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TMAO-ASE ACTIVITY IN TISSUES OF FISH SPECIES FROM THE NORTHEAST ATLANTIC

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Abstract—1. The influence of various parameters (evacuation; pH value; addition of Fe^{2+}, cysteine, ascorbic acid and methylene blue) on the activity of the trimethylamine oxide demethylase (TMAO-ase) in extracts from the kidney of saithe (*Pollachius virens L.*) is described.

2. Assay conditions resulting in maximum formaldehyde (FA) production were used to measure the TMAO-ase activity in kidney, spleen, liver, pyloric caeca, blood and muscle from Northern Atlantic fish species.

3. Only in organs from fishes of the order Gadiformes could TMAO-ase activity be detected.

4. The TMAO-ase was most active in kidney and spleen whereas in muscle the enzyme activity could be barely detected.

INTRODUCTION

Trimethylamine oxide (TMAO) is a main constituent of the non-protein N-fraction of the muscle of many species of marine fishes; during iced or frozen storage of fish muscle TMAO can be degraded to dimethylamine (DMA) and formaldehyde (FA) (Castell *et al.*, 1971; Mackie and Thomson, 1974; Hebard *et al.*, 1982). The formation of FA is of great importance for the deterioration of many fishery products, because reactions between FA and muscle proteins result in a poor texture of fillets or minces, characterized by toughening and decrease in water binding capacity (Sikorski *et al.*, 1976). The enzyme trimethylamine oxide demethylase (TMAO-ase), which catalyses the formation of DMA and FA, has been detected in organs of several fish species (Sikorski and Kostuch, 1982). The mechanism and the physiological significance of this reaction are unknown.

Intracellular localization and conditions for optimum activity *in vitro* depend on the origin of the enzyme: (1) A lysosomal TMAO-ase with a pH optimum at 5.0 has been isolated from the kidney of cod (*Gadus morrhua*); this enzyme needs methylene blue for optimum activity and is not influenced by the oxygen tension (Gill and Paulson, 1982). (2) In the kidney of cod and of blue whiting (*Micromesistius poutassou*) the activity is found in the supernatant after centrifuging a 1:10 (w/w) mixture of $(NH_4)_2SO_4$ and homogenate and it can be precipitated by adding another 15% (w/v) $(NH_4)_2SO_4$. The assay has been done in the presence of methylene blue and under flushing the test tubes with nitrogen (Svensson, 1980). (3) This separation procedure was first applied by Yamada *et al.* (1969) to the pyloric caeca of Alaska pollock (*Theragra chalcogramma*); they found a dialysable, heat stable cofactor in the supernatant of the $(NH_4)_2SO_4$ precipitation as well as in shark liver. Tomioka *et al.* (1974), using an acetone powder from the pyloric caeca of Alaska pollock, confirmed the need for this cofactor, the strict dependence on methylene blue found by Yamada and Amano (1965), as well as the considerable inhibition of the TMAO-ase by oxygen. The enzyme exhibits maximum activity at pH 5.0. (4) Tokunaga (1980) was able to purify the enzyme from the gall bladder and pyloric caeca of Alaska pollock partially (50-fold); in this case enzyme activity and stability show maxima at pH 7.0; the formation of DMA by the purified enzyme is markedly stimulated by myoglobin, haemoglobin and haematin. (5) From the muscle of red hake (*Urophycis chuss*) a microsomal TMAO-ase has been prepared, which needs Fe^{2+}, ascorbic acid and cysteine for maximum activity; furthermore the enzyme is activated by phenazine methosulphate or methylene blue; the pH optimum of this enzyme is about 6.5–7.0 (Parkin and Hultin, 1981, 1982). The occurrence of a particulate TMAO-ase activity in red hake muscle has been confirmed by Lundstrom *et al.* (1982a), who stated a high stimulation of activity of this enzyme by ascorbate plus methylene blue and to a smaller extent by Fe^{2+} plus cysteine and who demonstrated a particle size of $\geqslant 0.1\ \mu m$ for the active fraction. (6) Harada (1975) partially purified a TMAO-ase from the liver of lizard fish (*Saurida tumbil*); this enzyme exhibits optimum activity at pH 5.0 and a need for methylene blue, best in the reduced state. From the liver and midgut gland of various marine species a heat stable cofactor stimulating TMAO-ase activity can be isolated. The same author tested muscle, liver and viscera of around 300 marine species from the Southeast Asian seas for TMAO-ase activity, but fishes of the order Gadiformes have not been included in this examination. (7) In the viscera of a bivalvian mollusc (*Barbatia virescens*) Harada and Yamada (1971) found a TMAO-ase with differing properties, which does not need a cofactor and is inhibited to 50% by either 10 mM riboflavin, 50 mM oxaloacetate or 10 mM azide; the enzyme exhibits a pH optimum at 5.0 and has been assayed in the presence of 1 mM methylene blue under exclusion of oxygen.

We want to report here the occurrence and distribution of TMAO-ase in the organs of several com-

mercially important fish species from the Northeast Atlantic and some properties of TMAO-ase containing extracts from the kidney of saithe.

MATERIALS AND METHODS

With the exception of Alaska pollock the fish had been caught during two research cruises: the first cruise was carried out in the spring of 1982 leading to the waters south of Iceland and to the Rockall- and Lousy-Bank; the second cruise was undertaken in the summer of 1982 to the waters off Northern Norway and Spitzbergen. Immediately after hauling the fish were identified, measured and processed: at first the tail was cut off by the peduncle and the blood was collected in a plastic bottle; then liver, spleen, pyloric caeca, kidney and muscle were taken. All samples were frozen and stored at −25 to 30°C.

Deep frozen (−30°C) Alaska pollock (round fish, not eviscerated) was delivered by a fishing company. The fish had been caught in April 1982 off Alaska and frozen on board (Karl *et al.*, 1983). Before analysis Alaska pollock was thawed incompletely overnight in a refrigerator; next morning the slightly frozen fish could be dissected easily.

Preparation of extracts

From the fish tissues TMAO-ase was extracted by solutions of the non-ionic detergent Triton X-100, polyethylene glycolmono [*p*-(1,1,3,3-tetramethyl-butyl)-phenyl] ether. The tissue was homogenized in 4 parts (w/v) of 0.2% Triton X-100 solution in distilled water by using an Ultra-Turrax. After standing in a refrigerator for 30 min the homogenate was centrifuged at high speed (38,000 g_{max}, 30 min, 5°C); the supernatants were kept in iced water until assayed. In some experiments Triton X-100 was dissolved in 0.1 M sodium phosphate buffer pH 6.1 instead of distilled water.

TMAO-ase tests

TMAO-ase activity was measured in two ways; in the first run a modification of the method described by Tomioka *et al.* (1974) was used.

Method I: 3.25 ml 0.1 M sodium phosphate pH 6.1, 0.50 ml 0.1 M TMAO, 0.25 ml 1 mM methylene blue and 1.0x ml distilled water were put into Thunberg tubes; after the addition of x ml extract the tubes were evacuated to 40 mbar within 1 min by a water jet pump regulated by a Büchi 165 Vacuum Controller; during evacuation the tubes were vortexed. Then the tubes were closed and the reaction mixture was incubated for 10 min in a water bath at 25°C; then 1 ml was mixed with 9 ml 6% (w/v) precooled perchloric acid and about 1.3 ml of the samples were centrifuged at 8000 g for 1.5 min. One ml of the supernatant was used for analysis of FA according to Nash (1953); the concentration of FA was calculated using the extinction coefficient of 8000/M/cm (Werringloer, 1978). Blanks were performed by adding enzyme solution after perchloric acid.

Method II: the reaction mixture consisted of 3.25 ml 0.1 M sodium acetate pH 4.5, 0.5 ml 0.1 M TMAO, 0.25 ml 1 mM methylene blue, 0.2 ml 2.5 mM ferrous chloride, 0.15 ml 33.3 mM L(+)-ascorbic acid, 0.15 ml 33.3 mM L-cysteine and 0.5x ml distilled water; after addition of x ml extract the assay procedure was carried on as described above.

The protein content of the extracts was determined by the Biuret method with bovine serum albumin used as a reference; if necessary, turbidity controls were made (Rehbein *et al.*, 1978). Phosphate content was measured using the Fiske–Subbarow reaction (Merckotest 3331: inorganic phosphate).

RESULTS AND DISCUSSION

For the detection of TMAO-ase activity in fish tissues a very sensitive assay is necessary, because low activities have to be expected in many fish species and tissues. Most of the previous results obtained with different TMAO-ase preparations indicate, that for optimal enzyme activity *in vitro* besides the presence of both methylene blue and Fe^{2+} reducing conditions (cysteine, ascorbic acid, exclusion of oxygen) are necessary (Tomioka *et al.*, 1974; Parkin and Hultin, 1981; Lundstrom *et al.*, 1982a). The results shown in Fig. 1 and Table 3 demonstrate that the TMAO-ase activity in extracts from the kidney of saithe was greatly enhanced by a reducing environment; oxygen inhibited the enzyme activity, which strictly depended on the presence of methylene blue in the assay. Obviously this inhibition also takes place during the storage of fish muscle: during the frozen storage of fillets from the North American hake, *Merluccius bilinearis*, (Castell *et al.*, 1973) and during the iced

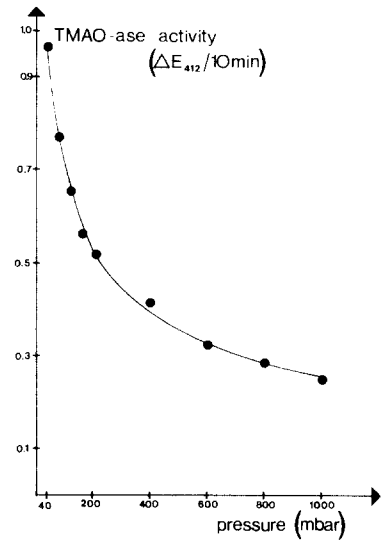

Fig. 1. Influence of atmospheric pressure in the reaction vessel on the TMAO-ase activity. For assay of TMAO-ase activity (method I) 100 µl of extract from the kidney of saithe (extraction with Triton X-100/phosphate buffer) with a protein content of 13.8 mg/ml was used. At first the Thunberg tubes were evacuated while being vortexed, then the tubes were incubated for 10 min at 25°C in a water bath still being connected with the vacuum control instrument. The time needed for evacuation varied between 84 sec pressure: 40 mbar) and 2 sec (pressure: 800 mbar).

Table 1. Phosphate content in extracts of tissues from cod and saithe

Organ	Content of phosphate in the extract (mM)* Cod	Saithe
Spleen	5.4 ± 14% (4)	5.1 ± 13% (2)
Liver	1.1 (1)	1.9 ± 64% (2)
Kidney	4.3 ± 9% (2)	2.6 ± 31% (2)†
Pyloric caeca	5.0 ± 13% (3)	6.8 ± 18% (2)
Muscle	11.0 ± 3% (4)	11.2 (1)
Blood	1.7 ± 26% (3)	n.d.

*Mean value and coefficient of variation; number of samples in brackets.

†The kidneys of saithe still contained great amounts of blood.

An equal volume of 20% (w/v) trichloric acid was added to the extracts and the mixture centrifuged for 5 min at 3000 rpm at room temperature. The supernatants were stored deep-frozen until analyzed for phosphate.

Table 2. Dependence of TMAO-ase activity on the presence of phosphate in the assay

Extract (μl)	Buffer	Concn of phosphate in the assay (mM)	TMAO-ase activity (Δ E_{412}/10 min) I	II	Mean
50	Sodium phosphate	65	0.56	0.54	0.55 ± 3%
100	100 mM, pH 6.1	65	1.27	1.30	1.29 ± 2%
50	Histidine	0.1	0.52	0.50	0.51 ± 3%
100	50 mM, pH 6.1	0.1	1.23	1.30	1.27 ± 4%

The TMAO-ase activity was determined using method I. Due to the low amount of extract used in the assay, its phosphate contribution could be neglected.

Table 3. Influence of various assay components on TMAO-ase activity

No.	Assay conditions	TMAO-ase activity [Δ E_{412}/20 min]*	[% control]
1	Control	0.46 ± 0.3%	100
2	+ 0.1 mM $FeCl_2$	0.46 ± 62%	100
3	+ 1 mM L-cysteine + 1 mM L-ascorbic acid	0.62 ± 3.4%	135
4	+ 0.1 mM $FeCl_2$ + 1 mM L-cysteine + 1 mM L-ascorbic acid	0.76 ± 1.6%	165
5	Additions as in No. 4, but without methylene blue	0.022 ± 64%	5
6	Additions as in No. 4, but without vacuum	0.46	100

*Mean values with coefficients of variation; $n = 2$.
Kidney of saithe was extracted; 50 μl of extract (12.5 mg protein/ml) were used for the TMAO-ase test (control performed according to method I).

storage of whole or minced fillets from the red hake exposed to N_2 or to vacuum (Lundstrom *et al.*, 1981, 1982b) TMAO was degraded to DMA and FA more rapidly compared to samples of both fish species which had been stored in ice in the presence of air (oxygen).

In contrast to Parkin and Hultin (1981) we did not observe any interference of the FA determination by Fe^{2+} in the Nash test. Furthermore, these authors report of the inhibition of TMAO-ase activity by inorganic phosphate (1981, 1982): DMA formation was reduced to 30% by 3 mM phosphate in the assay; this concentration was found in extracts from fish tissues (Table 1). The TMAO-ase activity in extracts from the kidney of saithe, however, was not inhibited by 65 mM phosphate (Table 2); this discrepancy to the results of Parkin and Hultin may arise from two causes: (1) different sources of the enzymes (red hake or saithe) and (2) pH dependence of the inhibition (Fig. 2). The TMAO-ase activity of the microsomes isolated from the muscle of red hake was measured at pH 7.0, whereas the extract from the kidney of saithe was tested at pH 6.1.

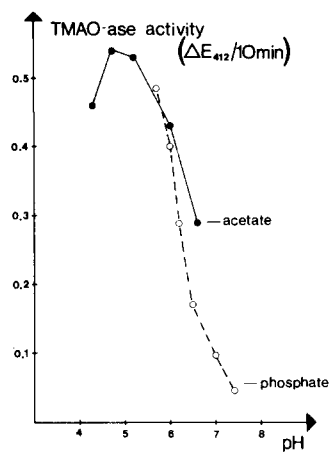

Fig. 2. pH optimum of TMAO-ase activity and comparison of the effects of acetate and phosphate on the enzyme activity at various pH values. 50 μl of extract (extraction with Triton X-100/phosphate buffer; 9.8 mg protein/ml) from the kidney of saithe were used. The pH values of the assay mixtures containing either 0.1 M sodium acetate or 0.1 M sodium phosphate had been determined after the end of the reaction.

Different pH optima are reported for TMAO-ase activity in various organisms (Sikorski and Kostuch, 1982). The enzyme in kidney extracts (saithe) was most active at pH 5.0 (Fig. 2); a comparison of the TMAO-ase activities at pH 4.5 and 7.0 in several tissues from cod (spleen, kidney, blood, pyloric caeca) demonstrated that the TMAO-ase activity was lower at neutral pH in all tissues (Table 4). These effects are in accordance with the results of Gill and Paulson (1982), who demonstrated that TMAO-ase is a lysosomal enzyme with a pH optimum in the acidic range.

Table 4. Comparison of TMAO-ase activities at different pH values in extracts from tissues of cod

Tissue	Ratio (%) of TMAO-ase activity at pH 7.0/pH 4.5*
Spleen	33 ± 2.2% (4)
Kidney	29 ± 2.8% (2)
Pyloric caeca	32 ± 4.2% (2)
Blood	39 ± 5.7% (2)

*Mean values, coefficients of variation and number of samples are given.
Enzyme activities were measured by method II replacing acetate buffer by 0.1 M histidine for the tests with pH 7.0 buffer.

Table 5. TMAO-ase activity in tissues of marine fishes

Fish species	Liver		Pyloric caeca		Kidney		Spleen		Blood		Muscle	
	I	II	I	II	I	II	I	II	I	II	I	II
Gadus morrhua (L.)	0.24 ± 80% (4)	12.7 ± 92% (4)	6.25 ± 55% (4)	352.5 ± 54% (4)	51.7 ± 34% (2)	3640 ± 26% (2)	22.1 ± 42% (4)	2072 ± 41% (4)	0.42 ± 19% (2)	27.5 ± 23% (4)	0.11 ± 112% (4)	3.49 ± 116% (4)
Pollachius virens (L.)	1.03 ± 32% (4)	28.0 ± 67% (4)	102.3 ± 47% (4)	5312 ± 48% (4)	21.7 ± 38% (3)	1610 ± 43% (3)	7.76 ± 52% (4)	619.2 ± 41% (4)	0.07* (1)	7.39* (1)	—† (4)	— (4)
Melanogrammus aeglefinus (L.)	0.09 ± 127% (4)	4.07 ± 116% (4)	3.77 ± 80% (4)	220.9 ± 85% (4)	48.0 ± 3% (2)	2984 ± 0.5% (2)	0.59 ± 177% (4)	46.8 ± 179% (4)	0.09 ± 141%* (2)	4.4 ± 141%* (2)	— (4)	— (4)
Molva molva (L.)	3.09 ± 93% (2)	77.8 ± 93% (2)	12.8 ± 21% (2)	750.9 ± 27% (4)	6.13 ± 34% (2)	452.4 ± 65% (2)	84.2 ± 19% (2)	6775 ± 22% (2)	n.d.‡	n.d.	0.095 ± 141% (2)	3.89 ± 141% (2)
Molva dypterygia (Pennant)	2.33 (1)	69.3 ± 67% (2)	9.49 (1)	729.5 ± 27% (2)	61.0 (1)	3140 (1)	108.7 (1)	7008 ± 17% (2)	3.5 (1)	234.7 (1)	0.23 (1)	6.61 ± 8% (2)
Brosme brosme (Ascanius)	2.39 ± 36% (2)	163.6 ± 59% (2)	31.8 ± 45% (2)	1425 ± 27% (2)	111.3 (1)	7591 (1)	104.4 ± 28% (2)	8252 ± 32% (2)	15.8 ± 22% (2)	595.4 ± 25% (2)	0.045 ± 141% (2)	1.55 ± 141% (2)
Micromesistius poutassou (Risso)	— (2)	— (2)	0.48 ± 104% (4)	22.2 ± 100% (4)	37.0 (1)	2255 (1)	7.06 ± 76% (3)	436.1 ± 48% (3)	0.24 ± 32% (2)	15.1 ± 23% (2)	0.06 ± 173% (3)	2.27 ± 173% (3)
Theragra chalcogramma (Pallas)§	0.95	24.3	4.35	268.3	55.0	3694	18.96	1633	n.d.	n.d.	—	—
Coryphaenoides rupestris (Gunnerus)	0.24 ± 0% (2)	5.44 ± 0% (2)	0.59 ± 26% (2)	39.7 ± 25% (2)	240.9 ± 22% (2)	16,184 ± 30% (2)	9.54 (1)	671.7 (1)	0.16 (1)	8.49 (1)	— (1)	— (1)

*Reaction time: 30 min; †no TMAO-ase activity detected ($\Delta E_{412} < 0.030$); ‡n.d.: not determined; §tissues of 5 fishes pooled.

Enzyme activities (mean values, coefficients of variation, number of samples) are given as I: nkat/mg protein, and II: nkat/g tissue; 1 nkat corresponds to the formation of $6 \cdot 10^{-2}$ μmole formaldehyde per min, i.e., $6 \cdot 10^{-2}$ U. The reaction time was 10 min, if not indicated otherwise. Enzyme assay was performed by method II.

A linear relationship between FA formation and reaction time ($t \leqslant 30$ min) or amount of extract ($x \leqslant 0.3$ ml), respectively, was found, when TMAO-ase activity was measured by method II. TMAO-ase activities determined by the optimized test system (method II) are given in Table 5 for tissues of nine marine fish species. All these species belong to the order Gadiformes, whereas in species from other orders very low (*Anarhichas* spp.: 0.07 nKat/mg protein, spleen; *Clupea harengus* L.: 0.05 nKat/mg protein, kidney) or no (*Sebastes marinus* L., *S. mentella* Travin: blood and kidney; *Scomber scombrus* L.: blood) TMAO-ase activity could be detected.

The comparison of the activities in a single tissue from different fish species of the order Gadiformes revealed a great variation of the values (Table 5). Nevertheless, some main features of the enzyme distribution should be depicted: the TMAO-ase was most active in spleen and kidney, from which follows that these tissues are suitable "indicator organs" for the presence of TMAO-ase in a fish. Muscle exhibited only very low activities with high coefficients of variation being due to the zero enzyme activity in some samples. The TMAO-ase activities in pyloric caeca, blood and liver were in the medium range. The distribution of TMAO-ase activity in the tissues of Alaska pollock are in agreement to the results reported by Tokunaga (1980); in addition he had found very high activities in the intestine, the stomach and especially in the gall bladder of this fish species.

Beside the basic information on the distribution of TMAO-ase activities in different species and in the different organs of one species, some importance for processing may be attached to these results: bleeding the fish carefully is advised for species possessing TMAO-ase (blood is rich in this enzyme), in order to avoid FA formation and texture deterioration. Another point is removing kidney tissues completely, especially before preparing minced fish flesh from the frames (compare the results by Svensson, 1980). Another interesting point is to be cautious while mixing the flesh of different species in the preparation of products: it has been demonstrated, that admixing of red hake muscle to flat fish which lack TMAO-ase activity starts FA formation in these mixtures (Lundstrom, 1982a).

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