Journal of Chromatography, 528 (1990) 305–314
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 5248

Measurement of adenine nucleotide levels with an adenine analyser as an index of freshness of porgy

HIROYUKI FUJIMORI*

Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotogecho, Hirakata, Osaka 573-01 (Japan)

TOORU SASAKI, KIYOKATSU HIBI and MASAAKI SENDA

Japan Spectroscopic Co., Ltd., 2967-5, Ishikawacho, Hachioji, Tokyo 192 (Japan)

and

MASANORI YOSHIOKA

Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotogecho, Hirakata, Osaka 573-01 (Japan)

(First received December 11th, 1989; revised manuscript received January 26th, 1990)

SUMMARY

In a prototype of an adenine analyser, adenosine and adenine nucleotides were derivatized with a fluorescent reagent, bromoacetaldehyde, after separation on a Hitachi gel No. 3012-N column by high-performance liquid chromatography. The previous analyser was improved by using a shorter reaction coil and by introduction of a Hitachi gel No. 3013-N with 5-μm particles of porous polystyrene–divinylbenzene, and applied to estimate the freshness of porgy. Total amounts of ATP, ADP and AMP in an isolated muscle just after death gradually decreased to 60% of the original amount after 5 h, and the ATP content rapidly decreased to 20% after 1 h. A good correlation was found between the levels of total adenine compounds and the energy charge values obtained from nineteen porgies frozen at a prerigour state. On the other hand, there existed no relationship between total adenine levels and the K values, which were indices for estimating freshness of fish. The analyser will be useful to evaluate the freshness of tissues and cells based on the higher contents of total adenine compounds, especially ATP.

INTRODUCTION

The adenine compounds, such as ATP, ADP, cAMP, AMP, adenosine (Ado)

0378-4347/90/$03.50

and adenine (Ade) are involved in a series of metabolic pathways in biological systems to produce carriers of high energy. They also play important roles as chemical mediators. The energy charge calculated from the concentrations of ATP, ADP and AMP has been shown to reflect the energy status between the generation and the consumption of ATP in various cells [1]. Therefore, the determination of the levels of ATP, ADP and AMP in biological materials might reflect the viability or freshness of various tissues and cells.

Saito et al. [2] reported that the freshness of raw or frozen fish could be expressed as *K* value, calculated from the proportion of the contents of inosine (HxR) and hypoxanthine (Hx) with respect to those of ATP, ADP, AMP, inosine monophosphate (IMP), HxR and Hx. The adenine compounds in fish muscle are known to be rapidly metabolized successively to IMP, HxR and Hx following death. At present, methods for measuring HxR and Hx rather than adenine compounds have been developed for estimating the freshness of fish [3–6], since the levels of adenine compounds in fish sold in circulation markets are too low for detection. To evaluate the real freshness, the adenine compounds in fish should be measured early after the death of the fish.

Yoshioka and co-workers [7–9] developed a high-performance liquid chromatographic (HPLC) method for a routine determination of adenine compounds by using bromoacetaldehyde as a fluorescent reagent. A prototype of an analyser of adenine compounds was also developed by using a small column packed with Hitachi gel No. 3012-N, particles of macroreticular anion-exchange resin with a mean diameter of 7 μm [10].

This paper describes the use of a prototype of the analyser, with a regular-size column packed with Hitachi gel No. 3013-N (5 μm), to determine the adenine compounds in porgy as a means of evaluating the freshness of tissues.

EXPERIMENTAL

Materials

Bromoacetaldehyde was prepared and crystallized according to the method of Schukovskaya et al. [11]. Hitachi gel No. 3013-N (5 μm) was kindly supplied by Hitachi (Tokyo, Japan). Asahipak GS-320H was purchased from Asahi Chemical Industry (Kanagawa, Japan). All adenine compounds were dissolved in 0.1 *M* phosphate buffer (pH 7.0), to make 0.5–10 μM standard solutions, and stored at $-80°C$ until HPLC analysis. The other chemicals of reagent grade were commercially obtained.

Analyser system for adenine compounds

The analyser was constructed according to the method described previously [10] with some modifications. The mobile phase was a 0.025 *M* citrate buffer (pH 4.0) containing 0.1 *M* bromoacetaldehyde, 0.1–0.2 *M* sodium chloride and 15% acetonitrile. The flow-rate was 0.6 ml/min from an Intelligent HPLC

pump 880-PU (Jasco, Tokyo, Japan). A Hitachi gel No. 3013-N column (125×4.6 mm I.D.) was maintained at 45°C. The eluate was heated in a reaction coil (10 m×0.25 mm I.D.) at 120°C with an FIU reaction unit RU-150 F (Jasco), which was filled with polyethylene glycol 400, and was detected by an Intelligent spectrofluorometer 820-FP (Jasco). The wavelengths of excitation and emission were set at 254 and 400 nm, respectively.

Optimization of length of reaction coil

The analyser system was practically the same as described above. The mobile phase was a 0.025 *M* citrate buffer (pH 4.0) containing 0.1 *M* bromoacetaldehyde. The flow-rate was 1.0 ml/min. An Asahipak GS-320H column (250 mm×7.6 mm I.D.) was maintained at ambient temperature. It was connected to a reaction coil of 1–15 m×0.25 mm I.D. maintained at 120°C. The eluate was detected as described above.

Analysis of adenine compounds in porgy tissues

A porgy was taken from seawater saturated with 95% oxygen and 5% carbon dioxide and killed as rapidly as possible. Immediately, ca. 0.5 g of tissue, such as muscle, liver, spleen or dark meat, was added to 10 volumes of 4 *M* perchloric acid and homogenized in a glass homogenizer. The homogenate was vigorously mixed by a vortex-mixer for 1 min and then chilled in ice for 1 min. These procedures were repeated seven times to extract the acid-soluble adenine compounds. After removal of the precipitate by centrifugation at 6000 *g* for 30 min, 2.0 ml of the supernatant were added to 1.82 ml of 4 *M* potassium hydroxide, and the neutralized mixture was centrifuged at 3500 *g* for 5 min. To 1.0 ml of the mixture was added 1.0 ml of 0.2 *M* phosphate buffer (pH 7.0). The solution was usually diluted 30- to 60-fold with water, and 10 μl of the diluted solution were injected into the analyser.

Determination of inorganic phosphate in porgy muscle

Inorganic phosphate in the muscle was extracted by the same procedure as described above, but without the addition of 0.2 *M* phosphate buffer (pH 7.0) after the neutralization of perchloric acid with potassium hydroxide. Inorganic phosphate was determined by the method described by Gomori [12].

Estimation of freshness of porgy with a freshness testing paper

A kit for the estimation of the freshness of porgy was obtained from the Environmental Analytical Center (Tokyo, Japan). The procedure was as described in the kit manual [13]. The muscle isolated with a cork bowler was added to 9 volumes of phosphate buffer containing EDTA, which was prepared and specially blended by the Center, and homogenized with a Polytrone for 10 s in an ice-bath. The freshness testing paper was immediately immersed into the homogenate and left in it at room temperature for 10 min. The correspond-

ing K value was estimated from the comparison of the colour developed on the paper with the colour table for the K value reduction. The K value, which increases from 0 to 70% in ten steps, rises as the total amount of HxR and Hx increases stepwise with 0.5 μmol/g of isolated muscle from 0 to 5 μmol/g. A lower K value means a lower total amount of HxR and Hx and a higher degree of freshness.

RESULTS

Because a prototype of the analyser previously designed [10] was equipped with a 30 m × 0.1 mm I.D. reaction coil, the coil often became blocked. To solve this problem, a reaction coil of 0.25 mm I.D. was introduced into the analyser, and then the effect of length of the reaction coil was studied. The adenine compounds were well separated. ATP, ADP, AMP and cAMP were eluted in this order from the Asahipak GS-320H column, which can be eluted at greater flow-rate than a Hitachi gel No. 3013-N column (data not shown). As the peak heights of the adenine compounds reached plateaus over 5 m of the coil, a 10-m length was chosen (Fig. 1).

The adenine compounds were also well separated by isocratic elution on Hitachi gel No. 3013-N, and the analysis time was ca. 26 min (Fig. 2). By changing the reaction coil and by introduction of a Hitachi gel No. 3013-N column, a good separation of adenine compounds was obtained and the analyser system functioned properly for more than one year. As the final concentration of sodium chloride in the mobile phase containing 15% acetonitrile changed from 0.1 to 0.2 M, the separations of Ado from AMP, AMP from

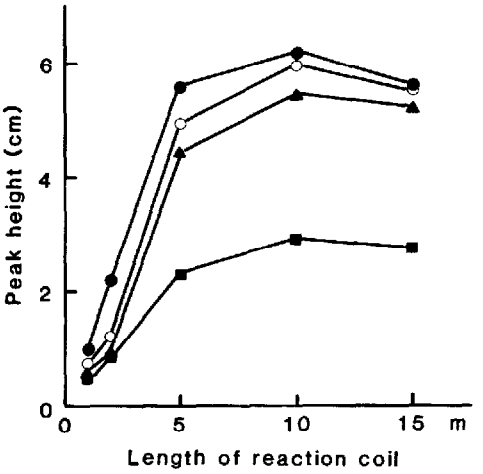

Fig. 1. Effect of the length of the reaction coil on peak heights: 20 pmol of adenines injected. (■) cAMP; (▲) AMP, (○) ADP; (●) ATP.

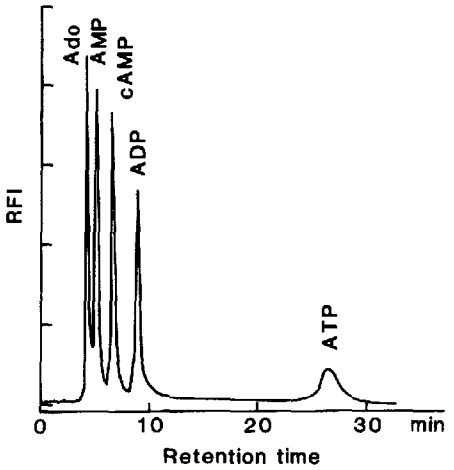

Fig. 2. Chromatogram of authentic adenine compounds on a column of Hitachi gel No. 3013-N: 10 μl of 2 μM adenine compounds in 0.1 M phosphate buffer (pH 7.0) were injected. The relative fluorescence intensity (RFI) of the peaks was drawn.

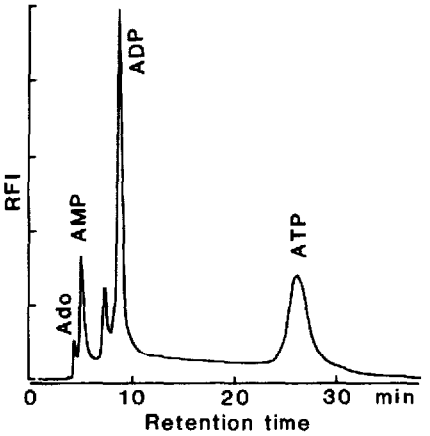

Fig. 3. Chromatogram of adenine compounds in tail muscle.

cAMP, cAMP from ADP, and ADP from ATP decreased. Although the separation of Ado from AMP was not good, a resolution of 1.6 was obtained with both 0.1 and 0.15 M sodium chloride. The other combinations of adenine compounds were completely separated with 0.1 and 0.15 M sodium chloride. The retention time of ATP was ca. 60 and 26 min at 0.1 and 0.15 M sodium chloride, respectively, hence 0.15 M sodium chloride was selected for the analysis.

The adenine compounds in porgy tail muscle were determined (Fig. 3). The amounts of ATP, ADP and AMP were 7.0, 2.8 and 0.4 μmol/g wet weight, respectively. The chromatogram of a liver sample is shown in Fig. 4. The amounts of ATP, ADP and AMP were 0.5, 1.0 and 5.7 μmol/g wet weight,

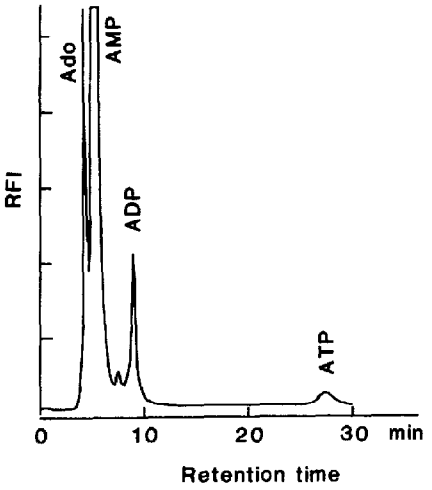

Fig. 4. Chromatogram of porgy liver.

TABLE I

ADENINE COMPOUND LEVELS IN VARIOUS TISSUES OF PORGY

Tissue	Concentration (μmol/g)				Energy charge[b]
	AMP	ADP	ATP	Total[a]	
Breast muscle	0.2	2.0	3.1	5.3	0.77
Abdominal muscle	0.2	1.7	3.2	5.1	0.79
Tail muscle	0.4	2.8	7.0	10.2	0.82
Liver	5.7	1.0	0.5	7.2	0.14
Spleen	2.4	1.0	0.4	3.8	0.24
Dark meat	2.6	0.8	1.1	4.5	0.33

[a]AMP + ADP + ATP.
[b](ATP + 0.5ADP)/(ATP + ADP + AMP).

respectively. The contents of the adenine compounds in various tissues of a porgy are summarized in Table I. The energy charge values from three different parts of muscles were higher than those of liver, spleen and dark meat, which were also excised immediately after death. The time-course of changes in adenine compounds of breast muscle kept in ice was as shown in Fig. 5. Total amounts of ATP, ADP and AMP in an isolated tissue gradually decreased to 40% during 5 h. The ATP content in the tissue rapidly decreased to 20% in 1 h and the levels were constantly low from 1 to 5 h. The amounts of ADP and of AMP increased 1–2 h after isolation and then decreased gradually between 2 and 5 h. The Ado levels increased after 1 h and gradually decreased as a

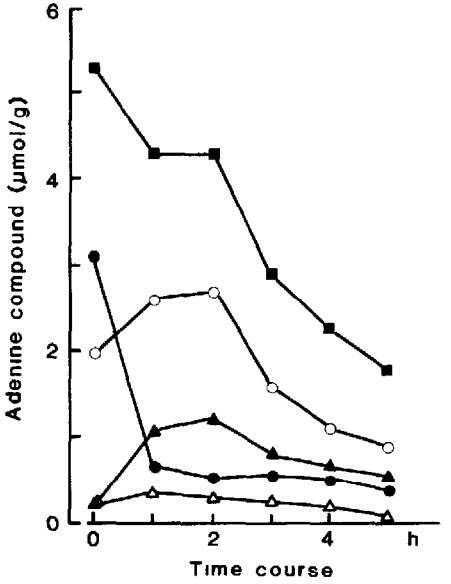

Fig. 5. Changes of adenine compound levels in muscle kept on ice. (△) Ado; (▲) AMP; (○) ADP; (●) ATP; (■) AMP+ADP+ATP.

TABLE II

CHANGES OF LEVELS OF INORGANIC PHOSPHATE AND *K* VALUE IN PORGY BREAST MUSCLE

Time (h)	Pi[a] (μmol/g)	*K* value (%)
0	68.7	<7
3	81.9	<7
7	85.9	7–14
24	116.4	14–21
48	103.9	21–28
72	81.5	28–35
96	83.0	>70

[a]Inorganic phosphate.

function of time. The energy charge value was as high as 0.8 at 0 h and became constant between 1 and 5 h.

Next, the changes of levels of inorganic phosphate and *K* value in breast muscle were examined. The inorganic phosphate levels increased significantly during 96 h, but no correlation between the decrease of total adenine compounds and the increase of inorganic phosphate was found. The *K* values were very low up to 3 h and then constantly increased between 3 to 96 h (Table II).

The levels of adenine compounds and the *K* and energy charge values in

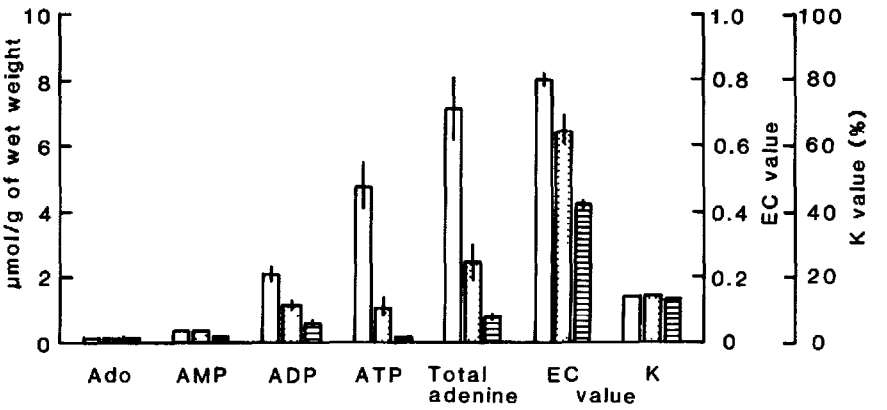

Fig. 6. Differences of adenine compound levels, and energy charge (EC) and K values in frozen porgies classified in three groups. Mean ± S.E. of high ($n=6$; ⊟), middle ($n=3$; ▩) and low ($n=10$; ▥) levels of adenine compounds.

nineteen frozen porgies, kept at −80 °C, are summarized in Fig. 6. They were classified into three groups based on the energy charge value, as shown in Fig. 6; the first one was above 0.80 ± 0.014 ($n=6$) of the mean ± S.E. of the energy charge value (high), the second was 0.64 ± 0.05 ($n=3$) of that (middle), and the third was 0.42 ± 0.012 ($n=10$) of that (low). A good correlation was found between the energy charge values and the content of total adenine compounds or ATP.

DISCUSSION

Two different methods for measuring adenine-related compounds were already established for the estimation of freshness of fish muscle. One is based on the separation of adenine compounds by ion-exchange chromatography [14,15], thin-layer chromatography [16] or HPLC [17] with UV detection, which is less specific and less reliable for peak identification. The other one is the estimation of the K value by an enzymic method [18], by colorimetry [19] or by using an enzyme sensor [20,21] in the presence of adenine-related compounds. Since fish at the state of postrigour in circulation market usually contain much higher levels of HxR and Hx than of the adenine compounds, the freshness of fish can be reasonably evaluated by measuring only HxR and Hx. The K value of fish muscle estimated by freshness testing paper is a good practical index for consumers. However, the method is not available for estimating the freshness of fish before circulation.

It is generally accepted that the contents of adenine compounds are high and those of HxR and Hx are low in the fresh muscle of fish [22,23]. In the present study, the K value of porgy estimated by freshness testing paper was below 7% at 0 h and 7–13% after 7 h (Table II), indicating that the isolated muscle in

an ice-bath contained low levels of HxR and Hx. The content of total adenine compounds gradually decreased. However, no significant increase of Ado was observed (Fig. 5), indicating that adenine compounds would be mainly metabolized from AMP to IMP by AMP deaminase. The energy charge value was very high when the muscle was isolated immediately after death and became low (0.42–0.45) after 1 to 5 h of incubation. These results suggest that the change of level of total adenine compounds would be a better index of the freshness at the prerigour state than the change of the energy charge value.

When codlings were killed in rested or exhausted states, the contents of AMP and ADP in both states were nearly the same, but the ATP level in the exhausted state was ca. 5% of that in the rested one [22]. On the other hand, the IMP level (5.86 μmol/g) in exhausted codlings was ca. 4.7-fold higher than that (1.26 μmol/g) in rested fish. The energy charge values calculated by us were 0.85 in the rested and 0.38 in the exhausted codling, although all the fish were fresh. When the muscle frozen in a prerigour state is thawed, the metabolism of glycogen and the degradation of ATP are facilitated compared with those in unfrozen muscle undergoing normal rigour [24–26]. Although the nineteen porgies were frozen as soon as possible after death, there were large differences in the contents of total adenine compounds, ATP and ADP, and in the energy charge values between the three groups, but with no significant change in the K value as shown in Fig. 6. The differences in the levels of total adenine compounds might be due to differences of exhaustion at the time of freezing and variation of occurrence of thaw rigour after muscle isolation.

Because the very fresh fish, which was not only raw but also frozen, contained very high levels of total adenine compounds and ATP in the muscle, the determination of adenine compounds, especially ATP, might be a critical factor for assessing the freshness of fish. The level of the adenine compounds may be influenced by the effect on fish muscle of the freezing and thawing techniques. This method will be very useful to determine adenine compounds not only for estimating freshness but also for developing the freezing and thawing techniques. Since the analyser does not need large amounts of tissue because the sensitivity and specificity are very high owing to the use of bromoacetaldehyde, the method will also be useful for assessing the freshness of various tissues for organ transplants.

ACKNOWLEDGEMENTS

We are grateful to Mr. N. Yuasa of this University Cafeteria for cooking porgies. We also thank Miss K. Kosaka, Mr. T. Ida and Mr. Y. Honda of this University for their technical assistance. The porgies were kindly donated by Mr. C. Kuroyanagi of CA Products, Tokyo, Japan.

REFERENCES

1 D.E. Atkinson (Editor), Cellular Energy Metabolism and its Regulation, Academic Press, New York, 1977.
2 T. Saito, K. Arai and M. Matsuyoshi, Bull. Jpn. Soc. Sci. Fish., 24 (1959) 749.
3 S. Ehira and H. Uchiyama, Bull. Jpn. Soc. Sci. Fish., 35 (1969) 1080.
4 N.R. Jones, J. Murray, E.I. Livingston and C K. Murray, J. Sci. Food Agric., 15 (1964) 763.
5 J. Spinelli, M. Eklund and D. Miyauchi, J. Food Sci., 29 (1964) 710.
6 M. Patashnik, Commer. Fish. Rev., 28 (1966) 1.
7 M. Yoshioka, K. Nishidate, H. Iizuka, A. Nakamura, M.M. El-Merzabani, Z. Tamura and T Miyazaki, J. Chromatogr., 309 (1984) 63.
8 M. Yoshioka, K. Yamada, M.M. Abu-Zeid, H. Fujimori, A. Fuke, K. Hirai, A. Goto, M. Ishii, T. Sugimoto and H. Parvez, J. Chromatogr., 400 (1987) 133.
9 M. Yoshioka, K. Yamada, M M. Abu-Zeid, H. Fujimori, A. Fuke, K. Hirai, A. Goto, M. Ishii, T. Sugimoto and H. Parvez, in M. Yoshioka, S. Parvez, T. Miyazaki and H. Parvez (Editors), Supercritical Fluid Chromatography and Micro-HPLC, VSP Press, Utrecht, 1989, p. 181.
10 M. Yoshioka, Z. Tamura, M. Senda and T. Miyazaki, J. Chromatogr., 344 (1985) 345.
11 L.L. Schukovskaya, S.N. Ushakov and N.K. Galania, Izv. Akad Nauk SSSR Otd. Khim. Nauk, (1962) 1692.
12 G. Gomori, J. Lab. Clin. Med., 27 (1942) 955.
13 The Manual of Freshness Testing Paper (in Japanese), Environmental Analytical Center, Tokyo, 1984.
14 K. Arai and T. Saito, Bull. Jpn. Soc. Sci. Fish., 29 (1963) 168.
15 R.B. Hurlbert, H. Schmitz, A.F. Brumm and V.R. Potter, J. Biol. Chem., 209 (1954) 23.
16 D.I. Fraser, J.R. Dingle, J.A. Hines, S.C. Nowlan and W.J. Dyer, J. Fish. Res. Board Can., 24 (1967) 1837.
17 E. Lee, T. Ohshima and C. Koizumi, Bull. Jpn. Soc. Sci. Fish., 48 (1982) 255.
18 H. Uchiyama and S. Ehira, Mod. Media, 24 (1978) 78.
19 F. Uda, E. Hayashi, H. Uchiyama and K. Kakuda, Bull. Tokai Reg. Fish. Res. Lab., 111 (1983) 55.
20 E. Watanabe, K. Ando, I Karube, H. Matsuoka and S. Suzuki, J. Food Sci., 48 (1983) 496
21 E. Watanabe, K. Toyama, I. Karube, H. Matsuoka and S. Suzuki, Appl. Microbiol. Biotechnol., 19 (1984) 18.
22 K. Kobayashi, Bull. Jpn. Soc. Sci. Fish., 32 (1966) 166.
23 D.F. Hiltz, L.J. Bishop and W J. Dyer, J. Fish. Res Board Can., 31 (1974) 1181.
24 R.K. Scopes and R.P. Newbold, Biochem. J., 109 (1968) 197.
25 D.F. Hiltz and W.J Dyer, J. Fish. Res. Board Can., 30 (1973) 45.
26 H Yamanaka, T. Nakagawasai, T. Kikuchi and K. Amano, Bull Jpn. Soc. Sci. Fish., 44 (1978) 1123.