ボラ卵巣のペプチダーゼ活性の存在について

誌名	日本水産學會誌
ISSN	00215392
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巻/号	57巻3号
掲載ページ	p. 491-494
発行年月	1991年3月

Nippon Suisan Gakkaishi 57(3), 491–494 (1991)

Existence of Peptidase Activities in Mullet *Mugil cephalus* Roe

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(Received July 11, 1990)

In order to understand the mechanism of a remarkable increase of free amino acids during the processing of dried mullet roe, hydrolyzing activities of various enzyme extracts from the fresh roe toward α-aminoacyl β-naphthylamides and α-*N*-benzoyl-DL-arginine β-naphthylamide as well as the effects of monoiodoacetic acid, puromycin, and EDTA on the hydrolyzing activities were examined. The results obtained indicated the existence in the roe of at least a few soluble aminopeptidases, cathepsin H-like enzyme, and more than two kinds of insoluble peptidases.

Previously, we reported the presence of proteolytic activities in the soluble fraction of mullet roe,[1] and the isolation and properties of an aminopeptidase that resembles soluble alanyl aminopeptidase (EC 3.4.11.14).[2] Those studies were conducted to understand the mechanism of a remarkable increase in free amino acids during the processing of dried mullet roe called "Karasumi" in Japan.[3] Although the increment in free amino acids could be accounted for partly by the proteolytic activity of the isolated aminopeptidase, but not fully by it.

It was reported that free amino acids increased during the autolysis at neutral pH in rabbit skeletal muscle,[4,5] fish muscle,[6,7] and krill,[8,9] and such an increase in free amino acids was supposedly due to the proteolysis by several exopeptidases in muscle[10] and both endo- and exopeptidases in krill.[9,11] The present study was carried out, therefore, to demostrate the existence of multiple peptidase activities in the soluble and insoluble fractions of mullet roe, using α-aminoacyl β-naphthylamides and α-*N*-benzoyl-DL-arginine β-naphthylamide as substrates.

Materials and Methods

Reagents

All the substrates used for assay contained amino acids in the L-configureation except where indicated. Leu-β-naphthylamide (Leu-NNap), Ala-NNap, Lys-NNap, Pro-NNap, Ile-NNap, Ser-NNap, Val-NNap, DL-Met-NNap, α-*N*-benzoyl-DL-Arg-NNap (BANA), puromycin, and DL-dithiothreitol (DTT) were from Sigma Chemical Co., St. Louis, MO. U.S.A. Glu-, Arg-, and Asp-NNap were from Bachem Feinchemikalien AG, Switzerland. Papain (1:350), Triton X-100, and 2-mercaptoethanol (MCE) were from Wako Pure Chemical Co., Japan. Other chemicals were analytical grade from commercial sources.

Roe Sample

Mullet *Mugil cephalus* roe used here was the same as described previously.[2]

Preparation of Crude Enzyme Extracts

Unless otherwise stated, all procedures for the preparation of enzyme extracts were done at 0–4°C.

1. Extract-1, -2, and -3. Twenty grams of mullet roe was homogenized with 3 volumes of 10 mM sodium phosphate buffer containing 2 mM MCE, pH 7.2 (buffer A) and centrifuged at 10,000×g for 20 min. The supernatant was divided into three parts and the residue was used for the extraction of insoluble enzymes as described later. One part of the supernatant (Ex-1) was subjected directly to the assay of enzyme activity. Another part was added with solid ammonium sulfate and brought to 80% saturation. The precipitated protein was collected by centrifugation, dissolved in a small amount of buffer A, and dialyzed against buffer A for 24 h. The dialysate was centrifuged again and the supernatant (Ex-2)

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was used for the assay. A third part was added with Triton X-100 to a final concentration of 0.2%. The mixture was then left at 4°C for 24 h, dialyzed against buffer A overnight, and centrifuged to obtain a supernatant (Ex-3).

2. Extract-4. Ten grams of mullet roe was homogenized with 3 volumes of buffer A. The homogenate was autolyzed at 4°C for 24 h and centrifuged to obtain a supernatant (Ex-4).

3. Extract-5. A procedure employed for the extraction of cathepsin H[12] was followed. Ten grams of mullet roe was homogenized with 3 volumes of 1% NaCl-2% *n*-butanol-10 mM EDTA solution. The homogenate had Triton X-100 added to a final concentration of 0.2%, adjusted to pH 4.5 with 2 N HCl, and centrifuged. The supernatant was incubated at 40°C for 14 h and centrifuged to obtain a supernatant (Ex-5).

4. Extract-6 and -7. The centrifugal residue (15.8 g, wet weight) obtained in the preparation of Ex-1, -2, and -3, was equally divided into two parts. One part was suspended in 0.1 M sodium acetate buffer containing 1% NaCl (pH 5.5) and added with Triton X-100 to a final concentration of 0.2%. The mixture was left at 4°C for 10 h and centrifuged. Then, the supernatant was dialyzed against 10 mM sodium phosphate buffer containing 0.2 mM DTT (pH 6.5) overnight and centrifuged to obtain a supernatant (Ex-6). The other part was suspended in 0.1 M sodium phosphate buffer containing 1% NaCl (pH 7.0). After the addition of papain (20 mg), the suspension was kept at 40°C for 20 min. The following procedures including centrifugation and dialysis were the same as those for Ex-6 (Ex-7).

Assay of Enzyme Activity

Assay of the hydrolyzing activities toward various α-aminoacyl-NNaps and BANA was done by the method of Matsutani *et al.*[13] as described previously[2] except that the assay of the activity of Ex-5 was essentially accorded with the method of Barret and Kirschke.[12] One enzyme unit is defined as the amount of enzyme which releases 1 nmol of β-naphthylamine per min.

Results and Discussion

The crude enzyme extracts prepared under above different conditions were examined for the hydrolyzing activities toward α-aminoacyl-NNaps and BANA. The results are summarized in Table 1.

Ex-1 which was an extract with buffer A could hydrolyze various α-aminoacyl-NNaps, but was inactive toward BANA. Furthermore, the hydrolyzing activity toward the substrates was almost the same as that of the extract with 40 mM Tris-HCl buffer containing 10 mM MCE, pH 7.2, which was used for extraction of the alanyl aminopeptidase in mullet roe.[2] The activities of Ex-2, which was prepared by ammoniun sulfate precipitation from Ex-1, toward the α-aminoacyl-NNaps decreased considerably. These results indicate that the aminopeptidase activity of Ex-1 is unstable during the treatment including dialysis in the presence of 2 mM MCE. In this connection, the alanyl aminopeptidase was successfully isolated from mullet roe in the presence of 10 mM MCE.[2]

Table 1. Peptidase activities of various extracts from mullet roe

(Units/g mullet roe)

Substrate (β-naphthylamide of)	Ex-1*	Ex-2	Ex-3	Ex-4	Ex-5	Ex-6	Ex-7
L-Alanine	474	283	135	409	1	137	106
L-Phenylalanine	378	220	60	332	3	58	47
L-Lysine	378	211	102	312	2	97	143
DL-Methionine	258	140	52	215	6	52	93
L-Arginine	254	140	98	211	2	99	127
L-Proline	174	101	75	139	0.3	97	20
L-Glutamic acid	141	77	57	112	0.3	44	8
Glycine	134	73	75	107	0.3	108	10
L-Leucine	127	84	29	94	5	26	87
L-Serine	91	49	43	72	0.7	65	12
L-Isoleucine	19	11	9	13	7	12	16
L-Valine	17	9	9	11	2	13	12
L-Aspartic acid	9	5	10	7	0.1	6	2
α-*N*-Benzoyl-DL-arginine	0	0	0	0	0.5	10	12

* See the text for preparations of Ex-1 to Ex-7.

The activities of Ex-4 which was an extract with buffer A from the autolyzed homogenate of mullet roe, were slightly lower than those of Ex-1. On the other hand, Ex-3 obtained from Ex-1 after autolysis in the presence of 0.2% Triton X-100, had a fairly low activity when compared with Ex-4 and Ex-1. Ex-3 and -4 did not hydrolyze BANA at all. The difference in relative activities toward α-aminoacyl-NNaps among the four enzyme extracts suggests the presence of a few kinds of aminopeptidases in the mullet roe.

Ex-5, which was prepared following the procedures for extraction of cathepsin H,[12] was able to cleave BANA and α-aminoacyl-NNaps, although the activities were very low (Table 1). As for the activity of Ex-5, monoiodoacetic acid (IAcOH) showed a strong inhibition on the hydrolysis of Arg-NNap, but did a slight inhibition on the hydrolysis of Leu-NNap, Ala-NNap, and BANA. On the other hand, puromycin activated the hydrolysis of the tested substrates, especially BANA (Table 2). These properties of Ex-5 are reminiscent of the presence of cathepsin H[14,15] or a cathepsin H-like enzyme and other unknown-peptidase judging from small inhibition of IAcOH on the hydrolysis of Leu-NNap and BANA.

Ex-6 (Triton X-100-treated) and Ex-7 (papain-treated), both of which contained insoluble enzymes, could hydrolyze various α-aminoacyl-NNaps and BANA at moderate and slow rates, respectively (Table 1). However, both extracts apparently differed from each other in their relative rate of hydrolysis of the substrates; Ex-6 cleaved Ala-NNap most rapidly, followed by Gly-, Arg-,

Table 2. Effect of monoiodoacetic acid and puromycin on the peptidase activity of Ex-5* from mullet roe

Substrate (β-naphthylamide of)	Relative activity (%)		
	Control	IAcOH (1 mM)	Puromycin (0.1 mM)
L-Alanine	100	89	143
L-Leucine	100	91	131
L-Arginine	100	22	139
α-*N*-Benzoyl-DL-arginine	100	91	218

* See the text for its preparation.

Table 3. Effect of chemicals on the peptidase activity of Ex-6* from mullet roe

Substrate (β-naphthylamide of)	Reclative activity (%)			
	Control	IAcOH (1 mM)	Puromycin (0.1 mM)	EDTA (1 mM)
L-Alanine	100	106	29	100
L-Lysine	100	133	60	104
L-Arginine	100	121	66	102
L-Proline	100	96	20	99
L-Glutamic acid	100	85	12	97
Glycine	100	89	8	99
α-*N*-Benzoyl-DL-arginine	100	100	100	100

* See the text for its preparation.

Table 4. Effect of chemicals on the peptidase activity of Ex-7* from mullet roe

Substrate (β-naphthylamide of)	Relative activity (%)			
	Control	IAcOH (1 mM)	Puromycin (0.1 mM)	EDTA (1 mM)
L-Alanine	100	103	31	93
L-Lysine	100	125	43	101
L-Argine	100	118	55	107
L-Methionine	100	110	40	102
L-Leucine	100	133	56	109
α-*N*-Benzoyl-DL-arginine	100	15	86	228

* See the text for its preparation.

and Pro-(=Lys-)NNap in decreasing order, while Ex-7 was the most active toward Lys-NNap, followed by Arg-, Ala-, DL-Met-, and Leu-NNap.

The effects of IAcOH, puromycin, and EDTA on the activities of Ex-6 and Ex-7 are shown in Tables 3 and 4, respectively. The BANA-hydrolyzing activity of Ex-6 was not affected by these chemicals, while that of Ex-7 was strongly inhibitited by IAcOH and strongly activated by EDTA. The inhibiting or activating effects of these chemicals on the hydrolysis of other substrates were also different between them. Furthermore, all these properties of the aminopeptidase activities in Ex-6 and Ex-7 disagreed with those of the purified alanyl aminopeptidase.[2)]

From the results obtained, we can conclude that the purified alanyl aminopeptidase, which we already characterized in the previous study,[2)] represents most of aminopeptidase activities in the soluble fraction of mullet roe and that at least a few other soluble enzymes with lower activity are also present. Moreover, the roe probably contains cathepsin H-like enzyme and more than two kinds of aminopeptidases or exopeptidases in the insoluble form. All of those enzymes, in combination with endopeptidases,[1)] might contribute more or less to the remarkable increase in free amino acids during the Karasumi processing. Purification of some of those mullet roe proteases are being attempted.

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