Bull. Fac. Fish. Mie Univ.
No. 11 : 51–56
October 1. 1984

Study of Lysosomal Enzymes in Fish Muscle Tissues–VI Distribution of β-N-Acetylglucosaminidase in Tissues of Several Fish Species

Ryuji UENO and Yoshishige HORIGUCHI
Faculty of Fisheries, Mie University

The present study was undertaken to examine the distribution of lysosomal and soluble β-N-acetylglucosaminidases in the white and red muscles of several fish species and in the internal organs of carp. Assays distinguishing between lysosomal and soluble β-N-acetylglucosaminidases were used. The additions of citrate buffer, pH 4.5 and of N-acetylgalactosamine to the enzyme solution prepared from fish tissues were for the assays of lysosomal and soluble β-N-acetylglucosaminidases, respectively. Eight species of fish : yellow tail, carp, mackerel, sardine, gizzard shad, grunt, stone flounder and yellowfin tuna were examined. The activity of both β-N-acetylglucosaminidase was found to be higher in red muscle than in white. The lysosomal β-N-acetylglucosaminidase was prominent in both muscles, but the white muscle of stone flounder had higher activity of soluble β-N-acetylglucosaminidase than that of the lysosomal. The greatest amounts of both β-N-acetylglucosaminidase activity were found in the spleen of carp. The lysosomal β-N-acetylglucosaminidase was also prominent in all organs but the blood of carp. The blood cells had approximately the same amounts of soluble β-N-acetylglucosaminidase as that of the lysosomal, and the plasma contained mostly lysosomal β-N-acetylglucosaminidase.

Key words : β-N-acetylglucosaminidase, lysosomal enzyme, fish, distribution

In previous papers (UENO *et al.* 1979 and 1984), it was reported that two types of β-N-acetylglucosaminidase (NAG), lysosomal and soluble, were found in the white and red muscles of carp. The former are the type B of NAG which has the optimum pH at 4.5, and the latter might be the type C like enzyme which has the optimum pH at 6–7.

The study reported here was undertaken to investigate the distribution of lysosomal and soluble NAGs in the tissues of several species of fish.

Materials and Methods

Fish : Yellow tail (2000 g), *Seriola quingueradiata*, and carp (500 g), *Cyprinus carpio*, were purchased from the Tado Fish Farm and the Sazara-Ura Fish Farm, Mie, respectively. Mackerel (107 g), *Scomber japonicus*, sardine (80 g), *Sardinops melanostictus*, gizzard

shad (170 g), *Clupanodon punctatus,* and grunt (130 g), *Parapristipoma trilineatum,* which had been caught by net in Ise Bay, Mie, were used. Stone flounder (100 g), *Kareius bicoloratus* and the frozen fillet of yellowfin tuna, *Neothunnes albacora,* were purchased from the Tsu Fish Market, Mie. All fish but yellowfin tunna were alive just before being used as experimental material.

Reagents: 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch-Light) was used as the substrate of NAG. The other reagents were obtained from the Wako Pure Chemical Industries, Ltd.

Preparation of enzyme: The muscles of yellowfin tuna, yellow tail, mackerel, sardine, grunt, gizzard shad, and carp were separated into white and red. In stone flounder, white muscle only was used because it has no red.

Carp tissues were prepared as follows: Carp was placed in an anesthetic of 3% ethyl carbamate. After a few minutes, about 5 ml of blood was collected from the balbus arteriosus by cylinge, which had been wetted with 0.25% heparin. The body was then dissected and the internal organs obtained were separated into heart, liver, spleen and spermary.

The tissues except the blood were rinsed with 1% NaCl and cut into small pieces. A portion of the tissues (1 g) was placed in a blender cup holding 10 ml of 0.01 M phosphate containing 50 mM EDTA, pH 7.2. The blood (5 ml) was centrifuged at 5,000 rpm for 10 min to separate the blood cells and plasma (about 3 ml). The blood cells were washed three times with 1% NaCl and suspended in 3 ml of the phosphate buffer.

The suspension obtained was homogenized by a Waring Blender (Sakuma Seisakusho Co. Ltd.) at maximum speed for 5 min. The homogenate was made to 0.1% Triton X 100 and allowed to stand at 4°C for 30 min. After centrifugation at 15,000 rpm for 30 min, the resulting supernatant was kept in a freezer at −80°C and used for the assay of NAG.

Substrate solution: 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (10 mM), which had been dissolved in ethylen glycol monoethyl ether, was kept in a refrigerator and used as the stock solution of substrate. The solution was diluted with acetate or citrate buffer to be 0.2 mM just before using.

Assays distinguishing between lysosomal and soluble NAGs in fish tissues were carried out as follows:

Assay of lysosomal NAG: To 0.05 ml of the enzyme solution, 0.05 ml of 0.2 M citrate buffer, pH 4.5, was added and preincubated at 37°C for 30 min. After preincubation, 0.1 ml of the substrate solution diluted with the buffer containing 0.3 M NaCl was added and incubated at 37°C for 30 min. Then, 3.3 ml of 50 mM glycine buffer containing 5 mM EDTA, pH 10.4, was added to the reaction mixture. The released 4-methylumbelliferone was measured at Em 460 nm and Ex 365 nm by a spectrofluorometer (Japan Spectronic Co. Ltd., Type FP−4).

Assay of soluble NAG: To 0.05 ml of the enzyme solution was added 0.05 ml of 80

mM N-acetylgalactosamine and preincubated at 37°C for 30 min. After preincubation, 0.1 ml of the substrate solution diluted with 0.2 M acetate buffer containing 0.3 M NaCl, pH 6.0, was added and incubated at 37°C for 30 min. Then, the glycine buffer was added to the reaction mixture, and the released 4-methylumbelliferone was measured by a spectrofluorometer.

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 and Discussion

Examination of assays distinguishing between lysosomal and soluble NAGs in fish tissues

In a previous paper (UENO *et al.* 1984), it was reported that lysosomal and soluble NAGs of carp muscle differed in pH stability, pH optima and inhibition behaviour by acetate and N-acetylgalactosamine. In the present experiment, assays distinguishing between both NAGs in carp muscle was examined by using the differences in the enzymatic property of both NAGs. An extract of carp red mucle was used as the enzyme solution. The assay conditions are described in Materials and Methods.

The acid treatment (the addition of 0.2 M citrate buffer, pH 4.5, to the enzyme solution) was used to denature soluble NAG, and the amino sugar (the addition of 80 mM N-acetylgalactosamine to the enzyme solution) was for the inactivation of lysosomal NAG. The activity ratio of lysosomal to soluble NAG in the red muscle of carp was about 5 : 1 when measured by these assays. This value was approximately the same as that of the ratio which was obtained from the elution pattern of both NAGs in carp red muscle by DEAE-cellulose chromatography (UENO *et al.* 1984).

Various solvents for extraction of NAG from fish tissues

Table 1 shows the extraction of lysosomal and soluble NAGs by various solvents.

Table 1. Various solvents for extraction of β-N-acetylglucosaminidase from carp red muscle

Solvents	Lysosomal NAG*2 activity		Soluble NAG activity	
	RF*1 x10/min/g tissue	RF/min/mg prot.	RFx10/min/g tissue	RF/min/mg prot.
Distilled water	174.7	120.4	3.2	2.2
50 mM EDTA	248.1	79.1	28.3	9.1
1% NaCl	235.3	83.1	3.4	2.1
1% NaCl + 50 mM EDTA, pH 7.2	307.2	67.8	34.4	7.6
0.01 M phosphate buffer, pH 7.2	288.3	111.8	25.9	10.0
0.01 M phosphate buffer + 50 mM EDTA, pH 7.2	303.9	86.0	40.3	11.5
0.25 M sucrose + 1 mM EDTA + 0.2 M KCl, pH 7.2	257.3	66.7	17.9	4.9

*1 Relative fluorescence, *2 β-N-acetylglucosaminidase.

The highest specific activity of lysosomal NAG was obtained from the extract with distilled water, but its yield was low. With 0.01 M phosphate buffer, pH 7.2, and the same buffer containing 50 mM EDTA, a high specific activity and yield of lysosomal NAG were obtained from their extracts.

The highest specific activity of soluble NAG was observed in the extract with 0.01 M phosphate buffer containing 50 mM EDTA, pH 7.2, and its yield was also high. It is suggested that the addition of EDTA to the solution may be needed not only for stabilizing the NAG activity to dialysis, which is described in a previous paper (UENO *et al.* 1984), but also to facilitate a good extraction from fish tissues. Thus, as the solvent for extraction of both NAGs, 0.01 M phosphate buffer containing 50 mM EDTA pH 7.2, was used in the following experiments.

Distribution of lysosomal and soluble NAGs in muscle of several fish species

Table 2 shows the enzymatic activity of lysosomal and soluble NAGs of muscle extracts from several fish species.

Table 2. Distribution of β-N-acetylglucosaminidase in muscle of several fish species

Fish species		Lysosomal NAG[*4] activity		Soluble NAG activity	
		RF[*3]/min/ml	RF/min/mg prot.	RF/min/ml	RF/min/mg prot.
Yellowfin tuna	WM[*1]	370.7	77.4	9.8	2.0
	RM[*2]	381.7	50.6	35.0	4.6
Yellow tail	WM	141.3	23.9	17.9	3.0
	RM	327.3	62.7	35.2	6.7
Mackerel	WM	213.2	40.8	10.9	2.1
	RM	432.1	82.8	52.8	10.5
Sardine	WM	131.0	36.4	6.9	1.9
	RM	273.3	99.9	17.2	6.2
Grunt	WM	99.8	19.0	8.2	1.6
	RM	571.8	123.0	42.7	9.2
Gizzard shad	WM	368.3	70.6	14.5	2.8
	RM	621.8	113.0	38.6	7.0
Stone flounder	WM	99.0	15.2	185.9	28.5
Carp	WM	485.3	98.0	57.7	11.6
	RM	567.8	145.2	120.7	30.9

[*1] White muscle, [*2] Red muscle, [*3] Relative fluorescence, [*4] β-N-Acetylglucosaminidase.

Lysosomal NAG: The high activity of lysosomal NAG was found in the red muscle of all species. The high order of the NAG activity in fish muscle was as follows: gizzard shad > grunt > carp > mackerel > yellowfin tuna > yellow tail > sardine in red muscle; and carp > yellowfin tuna > gizzard shad > mackerel > yellow tail > sardine > grunt > stone flounder in white muscle.

Soluble NAG: The high activity of soluble NAG was also found in the red muscle of all species but stone flounder. The high order of the NAG activity in fish muscle

was as follows: carp > mackerel > grunt > gizzard shad > yellow tail > yellowfin tuna > sardine in red muscle; and stone flounder > carp > yellow tail > gizzard shad > mackerel > yellowfin tuna > grunt > sardine in white muscle. A considerably higher activity of the NAG was in the white muscle of stone flounder.

The data also indicate that lysosomal NAG was prominent in the white and red muscles of fish species used here, but only the white musle of stone flounder had higher activity of soluble NAG than that of the lysosomal.

Generally, it appears to be difficult to compare the activity of enzymes in fish tissues because of the individual, seasonal, and environmental variations among fish species. In this experiment, it was difficult to determine the correlation in the muscle NAG activity among them.

Distribution of lysosomal and soluble NAGs in various organs of carp

As shown in Table 3, greater amounts of both NAG activity were found in spleen

Table 3. Distribution of β-N-acetylglucosaminidase in the internal organs of carp

Organs	Lysosomal NAG*2 activity		Soluble NAG activity	
	RF*1 x10²/min/ml	RF/min/mg prot.	RFx10²/min/ml	RF/min/mg prot.
Liver	852.0	134.0	8.3	1.3
Heart	52.2	14.2	2.1	0.6
Spleen	2012.0	175.0	16.7	1.5
Spermary	145.0	46.6	5.6	1.8
Blood cells	18.5	0.2	20.0	0.2
Plasma	1.5	0.05	0.3	0.001
White muscle	4.9	1.0	0.6	0.1
Red muscle	5.7	1.5	1.2	0.3

*1 Relative fluorescence. *2 β-N-Acetylglucosaminidase.

and liver which contain various mucopolysaccharides consisting of hexsoses, hexsosamines and so on. KOIZUMI and NAKAMURA (1973) reported that the spleen, liver, and kidney of rats contained higher activity of mucopolysaccharases than the other tissues. Therefore, it is suggested that NAG participates in the metabolisms of mucopolysaccharides in these tissues.

The data show that lysosomal NAG was prominent in all tissues but blood. The blood cells had approximately the same amount of soluble NAG as that of the lysosomal, and the plasma contained mostly lysosomal NAG activity. The presence of soluble NAG in the plasma might be due to contamination by hemolysis at the time of collecting the blood from the carp.

Several isoenzymes of NAG were found in human serum and named: types B, I, I_2 and A of NAG, listed in the order of decreasing isoelectronic points (ROBINSON and STIRLING 1968, PRICE and DANCE 1972). These enzymes have an acidic pH optima and

might be similar to lysosomal NAG of carp. On the other hand, it has been reported that type C of NAG having a more neutral pH optima is present in various tissues except the blood cells of human (SWALLOW *et al.* 1976, BEUTLER and KUHL 1977). This enzyme might be soluble NAG in the blood cells and the red muscle of carp. It is interesting that considerable amounts of soluble NAG are present in the blood cells but not in the plasma of carp. Further work on soluble NAG will be concerned with the elucidation of the biological function of the enzyme in various fish species.

References

BEUTLER, E. and W. KUHL, 1977. The tissue distribution of hexosaminidase S and hexosaminidase C. *Ann. Hum. Genet., Lond.*, **41**: 163－167.

KOIZUMI, T and N. NAKAMURA, 1973. Internal organs. The Experimental Methods of Mucopolysaccharides－II. (ed. by Z. YOSHIZAWA and E. HASEGAWA), *Kagaku no Ryoiki*, Nanko-Do, Tokyo, **99**: 45－60 (In Japanese).

LOWRY, O. H., N. J. ROSEBROUGH, A. J. FARR and R. J. RANDALL, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**: 265－275.

PRICE, R. G. and N. DANCE, 1972. The demonstration of multiple heat stable forms of N-acetyl-β-glucosaminidase in normal human serum. *Biochim. Biophys. Acta*, **271**: 145－153.

POBINSON, D. and J. L. STIRLING, 1968. N-Acetyl-β-glucosaminidase in human spleen. *Biochem. J.*, **107**: 321－327.

SWALLOW, D. M., L. EVANS, N. SAHA and H. HARRIS, 1976. Characterization and tissue distribution of N-acetylhexosaminidase C: suggestive evidence for a separate hexosaminidase locus. *Ann. Hum. Genet., Lond.*, **40**: 55－66.

UENO, R., T. MORISHITA and T. TAKAHASHI, 1979. Study of lysosomal enzymes in fish muscle tissue－I. β-N-Acetylglucosaminidase in carp muscle. This Bull., **6**: 209－216 (In Japanese).

————, ———— and ————, 1984. Study of lysosomal enzymes in fish muscle tissue-V. Partial purification of β-N-acetylglucosaminidase in carp red muscle and their general property. *Ibid*, 11: 41－48.