魚類血液における β-N- アセチルグルコサミニダーゼの分布

誌名	日本水産學會誌
ISSN	00215392
著者	上野, 隆二 袁, 崇生 堀口, 吉重
巻/号	54巻1号
掲載ページ	p. 95-101
発行年月	1988年1月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

AgriKnowledge

Nippon Suisan Gakkaishi 54(1), 95–101 (1988)

Study of the Distribution of β-N-Acetylglucosaminidase in Fish Blood*[1]

Ryuji Ueno,*[2] Chongsheng Yuan,*[2] and Yoshishige Horiguchi*[2]

(Accepted August 19, 1987)

A distribution of β-N-acetylglucosaminidase in the fish blood of various species was investigated. The following results have been obtained: 1) β-N-Acetylglucosaminidases, both a neutral form and acidic forms, were demonstrated to have a wide distribution in fish blood of various species. However, the neutral form could not be detected from human and mammalian blood. 2) Carp blood neutral β-N-acetylglucosaminidase was localized in the cytosol of the red blood cells and the acidic forms of the enzyme were in the white blood cells. 3) There existed at least three different forms of β-N-acetylglucosaminidase in carp blood, one neutral form and two acidic forms. 4) With respect to the electrophoretical mobility, behavior of the isoenzymes in DEAE-cellulose chromatography and heat-stability, the neutral form was confirmed to correspond to Hexosaminidase C, and the acidic forms-1 and -2 may be related to Hexosaminidases B and A, respectively.

In human and various other mammalian tissues, β-N-acetylglucosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) exists in two major forms named Hexosaminidases A and B, which are localized in lysosome, have a common activity optimum at pH 4.5 and similar molecular weights, but they could be distinguished by electrophoretical mobility and thermostability. The form A shows greater anodal mobility and more heat-labile than the form B. These two forms have been extensively studied because of their involvement in the GM_2-gangliosidoses. In Tay-Sachs disease, the form A is deficient[1] and in Sandhoff's disease, both A and B are missing.[2] Besides the two forms, a neutral form named Hexosaminidase C has been from time to time reported.[3,4] This neutral form is described to be present in cytosol, have a pH optimum between 6 and 7 and migrate more faster than form A.[5,6] However, up to now, less has been known about its physiological significance and natural substrate. The interrelationships between the isoenzymes are still less certain though it could be detected in the tissues of a patient with Sandhoff's disease.

In our previous communications,[7,8] we have reported that a neutral β-N-acetylglucosaminidase in carp blood has been purified and characterized. The enzyme was inferred to correspond to Hexosaminidase C with respect to its optimum pH of 6.5 and some other characteristics. However, its further informations in physiology or in pathology have not been obtained yet. Though it has been known that in carp tissues, as in human and other mammalian tissues, β-N-acetylglucosaminidase also exists in multiple forms (acidic form and neutral form),[9] the interrelationships between the isoenzymes remain unclear. Systematic exploration for clarifying the above problems is therefore becoming increasingly desirable. The present work, as the first step for elucidating the biochemical function of the isoenzymes and their interrelations, describes the investigations on distribution of the isoenzymes in the blood of fish species and their subcellular locations in the carp blood. The electrophoretic mobility and heat stability of the isoenzymes were also compared.

Materials and Methods

Carp *Cyprinus carpio* with average body weight of 1 kg, was from Tado Carp Farm, and other fish species used were purchased from Tamafuku Fishery Group, Mie, Japan. All of the fish species were alive before being used. Mammalian blood were from the Domestic Animal Center, Mie, and normal human blood was obtained from the Blood Test Center, Faculty of Medicine, Mie University. Percoll for density gradient centrifugation, Density Marker Beads for calibration of

*1 Studies of Lysosomal Enzymes in Fish Muscle Tissue—IX.

*2 Faculty of Fisheries, Mie University, Tsu, Mie 514, Japan (上野隆二, 袁 崇生, 堀口吉重: 三重大学水産学部).

gradients of Percoll were purchased from Pharmacia Fine Chemicals Co., Ltd. DEAE-Cellulose was from Seikagaku Kogyo, Co., Ltd. 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside was purchased from Koch-Light, Ltd. All other reagents used were of analytical grade or of the best grade available.

Preparation of Blood Samples

A) For blood survey, blood samples were taken from dorsal aorta of various fish with heparinized plastic syringes and used for enzyme assay immediately. B) For separation of blood cells, a volume of the carp blood was withdrawn into a plastic syringe already containing an equal volume of heparine solution in 0.15 M NaCl (30 U/m*l*), resulting in a 50% heparinized blood. C) For chromatography on DEAE-cellulose, the carp blood taken from the dorsal aorta with heparinized plastic syringe was homogenized in an equal volume (v/v) of 10 mM sodium phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol with the presence of 0.2% Triton X-100 using a Waring Blender (Sakuma Seisakusho, Japan). The homogenate was then centrifuged at $18{,}000 \times g$ for 30 min and the supernatant was used.

Separation of Blood Cells on Density Gradient Centrifugation

The method described by Pertoft *et al.*[10] was followed. A stock isoosomotic Percoll solution was first made by mixing 9 vol of Percoll with 1 vol of 1.5 M NaCl. Eleven volumes of this stock solution were then mixed with 5 vol of 0.15 M NaCl. The density of the resulting Percoll solution was 1.086 g/m*l*. The centrifuge tubes (1.6×7.5 cm) were filled with 8 m*l* of the Percoll solution and the gradient was performed by spinning in a 26° angle rotor at $20{,}000 \times g$ for 20 min. One and a half m*l* of 50% heparinized blood was laid on the top of gradient and centrifuged for 10 min at $400 \times g$. The upper layer (plasma layer) of 1.5 m*l* was then removed and replaced by 1.5 m*l* of Percoll solution with lower density ($\rho=1.040$ g/m*l*) and the centrifugation was continued at $800 \times g$ for a further 15 min. The position and densities of the banded cells were monitored using Density Marker Beads in an identical gradient contained in a second centrifuge tube. The gradient was fractionated by aspiration from above in a fraction size of 0.5 m*l*. The number of cells in each fraction was determined in a electronic cell-counter (ACCU-STAT Blood Cell Counter, Clay Adams, U.S.A.).

DEAE-Cellulose Chromatography

The enzyme extract (4 m*l*) prepared as described above was added to a column (2.1×12 cm) of DEAE-cellulose equilibrated with 10 mM phosphate buffer pH 7.0, containing 10 mM 2-mercaptoethanol. After eluting the column with 2 bed-volumes of the same buffer at a flow rate of 35 m*l*/h, a linear NaCl gradient from 0 to 0.3 M in the same buffer was then used to develop the column. The fractions of 4.3 m*l* were collected. The protein was monitored at 280 nm.

Sample Pretreatment for Electrophoresis

A) The unadsorbed fractions from DEAE-cellulose chromatography were pooled and adjusted to pH 4.0 by 1 M citrate acid and then incubated at 37°C for 30 min. The hemoglobin detrimental to electrophoresis was aggregated and removed by centrifugation. The supernatant was readjusted to pH 7.0 with the phosphate buffer. B) The white blood cell fractions from the density gradient centrifugation were diluted with saline and centrifuged at $18{,}000 \times g$ for 30 min to collect the cells and remove the particles of Percoll. The white blood cell suspension was sonicated with a sonicator (Model W-375, Heat Systems Ultrasonics Inc., New York) for 2 min bursts and then centrifuged at $25{,}000 \times g$ for 30 min, the supernatant was used for electrophoresis. C) The red blood cell fractions were hemolyzed hypotonically at 4°C for 15 min and centrifuged at 18,000 $\times g$ for 30 min. The supernatant was passed through a small DEAE-cellulose column (1.6×5 cm) equilibrated with the phosphate buffer, pH 7.0, to eliminate the unadsorbed hemoglobin fraction which showed no activity both at pH 4.5 and 6.5. The adsorbed fractions were then recovered by washing the column with the same buffer containing 2 M NaCl and desalted by gel filtration on a column (1.6×5 cm) of Sephadex G-25 before used for electrophoresis.

Electrophoresis

Polyacrylamide disc gel electrophoresis was carried out as described by Davis and Ann.[11] The run was made in a 5.6% polyacrylamide gel at a current of 2 mA per disc column with Tris-glycine buffer, pH 8.3. For each sample, two disc gels were run stimultaneously, the one for activity stain at pH 4.5 and the other at pH 6.5. After electrophoresis, the isoenzymes were located by staining with 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside as substrate. For the neutral form, the gels were soaked in 0.8 mM

substrate of pH 6.5 and for acidic forms, soaked in the substrate of pH 4.5. After incubation at 37°C for 30 min, the substrate solution was replaced with 50 mM glycine buffer, pH 10.4, and then the fluorescent bands were visualized under a compact 4-WATT UV lamp (San Gabriel, U.S.A.).

Enzyme Assays

4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside was used as substrate as described in our previous papers.[7,8] For the neutral form, the activity was assayed in a total volume of 0.2 m*l* mixture containing 0.02 m*l* of enzyme source, 0.08 m*l* of 80 mM *N*-acetylglucosamine and 0.1 m*l* of 0.4 mM substrate solution with pH 6.5. For the acidic forms, to 0.02 m*l* of the enzyme source were added 0.08 m*l* of 0.2 M citrate buffer, pH 4.5, and 0.1 m*l* of 0.4 mM substrate solution with pH 4.5. The reaction mixtures were incubated for 30 min at 37°C and then terminated by the addition of 50 mM glycine buffer pH 10.4, containing 50 mM EDTA. The liberated 4-methylumbelliferones were determined at Ex 365 nm and Em 460 nm by a spectrofluorometer (Japan Spectroscopic Co., Ltd. Type FP-4). When intact cells were required in the enzyme assays, sodium chloride was used to adjust the osmotic pressure of the reaction mixtures to isoosmotic condition.

Results and Discussion

The distribution of β-*N*-acetylglucosaminidase, both neutral and acidic forms, over carp tissues has been investigated, and the blood was found to contain much more amounts of neutral form than other internal organs.[12] In the present work, a further study on the distribution of the isoenzymes over the blood of various fish species was carried out. The result demonstrated that both neutral and acidic β-*N*-acetylglucosaminidases had a wide distribution in most of the tested fish blood (Table 1). In some of fish blood like carp and crucian carp, the neutral form accounted for more than 50% of the total activities, this was much greater than that of 2.5% in human brain tissues.[3] As a comparison, human and some other mammalian blood were also examined. But no neutral β-*N*-acetylglucosaminidase activity could be detected from these blood.

Table 1. Relative amounts of neutral and acidic β-*N*-acetylglucosaminidases in fish and mammalian blood

Species	Neutral NAG*1 activity R.F*2 ×10³/m*l*	Acidic NAG activity R.F ×10³/m*l*
Crucian carp *Carassius auratus*	182.5	53.8
Grunt *Plectorhynchus cinctus*	160.5	310.0
Carp *Cyprinus carpio*	145.5	115.1
Crescent *Theraton oxyrhynchus*	133.5	140.7
Stone flounder *Kareius bicoloratus*	122.5	185.0
Rabbitfish *Siganus fuscescens*	116.1	320.7
Red sea bream *Chrysophrys major*	107.5	139.0
Bambooleaf wrasse *Pseudolabrus japonicas*	103.8	281.3
Javelinfish *Parapristeipoma trilineatum*	90.0	312.5
Thornfish *Terapon jarbua*	87.5	87.2
Yellow tail *Seriola quinqueradiata*	67.5	109.1
Filefish *Sdephanolepis cirrhifer*	46.0	227.1
Horse mackerel *Trachurus japonicus*	45.2	99.3
Greenlings *Hexagramos otakii*	43.8	46.0
Truebass *Epinephelus septemfasciatus*	42.5	62.0
Bovine	—*3	127.5
Pig	—	86.3
Sheep	—	25.5
Human	—	25.2

*1 β-*N*-acetylglucosaminidase; *2 relative fluorescence; *3 not detected.

Enzyme Localities in Carp Blood

In order to determine the locations of the isoenzymes in fish blood, carp blood was used as a represent for localization test. The blood cells were separated into 3 main bands on density gradient centrifugation (Fig. 1). The upper band (ρ=1.033−1.042 g/m*l*) contained mainly mononuclear cells, like lymphocytes, the middle band mainly the granulocytes (ρ=1.076−1.088 g/m*l*) and the densest the erythrocytes (ρ=1.10−1.118 g/m*l*). The superficial fraction, *i.e.* the plasma layer, showed no activity both at pH 4.5 and 6.5. The neutral form of the enzyme was located in the red blood cells. Moreover, the enzyme was found in their cytosol because the enzyme was completely released from the cells by hypotonical hemolysis. The result coincides with that for Hexosaminidase C reported to be localized in cytosol of human and bovine brain tissues.[3,4] On the other hand, the acidic forms were only localized in the white blood cells. The acidic forms seemed

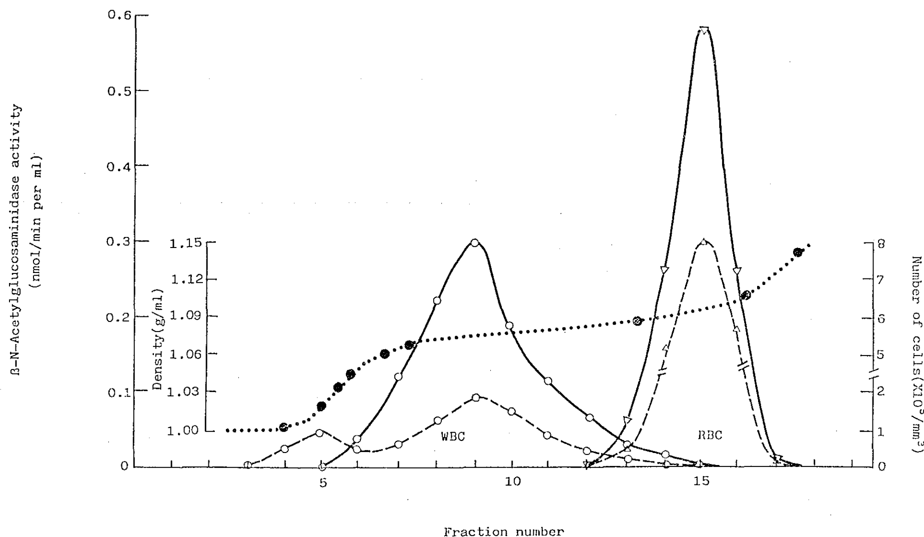

Fig. 1. Separation of carp blood cells in a gradient of Percoll. The tubes were filled with 8 m*l* of Percoll (ρ=1.086 g/m*l*), the gradient was preformed by spinning in a 26° angle rotor at 20,000 ×g for 15 min, and then 1.5 m*l* of 50% heparinized blood was layed on top of the gradient. Centrifugation was continued as described in the text. ○——○ acidic activity, ▽——▽ neutral activity, ○— — —○ white blood cells, △— — —△ red blood cells, ●------● density.

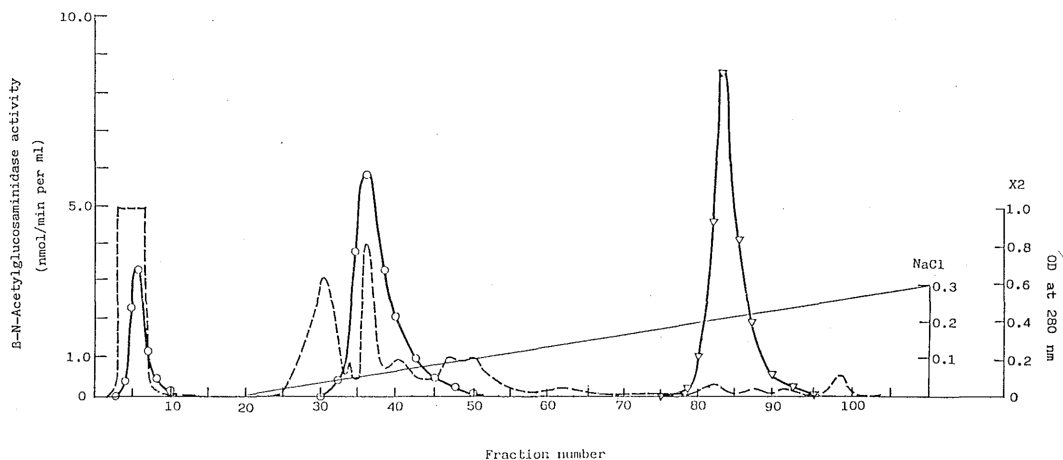

Fig. 2. DEAE-Cellulose chromatography of carp blood *β-N*-acetylglucosaminidases. Four m*l* of carp blood homogenate was added to the column of DEAE-cellulose (2.1 × 12 cm) equilibrated with 10 mM phosphate buffer, pH 7.0, containing 10 mM 2-mercaptoethanol. A linear gradient of NaCl from 0 to 0.3 M was introduced. The elution rate was 35 m*l*/h and 4.3 m*l* fractions were collected. — — — protein at 280 nm, ○——○ acidic activity, ▽——▽ neutral activity.

not to be localized in the cell organelle like lysosome in which Hexosaminidases A and B are reported to be localized.[13,14] but appeared to be cell membrane-bound enzymes as described by Munn *et al.*[15] This is in the view of the fact that the acidic forms of the enzyme couldn't be released easily from the white blood cells by hemolysis even for a long time. Sonication or homogenization was necessary for their release. In addition, the activity of the acidic forms of the enzyme was expressible from the intact white blood cells while the neutral form was inexpressible from the intact red blood cells. This phenomenon is considered to be of some physiological significance to be studied.

The isoenzymes of *β-N*-acetylglucosaminidase

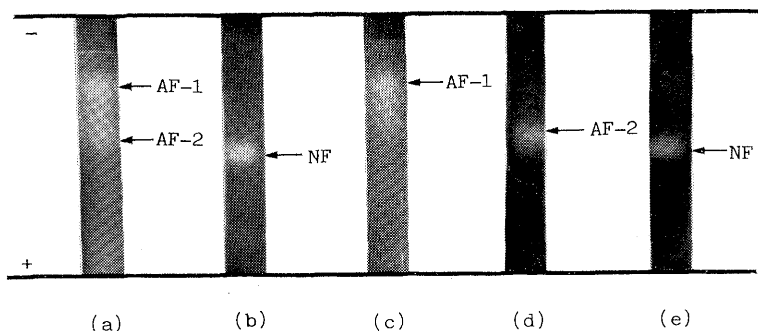

Fig. 3. Polyacrylamide disc electrophoresis of carp blood β-*N*-acetylglucosaminidase. The enzymes were visualized under a UV lamp after incubating the gels in 4-methyl-umbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside at pH 4.5 and 6.5. a) white blood cell extract, b) red blood cell extract, c) acidic form-1, d) acidic form-2, e) neutral form. AF-1: acidic form-1, AF-2: acidic form-2, NF: neutral form.

were usually separated and identified by DEAE-cellulose chromatography, electrophoresis and heat stability.[14,16,17,18] In this experiment, the multiple form existances of the enzyme in carp blood were also examined and identified by the above methods.

Chromatography on DEAE-Cellulose

As shown in Fig. 2, DEAE-cellulose resolved the carp blood β-*N*-acetylglucosaminidase into 3 main forms, two acidic forms and one neutral form. One of the acidic form was not adsorbed by the column and was named acidic form-1. The other was eluted at a NaCl concentration from 0.05 to 0.1 M and was named acidic form-2. The neutral form was eluted at a higher NaCl concentration from 0.2 to 0.22 M. The content ratio of the three forms was that acidic form-1: acidic form-2: neutral form was equal to 1: 6: 7.5. The elution pattern of the acidic forms was very similar to those of Hexosaminidases A and B.[19,20]

Electrophoresis on Polyacrylamide Gel

White blood cell and red blood cell fractions from density gradient centrifugation, and acidic forms-1, -2 and neutral form from DEAE-cellulose chromatography were all submitted to electrophoresis. As seen in Fig. 3, in the extract of the white blood cells, it showed two discrete fluorescent bands at pH 4.5 (Fig. 3a), but no fluorescent spot could be detected when stained at pH 6.5. However, in the red blood cell extract, it showed only one fluorescent band at pH 6.5 (Fig. 3b) and no fluorescent spot at pH 4.5. Both the acidic forms-1 and -2 exhibited one main fluorescent band at pH 4.5 (Fig. 3 c, d). The form-1 corresponded to the slower band shown in the white

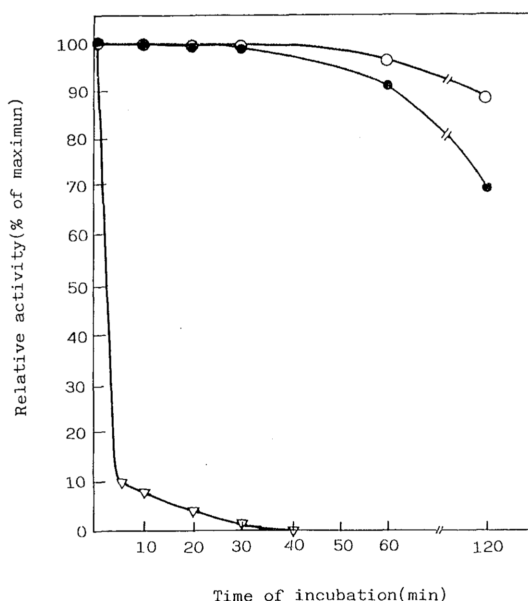

Fig. 4. Effect of temperature on the stability of neutral and acidic β-*N*-acetylglucosaminidases in carp blood. The enzymes were incubated at 50°C without the presence of substrate for durations as indicated. At the end of each incubation time, the tubes were placed in ice and then the residual activities were assayed as described in the text. ▽—▽ neutral activity, ○—○ acidic form-1 activity, ●—● acidic form-2 activity.

blood cell extract, and the form-2 to the faster band. The neutral form showed only one fluorescent band (Fig. 3e) corresponding to the band shown in the red blood cell extract. The relative electrophoretic mobilities of the three forms showed a strong resemblance to those of the isoenzymes in human and other tissues.[3,18] That is, the form-2 migrated faster than form-1 while

the neutral form was more anodic than both the two acidic forms.

Heat Stability

As shown in Fig. 4, the neutral form was very heat-labile and when heated at 50°C for 10 min, it lost more than 90% of its activity. In the contrary, both acidic form-1 and form-2 were more heat tolerable. When heated at 50°C for 30 min, they didn't suffer a loss in activities, but for 120 min, the form-2 lost more activity (about 30%) than form-1 (about 10%). Hexosaminidase B has been reported to be more heat stable than Hexosaminidase A which loses 90% of its activity when heated at 50°C for 120 min.[21,22]

With respect to the above results (behaviors in DEAE-cellulose, electrophoretic mobility and heat stability), the carp blood neutral β-*N*-acetylglucosaminidase (in red blood cells) is confirmed to be related to Hexosaminidase C, and the form-1 and form-2 (in white blood cells) are considered to be Hexosaminidase B- and Hexosaminidase A-like enzymes, respectively, though the form-2 showed to be more heat stable than Hexosaminidase A. This difference could conceivably be due to the different species.

Many authors have reported from time to time that the neutral β-*N*-acetylglucosaminidase is under separate genetic control from its acidic isoenzymes with respect to their different properties such as activity pH optimum, molecular weight, electrophoretic behavior, substrate specificity and so on.[5,13,17] Our results provided further clear evidence supporting and expanding the notion that the neutral form is indeed coded at a separate gene locus from its acidic isoenzymes and performs an independent function based on the fact the neutral form is localized in the red blood cells while the acidic forms are localized in the white blood cells which play a completely different role from red blood cells on physiology. Furthermore, the neutral β-*N*-acetylglucosaminidase is absent in some mammalian blood in which do exist the acidic forms.

An interesting and significant problem resulted from this study is why the neutral β-*N*-acetylglucosaminidase exists in most of fish blood but is not present in human and other mammalian blood. On the other hand, in white blood cell of human, there also exists two acidic forms, Hexosaminidases A and B as reported by Orlacchio *et al.*[23] These facts indicate that the differences between fish blood and mammalian blood may be mainly in red cells other than in white cells which may be similar in physiological functions both in fish blood and mammalian blood.

Further research approaching to elucidation of the biological functions of the isoenzymes would be necessary and desirable.

Acknowledgement

We express our heartfelt thanks to Mr. Kazuaki Uno, Faculty of Fisheries, Mie University, for his helping us with photograph of electrophoresis.

References

1) S. Okada and J. S. O'Brien: *Science*, **165**, 698–700 (1969).
2) K. Sandhoff, U. Andreae, and H. Jatzkewitz: *Life Sci.*, **7**, 283–288 (1968).
3) E. Penton, L. Poenaru, and C. Dreyfus: *Biochim. Biophys. Acta*, **391**, 162–169 (1975).
4) B. Overdijk, W. M. J. Van Der Kroef, G. J. Van Steijn, and J. J. W. Lisman: *Biochim. Biophys. Acta*, **659**, 255–266 (1981).
5) I. Braidman, M. Carroll, N. Dance, and D. Robinson: *Biochem. J.*, **143**, 295–301 (1974).
6) T. Izumi and K. Suzuki: *Biochim. Biophys. Acta*, **615**, 402–413 (1980).
7) R. Ueno, C. Yuan, and Y. Horiguchi: *Nippon Suisan Gakkaishi*, **53**, 1009–1016 (1987).
8) R. Ueno, C. Yuan, and Y. Horiguchi: *Nippon Suisan Gakkaishi*, **53**, 1017–1024 (1987).
9) R. Ueno, T. Morishita, and T. Takahashi: *Bull. Fac. Fish. Mie Univ.*, **6**, 209–216 (1979).
10) H. Pertof, M. Hirtenstein, and L. Kagedal: *Biochemistry*, **9**, 67–80 (1979).
11) B. J. Davis and N. Y. Ann: *Acad. Sci.*, **121**, 404 (1964).
12) R. Ueno and Y. Horiguchi: *Bull. Fac. Fish. Mie Univ.*, **11**, 51–56 (1984).
13) J. E. Wiktorowicz, Y. C. Awasthi, A. Kurosky, and S. K. Srivastava: *Biochem. J.*, **165**, 49–53 (1977).
14) D. Robinson and J. L. Stirling: *Biochem. J.*, **107**, 321–327 (1968).
15) E. A. Munn, C. A. Greenwood, A. Olacchio, and G. Porcellatic: *Int. J. Biochem.*, **10**, 1045–1052 (1979).
16) B. Ovrdijk, W. M. K. Vanderkroef, Veltamp, and G. J. M. Hooghwinkel: *Biochem. J.*, **151**, 257–261 (1975).
17) S. Srivastava, J. Wiktorowicz, R. Klebe, and Y. Awasthi: *Biochim. Biophys. Acta*, **397**, 428–436 (1975).
18) D. A. Wenger, S. Okada, and J. S. O'Brien: *Archiv. Biochem. Biophys.*, **153**, 116–129 (1972).
19) J. E. S. Lee and A. Yoshida: *Biochem. J.*, **159**,

535–539 (1976).
20) D. Marinkovic and J. N. Marinkovic: *Biochem. J.*, **163**, 133–140 (1977).
21) D. M. Swallow, L. Evans, N. Saha, and H. Harris: *Ann. Hum. Genet.*, **40**, 55 (1976).
22) R. J. Pierce and R. G. Price: *Biochem. J.*, **175**, 859–860 (1978).
23) A. Orlacchio, M. Claudio, E. Caria, R. Pietro, and D. Stepehn: *Biochem. Biophys. Res. Comm.*, **122**, 966–973 (1984).

Nippon Suisan Gakkaishi : Formerly *Bull. Japan. Soc. Sci. Fish.*