assay, 0.2 M acetate buffer, pH 6.0, was used. Incubation was at 37 °C for 60 min.

The preliminary experiment showed that the activity of soluble NAG but lysosomal one was lost rapidly by dialysis through a cellophane bag. Table 1 indicates the effect of dialysis on soluble NAG activity with various solutions. The activity of soluble NAG after dialysis against 1% NaCl decreased to only 2% when compared to that of the enzyme before it. High recovery of soluble NAG activity was obtained by the addition of 2-mercaptoethanol and EDTA to the outer liquid. These values were 50% for 1% NaCl containing 2 mM 2-mercaptethanol and 100% for 1% NaCl containing 2 mM 2-mercaptoethanol and 50 mM EDTA, pH 7.0.

Discussion

Regarding the heterogeneity of NAG, ROBINSON and STIRLING (1968) reported that two types of NAG, A and B, were found in human serum and kidney. Both NAGs had the optimum pH at 4.5−5.0 with citrate buffer and 5.5 with acetate buffer and were inhibited about 80% by 20 mM acetate at final concentration. Also the amino sugars, N-acetylglucosamine and N-acetylgalactosamine, inhibited strongly the activity of both NAGs. However, there was a significant difference of heat stability between types A and B. The former was more unstable than the latter when exposed to incubation at 50 °C for 180 min. They also suggested that types A and B of NAG were present in lysosomal fraction, whereas the supernatant contained type A only. On the other hand, type C of NAG was found in human and bovine brain by electrophoresis (BRAIDMAN *et al.* 1974, OVERDIJK *et al.* 1975). This enzyme had optimum activity at pH 6−7 and little inhibition of acetate. It was also easily denatured by heat.

Our results indicate that NAG in carp red muscle is separated by DEAE-cellulose chromatography into 4 components, peaks I − IV. It is suggested that the NAGs in peaks I − III might be isoenzymes. This might be explained by the fact that some property of these enzymes are very similar to each other, that is, they have the optimum pH at 4.5 and are inhibited by acetate, N-acetylglucosamine, and N-acetylgalactosamine. On the other hand, the property of NAG in peak IV is quite different from that in peaks I − III, that is, it has optimum pH at 6−7 and is inhibited strongly by N-acetylglucosamine but not by acetate and N-acetylgalactosamine.

Our data also show that the NAG in peaks I − III is mostly present in the lysosomal fraction and the final supernatant, and that in peak IV it is only in the final supernatant. This observation agrees with the results for carp white muscle (UENO *et al.* 1979).

From these experiments described above, it might be concluded that carp red muscle contains two major forms of NAG, lysosomal and soluble ones. The former might be very similar to the type B of human kidney NAG reported by ROBINSON and STIRLING (1968). The latter might be the type C like enzyme. Further work is needed to elucidate some analogy of soluble NAG in carp muscle with the type C of brain NAG in human and bovine (BRAIDMAN *et al.* 1974, OVERDIJK *et al.* 1975).

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