Aspolin, a Novel Extremely Aspartic Acid-rich Protein in Fish Muscle, Promotes Iron-mediated Demethylation of Trimethylamine-N-oxide*

Kazuharu Takeuchi‡§, Akimasa Hatanaka‡, Meiko Kimura‡, Nobuo Seki‡, Ikuo Kimura‡,
Shoichi Yamada‡, and Shinya Yamashita‡

From the ‡Central Research Laboratory, Nippon Suisan Kaisha, Ltd., Kitanomachi, Hachioji,
Tokyo 192-0906, Japan and ¶Laboratory of Food Biochemistry, Graduate School of Fisheries Science,
Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

Trimethylamine-N-oxide (TMAO) is abundant in marine fish. Formaldehyde synthesis by TMAO demethylation during storage markedly deteriorates fish meat. In the present work, we cloned the extremely aspartic acid-rich proteins from skeletal muscle of a commercially important species, walleye pollack, in the course of molecular identification of trimethylamine-N-oxide demethylease (TMAOase). One of the cDNAs, designated as aspolin1, encodes an extremely aspartic acid-rich protein of 228 amino acids which is converted to the TMAOase after processing between Ala42 and Asp43. Mature aspolin1/TMAOase protein contains 179 Asp in 186 total amino acids. The other cDNA, designated as aspolin2, has a common nucleotide sequence with aspolin1 in the 5′ part and encodes a protein which has an additional Asp polymer and a C-terminal cysteine-rich region. The amino acid sequence of the C-terminal cysteine-rich region of aspolin2 is highly homologous to the mammalian histidine-rich Ca2+-binding protein. Aspolin1/TMAOase and aspolin2 mRNA was most abundant in the skeletal muscle. A lower level of the mRNA was also detected in kidney, heart, spleen, and brain. Synthetic Asp polymer showed marked TMAOase activity in the presence of Fe3+, whereas a monomer and oligomers did not. Purified TMAOase protein bound to Fe3+ with low affinity, which may be responsible for the catalytic activity. Poly aspartic acid-Fe3+ complex generated after death would be involved in formaldehyde synthesis by the demethylation of TMAO during the storage of fish meat.

Trimethylamine-N-oxide (TMAO)† is widely distributed among various tissues of sea fish and invertebrates (1–3). Sev-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: TMAO, trimethylamine-N-oxide; DMA, dimethylamine; TMAOase, trimethylamine-N-oxide formaldehyde-lyase; HRC, histidine-rich calcium-binding protein; RT-PCR, reverse transcriptase-PCR.

†† To whom correspondence should be addressed: Central Research Laboratory, Nippon Suisan Kaisha, Ltd., 559-6 Kitanomachi, Hachioji, Tokyo 192-0906, Japan. Tel.: 81-426-56-5195; Fax: 81-426-56-5188; E-mail: k-takeuchi@nissui.co.jp.

§ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB117517 (aspolin1) and AB117518 (aspolin2).

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

The occurrence of formaldehyde markedly deteriorates the fish meat by the crosslinking of muscle protein. As a result, fish fillet loses elasticity and develops a sponge-like texture that is unsuitable for surimi products (seafood items that are made of fish-meat gel and sometimes look like crab, scallop, etc.) owing to the decrease in the solubility of myofibril at high-salt concentrations (5, 6).

TMAO demethylase (TMAOase, or trimethylamine-N-oxide formaldehyde-lyase, EC 4.1.2.32) has been proposed to be responsible for the formaldehyde synthesis by TMAO demethylation during frozen storage of fish meat (3). TMAOase activity has been detected in kidney, spleen, pyloric caecum, liver, dark muscle, and ordinary muscle of gadoid and non-gadoid fish (3). Some cofactor systems have been developed for the separation of the enzyme; flavin-NAD(P)H and Fe2+-ascorbate-cysteine with or without methylene blue under aerobic or anaerobic conditions (3). Despite the efforts to purify the enzyme, it has not been possible to identify the detailed component(s) of the enzyme, primarily because of the insolubility and the complex cofactor requirement of this enzyme (7–9).

Walleye pollack is caught in the northern Pacific and is the most important source of surimi products. Recently, TMAOase was detected and purified from the myofibrillar fraction of walleye pollack (10, 11). The walleye pollack enzyme was characterized as a single acidic protein with an apparent molecular mass of 25 kDa. This enzyme required Fe2+ for its activity and showed an optimum pH at 7.0; the Km value for TMAO was 30 mmol/kg. The activity of this enzyme was stable in the presence of SDS. Furthermore, the enzyme activity was retained after heating at 80 °C for 30 min, suggesting an extremely high thermal stability (12).

In this paper, we report the novel extremely aspartic acid-rich proteins found in the course of the molecular identification of walleye pollack muscle TMAOase. Our cDNA cloning revealed that this enzyme is one of the two homologous proteins containing the extremely long Asp polymer, an uninterrupted stretch (except for a single Glu residue) of 171
Asp residues. We demonstrate that the TMAO demethylation activity of this protein arises from the interaction of poly Asp and Fe²⁺.

**EXPERIMENTAL PROCEDURES**

**Materials**—Walleye pollock *Theragra chalcogramma* caught off the northern Hokkaido coast was used for protein and RNA preparation. Aspartic acid monomer, oligomer, and polymer were obtained from Sigma. Poly glutamic acid was obtained from ICN. TMAO was obtained from Aldrich.

**Purification and Analysis of Walleye Pollack TMAOase**—The TMAOase was purified from the myofibrillar fraction of walleye pollock skeletal muscle according to the method reported previously (11). After dialysis against distilled water, the protein was lyophilized. Phosphate was detected by staining with Coomassie Brilliant Blue and aluminum nitrate (13). Carbohydrate was detected by staining with periodic acid Schiff staining, or staining with a GelCode glycoprotein staining kit (Pierce). Amino acid composition analysis of the purified protein, about 180 amino acids are contained in the mature aspolin1 protein deduced from nucleotide sequence was performed by high pressure liquid chromatography. The amino-terminal amino acid sequence was analyzed with a Hewlett-Packard G1005A protein sequencing system. Molecular mass of the purified protein was measured by electrospray-ization mass spectrometry using a Sciex API300 triple quadrupole mass spectrometer (PerkinElmer Life Sciences).

**cDNA Cloning and Sequencing—**Poly(A)⁺ RNA was isolated from walleye pollock skeletal muscle using Quik-Prep micro mRNA purification kit (Amersham Biosciences). Double-strand cDNA was synthesized by using the SuperScript system (Invitrogen) and ligated to pExCell vector (Amersham Biosciences). An oligonucleotide corresponding to a stretch of Asp hexamer (5'-GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
purified protein. According to this assumption, the number of each amino acid was estimated as shown in Table II.

cDNA Cloning of Walleye Pollack TMAOase—The results of amino acid sequencing and composition analysis suggested that a large part of the amino acid sequence of the protein contains an Asp repeat. Therefore, we carried out the screening of the cDNA library of walleye pollack skeletal muscle using an oligonucleotide probe corresponding to the amino acid sequence, DDDDDD. As a result, two species of clone, designated as aspolin1 and aspolin2, containing open reading frames encoding extremely Asp-rich proteins, were obtained. To confirm the number of GAY repeats in these clones, RT-PCR was performed, and amplified products were cloned and sequenced (data not shown). Finally, the nucleotide sequence of two species of cDNA was determined (Fig. 1). As shown in Fig. 1, the 5’ noncoding region and the former part of coding region of two clones have a common nucleotide sequence. Deduced amino acid sequences of aspolin1 and aspolin2 are aligned in Fig. 2. Amino acid sequences of aspolin1 and aspolin2 contain 182 and 225 amino acids of Asp polymer, respectively, with a few inserts of other amino acids.

The N-terminal amino acid sequence of purified protein corresponds to the peptide stretch from Asp43 to Asp57 in aspolin1 and aspolin2. Assuming that the processing of the peptide occurs between Ala 42 and Asp43, the molecular weight of the mature aspolin1 protein is calculated as 21,383 (Table I). This value is almost identical to the molecular weight of the purified protein obtained by mass spectrometry, 21,389. Furthermore, the number of each amino acid in mature aspolin1 is almost identical with that estimated from amino acid analysis.
except for His and Lys (Table II). Thus, we concluded that aspolin1 encodes the precursor of the purified walleye pollack TMAOase.  

### Primary Structure of Aspolin1/pre-TMAOase and Aspolin2—
Proteins encoded by aspolin1/pre-TMAOase and aspolin2 cDNAs have 228 and 347 amino acids, respectively. It seems that both proteins contain 42 amino acids of pre-peptide at the N terminus. Mature aspolin1/TMAOase protein consists of a 182-amino acid stretch of Asp polymer and a C-terminal of 4 amino acids, His-Glu-Glu-Leu. In the Asp polymer region, there are three amino acids other than Asp, namely Ala52, Gly53, and Glu81. The deduced amino acid sequence of aspolin1/pre-TMAOase did not show homology to any known proteins in the data base. On the other hand, putative mature aspolin2 protein consists of a 225 amino acid stretch of Asp polymer and a C-terminal cysteine-rich region. The Asp polymer region of aspolin2 contains Ala52, Gly53, and Glu81, as well as aspolin1, and a His-Glu-Ala-Gly sequence at the amino acid position 225. The C-terminal region contains 14 Cys residues, and each of the Cys residues is spaced with 1–3 amino acid(s) other than Cys. The data base search revealed that the Cys-rich region of aspolin2 is highly homologous to mammalian histidine-rich calcium-binding protein (HRC) (Fig. 3; Ref. 15).  

### Tissue Distribution of Aspolin1/TMAOase and Aspolin2 mRNA—
The tissue distribution of aspolin1/TMAOase and aspolin2 mRNAs was analyzed by RT-PCR (Fig. 4). Both aspolin1/TMAOase and aspolin2 mRNAs were most abundant in skeletal muscle. Moderate amounts of both mRNAs were detected in kidney, and traces of the mRNAs were also detected in heart, spleen, and brain. Neither mRNA was detected in liver, stomach, pyloric caecum, intestine, and fin. None of the amplified band was detected in the control without reverse transcription (data not shown).  

### TMAO Demethylation Activity of Poly Asp—
To identify the region of mature aspolin1/TMAOase responsible for the TMAOase activity, we attempted to have aspolin1/TMAOase cDNA expressed in E. coli, COS-7 cells, and a wheat germ cell-free system. Probably because of the special characteristic of the primary structure of aspolin1/TMAOase, all of these trials were unsuccessful. Therefore, we analyzed the TMAO
demethylation activity of synthetic poly Asp. Surprisingly, synthetic poly Asp showed a marked TMAO demethylation activity. As shown in Fig. 5, the relative TMAO demethylation activity of poly Asp 8k was as much as that of purified TMAOase. The activity of poly Asp 35k was 1.7 times as much as that of purified TMAOase. To examine whether polymerization of Asp is required for TMAO demethylation activity, the activity of L-Asp monomer, dimer, and tetramer were measured. None of these small molecules, however, showed definite activity (Fig. 5). To examine whether carboxyl groups in the side chains of poly Asp are responsible for TMAO demethylation activity, the activity of poly Glu was measured. As shown in Fig. 5, poly Glu showed TMAO demethylation activity, although its level was relatively low.

Because TMAO demethylation in muscle of walleye pollack occurs during frozen storage, TMAO demethylation activity of poly Asp in the frozen state was measured. As shown in Fig. 6, poly Asp promoted TMAO demethylation both at −20 °C and −50 °C. The plateau level of DMA synthesis at −20 °C was more than twice as high as that at −50 °C.

Iron Binding of Purified TMAOase—A previous report (11) demonstrated that TMAO demethylation catalyzed by the walleye pollack TMAOase required an Fe²⁺ as a cofactor. To examine the binding capacity of purified TMAOase to Fe²⁺, an iron binding assay was performed by an ultrafiltration-based method. Iron binding of purified TMAOase exhibited a sigmoidal dependence on Fe²⁺ concentration (Fig. 7). The bound/free ratio of Fe²⁺ was not more than 0.25. At the concentration of Fe²⁺ used in the TMAO demethylation assay, bound Fe²⁺ amounted to about 1/3 of the carboxyl groups of the Asp side chain of TMAOase. At higher concentrations of Fe²⁺, however, bound Fe²⁺ continued to increase.

**DISCUSSION**

Nucleotide sequences of aspolin cDNAs contain long GAY repeats encoding poly Asp. In general, a correct analysis of such a repeat sequence is difficult because of the problem of sequencing chemistry. For this reason, we carefully analyzed nucleotide sequences to confirm the accuracy of the presented sequences. In the nucleotide sequences of the aspolin cDNAs, two kinds of codons for Asp, GAC, and GAU, appear randomly. Because this random mixture of GAC and GAU ensured the specificity of sequence and avoided slippage, the sequencing pattern of the GAY-repeat region was very stable (data not shown). On the other hand, cDNA in cloning vector
and PCR product were rather unstable (data not shown). Therefore, we cloned the RT-PCR product containing the GAY region and sequenced several clones to confirm the correct sequences. As for aspolin1/TMAOase, the calculated molecular weight from deduced amino acid sequence and the actual value from mass spectrometry were almost identical (Table 1), supporting that the idea that cDNA was derived from a functional mRNA.

Nucleotide sequences upstream from GAA, corresponding to Glu226, are common to aspolin1/pre-TMAOase and aspolin2 (Fig. 1). This sequence identity indicates that each mRNA is generated by alternative splicing. According to the N-terminal amino acid sequence analysis, aspolin1/pre-TMAOase preprotein is processed between Ala142 and Asp88. If the processing of aspolin2 protein depends on the N-terminal amino acid sequence, aspolin2 is processed at the same position as aspolin1/pre-TMAOase.

The amino acid sequence of the C-terminal region of aspolin2 is very similar to that of mammalian HRC (Fig. 3). HRC is known as a Ca2+-binding protein of sarcoplasmatic reticulum (16). Because aspolin2 mRNA is also abundant in skeletal muscle, aspolin2 may appear to be a fish homologue of HRC. However, the molecular mass of HRC, 165 kDa, is much higher than that of aspolin2, 35 kDa. Furthermore, HRC contains 9 tandem repeats of a 29-residue sequence consisting of a stretch of 10–11 acidic amino acids and a histidine-rich sequence (15). Although both HRC and aspolin2 have C-terminal cysteine-rich regions and are rich in acidic amino acids, the overall structures of these molecules are quite different.

Poly Asp in aspolin is reminiscent of another Ca2+-binding protein of sarcoplasmatic reticulum, calsequestrin. Skeletal muscle calsequestrins have a cluster of 10–45 acidic residues, typically aspartates, at C terminus (17–21). Calsequestrin binds Ca2+ with high capacity (40–50 Ca2+/molecule) and moderate affinity (22, 23). Negatively charged carboxyl groups of acidic residues are thought to be responsible for Ca2+-binding. A recent study (24) showed that binding of Ca2+ is highly dependent on the C-terminal Asp-rich region. It has been proposed that the length of the C-terminal Asp-rich region may be important for the Ca2+-binding capacity of calsequestrin (22). According to this report, mature aspolin1/TMAOase and aspolin2 are expected to have high Ca2+- binding capacity. Therefore, aspolins may be a Ca2+-binding protein in fish muscle.

Although no aspolin ortholog was found in the protein data base, a similar nucleotide sequence was found in the genomic data base of tiger puffer (data not shown). We also found some aspolin-like nucleotide sequences in amphibian expressed sequence tag (GenBankTM/EBI accession numbers BQ731479, BJ073162, and BQ524571). However, none of the aspolin-like sequences was found in sequences of the other groups of vertebrates, such as oxalic acid, malic acid, and aspartic acid, catalyzing the TMAO demethylation in fish muscle. Therefore, aspolins may be a Ca2+-binding protein in fish muscle.

Acknowledgments—We thank Dr. N. Takamatsu (Kitsatomo University) for cDNA library screening and courteous advice.

REFERENCES
1. Dyer, W. J. (1952) J. Fish. Res. Board Can. 8, 314–324
2. Tokunaga, T. (1975) Nippon Suisan Gakkaishi 41, 502–509
3. Sotoie, C. G., and Rebhein, H. (2000) in Seafood Enzymes (Haard, N. F., and Simpson, B. K., eds) pp. 167–190, Dekker, New York
4. Castell, C. H., Neal, W. E., and Dale, J. (1973) J. Fish. Res. Board Can. 30, 1246–1248
5. Tokunaga, T. (1974) Nippon Suisan Gakkaishi 40, 167–174
6. Castell, C. H., Smith, B., and Dyer, W. J. (1973) J. Fish. Res. Board Can. 30, 1205–1213
7. Gill, T. A., and Paulson, A. T. (1982) Comp. Biochem. Physiol. 71B, 49–56
8. Parkin, K. L., and Hultin, H. O. (1986) J. Biochem. 100, 87–97
9. Joly, A., Cottin, P., Han-Ching, L., and Ducastagné, A. (1992) J. Sci. Food Agric. 59, 261–267
10. Kimura, M., Seki, N., and Kimura, I. (2000) Fish. Sci. 66, 725–729
11. Kimura, M., Seki, N., and Kimura, I. (2000) Fish. Sci. 66, 967–973
12. Kimura, M., Kimura, I., and Seki, N. (2003) Fish. Sci. 69, 414–420
13. Hegenauner, J., Ripley, L., and Nace, G. (1977) Anal. Biochem. 78, 308–311
14. Dyer, W. J., and Mounsey, Y. A. (1949) J. Fish. Res. Board Can. 6, 359–367
15. Hofmann, S. L., Goldstein, J. L., Orth, K., Mosnau, C. R., Slaughter, C. A., and Brown, M. S. (1989) J. Biol. Chem. 264, 18083–18090
16. Hofmann, S. L., Brown, M. S., Lee, E., Pathak, R. K., Anderson, R. G. W., and Goldstein, J. L. (1989) J. Biol. Chem. 264, 8280–8270
17. Fliegel, L., Ohnishi, M., Carpenter, M. R., Khanna, V. K., Reithmeier, R. A. F., Simpson, W., and MacLennan, D. H. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1167–1171
18. Fujii, J., Willard, H. F., and MacLennan, D. H. (1990) Somatic Cell Mol. Genet. 16, 185–189
19. Choi, E. S. H., and Clegg, D. O. (1990) Dev. Biol. 142, 169–177
20. Treves, S., Vilsen, B., Chozzi, P., Andersen, J. P., and Zorzato, F. (1992) Biochem. J. 283, 767–772
21. Park, K. W., Goo, J. H., Chung, H. S., Kim, H., Kim, D. H., and Park, W. J. (1998) Gene 217, 25–30
TMAO Demethylation by Asp-rich Protein in Fish Muscle

22. Yano, K., and Zarain-Herzberg, A. (1994) Mol. Cell. Biochem. 135, 61–70
23. Berchtold, M. W., Brinkmeier, H., and Muntener, M. (2000) Physiol. Rev. 80, 1215–1265
24. Shin, D. W., Ma, J., and Kim, D. H. (2000) FEBS Lett. 486, 178–182
25. Craig, J. C., Dwyer, F. P., Glazer, A. N., and Horning, E. C. (1961) J. Am. Chem. Soc. 83, 1871–1878
26. Ferris, J. P., Gerwe, R. D., and Gapski, G. R. (1967) J. Am. Chem. Soc. 89, 5270–5275
27. Ferris, J. P., Gerwe, R. D., and Gapski, G. R. (1968) J. Org. Chem. 33, 3493–3498
28. Richardson, D. R., and Ponka, P. (1997) Biochim. Biophys. Acta 1331, 1–40
29. Monkovic, I., Wong, H., and Bachand, C. (1985) Synthesis, 770–773