Ca^{2+}-ACTIVATED NEUTRAL PROTEINASE IN SOME FISH ERYTHROCYTES

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In order to examine the type of calpain in fish tissues, some freshwater fish (trout and sturgeon) erythrocytes were chosen as the experimental material, since only calpain I, a low (micromolar)-Ca^{2+}-requiring form of calpain is known to exist in mammalian erythrocytes. The fish erythrocytes was found to contain a cytosol Ca^{2+}-activated proteinase (enzyme of calpain family). By Ultrogel chromatography, calpain and calpastatin (specific inhibitor for calpain) were separated from fish erythrocyte hemolysate. Studied effect of serine, carboxyl and thiol protease inhibitors on calpain revealed that it is a cystein proteinase. Trout and sturgeon erythrocyte calpain was classified as calpain II. That erythrocyte calpain showed maximal activity at 3 mM Ca^{2+}. It was inactivated by heating at 58°C for 5 min (less than half of maximal activity) at pH 7.5, the optimal pH for neutral proteinases. These properties are substantial characteristics of calpain II appear to exist in fish erythrocytes.

Calpain (Ca^{2+}-dependent cysteine proteinase, E.C.3.4.22.17) is known to be distributed ubiquitously in mammalian tissues (Murachi, 1983; Suzuki et al., 1988). There are at least two types of the enzyme, to be called calpain I and calpain II and show higher and lower sensitivities, respectively, to Ca^{2+} concentration. Calcium has been found to play an important role in the maintenance of cell conformation in the erythrocyte membrane of mammalians (Murakami et al., 1981). One notable Ca^{2+}-activated event in erythrocytes is the proteolytic or aggregative change in the membrane constituents, particularly in connection with the deformability or flexibility of cell membranes. The aggregation formation has been reported to be caused by action of transglutaminase, a cytosolic enzyme.

The relative amounts of calpains differ among tissues and cells. In particular, mammalian erythrocytes are known to contain only calpain I (Murachi et al., 1981). At the same time Ca^{2+}-activated proteinases in carp erythrocytes are represented by another enzyme form, calpain II (Toyohara et al., 1985). This paper describes the detection and characterization of cytosolic calpain from some freshwater fish erythrocytes in order to estimate it type.

Experimental Procedures

Materials. Trout (*Salmonidae*), Russian sturgeon and beluga (*Acipenseridae*) was used as objects of research. Blood (50 ml) was collected from fish by cutting their caudal fins. After washing thoroughly with 0.15 M NaCl solution, erythrocytes were collected by centrifugation and hemolyzed for 10 min in 100 ml of buffer A (20 mM Tris-HCl buffer, containing 1 mM EGTA and 5 mM 2-mercaptoethanol, pH 7.5). The hemolysate was centrifuged at 105.000 g for 60 min.

Methods. No proteolytic activity toward casein was detected in the unfractionated hemolysate. Gel chromatography of samples was done on columns with Ultrogel AcA 34 to separate calpastatin, a cytosol inhibitor of calpains, at elution rate 22 ml/h with buffer B (10 mM Tris-HCl, containing 50 mM NaCl, 1 mM EDTA, 5 mM 2-mercaptoetanol, pH 7.5). Calpain activity was determined in 3.5—4.0 eluent fractions with casein as substrate according to the method of Yoshimura et al. (1983). Reaction mixture for both calpains contained 0.4% casein, 0.5 mM cystein or dithiotreitol, 50 mM imidasole-HCl buffer at pH 7.5, and enzymatic solution. Its total volume was 2.5 ml and 3 mM Ca^{2+} ($CaCl_2$). After 30 min incubation at 30°C the reaction was terminated by adding 2.5 ml 10% trichloracetic acid. The concentration of acid-soluble products was determined spectrophotometrically at 280 nm. Blank had the same composition, but Ca^{2+} was added after termination of the reaction. Enzymatic activity was estimated in unites of activity in 1 ml eluent from Ultrogel column (absorbance units at 280 nm per ml eluent). One unit of caipain activity was defined as the amount that caused an increase in the absorbance at 280 nm of 1.0 for 30 min according to Murachi et al. (1981).

Results and Discussion

Separation of calpain and calpastatin. We could not detect any calpain activity in the hemolysate of trout and sturgeon erythrocytes. This could possibly be ascribed to the excess amount of calpastatin compared with calpain in the hemolysate, so gelchromatography was performed to separate calpain and calpastatin from the hemolysate. The hemolysate after dialysis against buffer B was applied on a column (2.6×60 cm) of Ultrogel AcA 34 equilibrated with buffer B and the column was washed with the same buffer.

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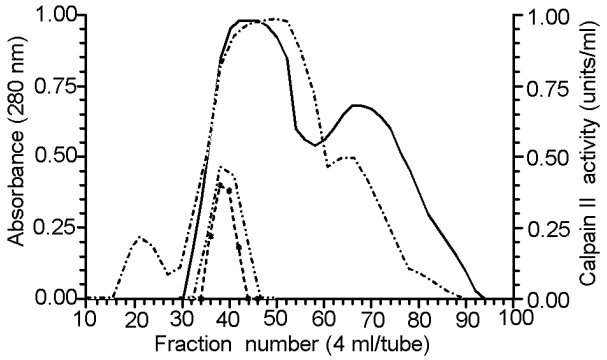

Fig. 1. Separation of calpain II from Russian sturgeon and trout erythrocyte hemolysate by Ultrogel AcA 34 chromatography. A column (2.6 × 60 cm) of Ultrogel AcA 34 was loaded with hemolysate. The elution was carried out with 20 mM Tris-Hcl buffer, containing 50 mM NaCl, 1 mM EDTA, 5 mM 2-ME, ph 7.5: ——, absorbance at 280 nm; – – –, calpain II activity for Russian sturgeon erythrosyte; – · – · –, absorbance at 280 nm, – ·· – ·· –, calpain II activity for trout erythrocyte.

As is shown from Fig. 1, at around fraction No. 40 calpain activity was eluted. Study of chromatografic distribution of calpain activity from hemolysates of fish erythrocytes detected only one form of enzyme with molecular mass approximately 70 kDa.

The fractions showing caseinolytic activity (fraction Nos 35-43) were pooled and condenced. This partially purified enzyme preparation was used for the further experiments.

Properties of calpain. Figure 2, *A* shows the effect of Ca^{2+} concentration on the activity. The caseinolytic activity was detected with millimollar concentrations of Ca^{2+} and was not detected at micromollar concentrations of Ca^{2+}. Therefore we concluded that this activity was due to calpain II, the high-Ca^{2+}-requiring form of calpain (Murachi et al., 1981). Trout and sturgeon erythrocyte calpains showed maximal activity at 3 mM Ca^{2+} (Fig. 2, *A*). The activity was decreased to some extent in the presence of higher concentration of Ca^{2+} in the case of trout erythrocyte calpain and stayed the same maximal level in the case of sturgeon erythrocyte calpain.

Both trout and sturgeon erythrocyte calpains showed maximal activity at pH 7.5 (Fig. 2, *B*). These facts indicated that there are neutral proteinases. Serine protease in-

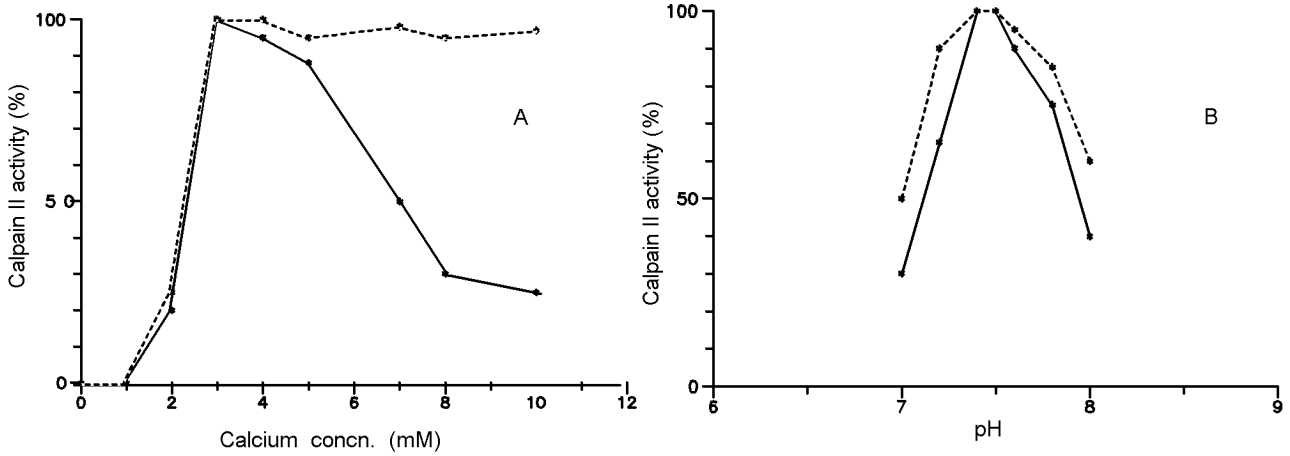

Fig. 2. Effect of calcium concentration (A) and pH (B) on the activities of Russian sturgeon (– – –) and trout (——) erythrocyte calpain II. The reaction mixture contained 0.5 ml of the enzyme solution, 5 mM DTT, 0.4% casein, 50 mM imidazole-HCl buffer, pH 7.5, and the indicated concentration of calcium in total volume of 2.5 ml. Calcium was added as calcium chloride.

Table 1

Effect of inhibitors on the activity of trout erythrocyte calpain II (Ca^{2+}-activated cystein proteinase)

Inhibitors	Concentration	Remaining activity (%)
None		100
Effect on cystein proteinases:		
Iodoacetamide 4-chloromercuribenzoic Na salt HgJ_2	1 mM 1 mM 5 mg/ml	20 30 10
Agent of bivalent ions binding: EGTA	 3 mM	 0
Effect on serine proteases:		
Phenylmethyl sulfonylfluoride PMSF	2 mM	90
Effect on acid proteinases:		
Pepstatin	20 mg/ml	90
Effect of enzyme substance heating:	Time of heating:	
	55℃ × 5 min	35

hibitor such as phenylmethylsulfonyl fluoride, and carboxyl protease inhibitor pepstatin did not show sufficient effect on the enzyme activity, whereas thiol reagents (iodoacetamide and *p*-chlormercuribenzoate) almost completely abolished it (Table 1). These data indicate that fish calpain is a cystein protease.

After heating of enzyme substance (55 °C × 5 min), calpain activity is 50% less comparing to reference (Table 1). That is known to be a good criterion of differentiating of calpain I and calpain II (Toyohara et al., 1985). Activity of calpain II usually reduces after this treatment, at the same time activity of calpain I remains unchanged.

These properties supported the view that trout and sturgeon erythrocytes Ca^{2+}-activated neutral cystein proteinase clearly belongs to the calpain family and is calpain II type.

It is very interesting that calpain II was detected in fish erythrocytes while only calpain I is known to exist in mammalian erythrocytes (Murakami et al., 1981., Hatanaka et al., 1983) and both calpains I and II—in chicken erythrocytes (Murakami et al., 1982). These facts are likely to imply the molecular evolution of calpains among fish, avian and mammalian erythrocytes (Toyohara et al., 1985).

The data obtained in this research concerning fish erythrocytes calpain in normal state offer possibilities to study of their properties and function in pathology. Our previous results of calpain activity study shows effect of different toxic agents on calpains of fish tissues (Käiväräinen et al., 1998). The same effect was observed in the tissues of the Russian sturgeon *Acipenser gueldenstaedti* caught in the Volga river delta. This species displayed so-called "muscle stratification" caused by industrial sewage (Nemova et al., 1992).

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