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Isolation and Characterization of Two Cathepsins from Muscle of *Carassius auratus gibelio.*

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Abstract

Two cathepsins were identified in the white skeletal muscle from Carassius auratus gibelio. One of these, cathepsin D, has optimal activity at pH 3.5 with hemoglobin as substrate and a molecular weight 38,200 Da. The second, cathepsin E, is a protein with a molecular weight of 82,000 Da and an optimum pH 2.5. Both of the enzymes were strongly inhibited by pepstatin, a specific inhibitor for aspartic proteinases.

Keywords: cathepsin D, cathepsin E, Carassius auratus gibelio, pepstatin.

Introduction

Proteolytic cleavage of peptide bonds is one the most frequent and important enzymatic modifications of proteins. Proteolytic processing is the final step in the expression of activity of a great variety of proteins. Processing occurs in many different ways and is triggered by many different kinds of proteases. However, in every known case, proteolysis is directed and limited to the cleavage of specific peptide bonds in the target protein. The key to this selectivity is limited proteolysis, which depends on the accessibility of the scissile peptide bond to the processing protease and on its specificity. Compact protein domains are usually resistant to proteolysis, in contrast to more flexible surface loops and interdomain regions that can adapt themselves to the active site of the protease [1].

Proteolytic enzymes are ubiquitously distributed in all biological tissues and fluids. The best characterized are mammalian digestive proteases such as pepsin, trypsin, chymotrypsin and elastase. The digestive proteases are involved in the hydrolysis of dietary proteins and do not play a role in protein turnover within an organism. Much less is known, by comparison, about intracellular tissue proteases, their enzymatic specificity and physiological substrates [2]. The repertoire of proteases that are integral components of cells is enormous and in part unexplored. To mention a few, it includes the entire class of lysosomal proteases (cathepsins), membrane-bound proteases and proteases of specialized tissues such as the reproductive tracts, muscle, skin, lens and adrenals.

The purpose of the present study was to investigate the proteolytic enzyme from white skeletal muscle of *Carassius auratus gibelio.* Many kinds of proteolytic enzymes were reported in fish. A human-like cathepsin D protease was isolated from the salmon (*Oncorhynchus masou)* ovary [3]. A caspase, which plays an important role in apoptosis in

fish cells and zebrafish (*Danio rerio)* embryo, was reported [4]. From chum salmon (*Oncorhynchus Keta),* an anionic trypsin acting on p- aminophenyl ester, was isolated [5]. In rainbow trout matrix was detected a metalloproteinase capable of degrading type I collagen [6].

Materials and Methods

Enzyme's extraction

After the fish killed, the white skeletal muscle was quickly dissected and rinsed with cold saline solution to remove blood. The tissue was suspended in demineralised water and was disrupted with a Polytron homogenizer at 0-4^{o}C. The disrupted cells were submitted at a subcellular fractionation by differential centrifugation. In this procedure the homogenate was centrifuged at 600 g for 10 minutes, than the supernatant obtained was centrifuged for 10 minutes at 10,000 g. The pellets were suspended in 0,1 M TRIS-HCl buffer, pH 7.4 and centrifuged at 4,000 g. The clear supernatant obtained continents mainly proteins from mitochondria and lysosomes and was utilized for investigation.

Assay of proteolytic activity

The proteolytic activity (cathepsin activity) was assayed according to Barret [7], using acid-denatured hemoglobin as a substrate. A 8% hemoglobin solution was diluted about 3-fold with distilled water, then acidified to pH 2.0 with 1 N HCl, and the final concentration of hemoglobin was brought to 2% with distilled water. One milliliter of the substrate solution is incubated with an appropriate amount of enzyme solution at 37^{0}C. The reaction is stopped by addition of 2 ml of 5% trichloroacetic acid. The mixture was filtered and the soluble reaction products were treated with Folin reagent and measured at 750nm.

Units of activity

There is no international agreement about units obtained by the hemoglobin-digestion method. Some investigators, including Anson and Mirsky [8] used the amount of tyrosine-equivalent in trichloroacetic acid-soluble peptide. In the current study, one unit of proteolytic activity (U) was defined by the amount of enzyme will remove 1μmol of tyrosine in a minute from the acid-denatured hemoglobin.

Determination of protein

Protein concentration was determined using the Bradford method [9], with bovine serum albumin as standard, and, during the course of enzyme purification, by measurement of A_{280}.

Testing of potential inhibitors

Portions of the enzyme solution were mixed with potential inhibitors and incubated for 30 minutes at 37^{0}C. Mixtures were then incubated with acid-denatured hemoglobin. Reagents blanks were also run for each potential inhibitor. The percentage inhibition was determined by comparing the activities with those measured for positive controls that contained no inhibitor.

Results and Discussions

The crude extract of white muscle of *Carassius auratus gibelio* was fractionated with acetone. The 40 – 70% acetone precipitate was collected by centrifugation at 15,000 g for 15 minutes at –5^{0}C. This was redissolved in 9% NaCl and applied on a Bio-Gel P-100 column (1.6 x 72 cm) preequilibrated with the same solution. The column was run at 10 ml/h with a solution 9% of NaCl. The typical elution profile is shown in **Figure 1**. Proteolytic activity against hemoglobin was detected as two peaks. We designated these two peaks as cathepsin I

and cathepsin II because the cathepsins are the only proteolytic enzymes founded in lysosomrs.

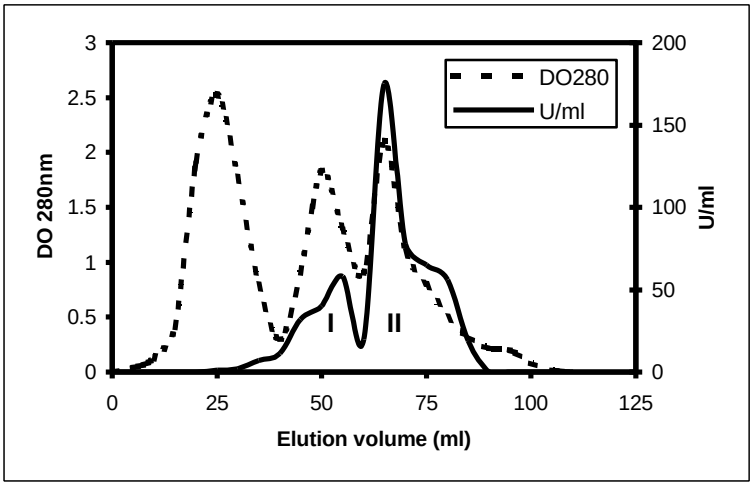

Figure 1. Separation of cathepsins by gel-filtration on Bio-Gel P-100.

The molecular weights of the two proteolytic enzymes were estimated using a calibration kit with: aprotin (6,500), citocrom c (12,400), carbonic anhydrase (29,000), albumin (66,000) and alcohol dehydrogenase (150,000). The elution volume of cathepsin I matched a molecular weight of 82,000 Da. For the cathepsin II, the molecular weight was estimated to 38,200 Da (**Figure 2**).

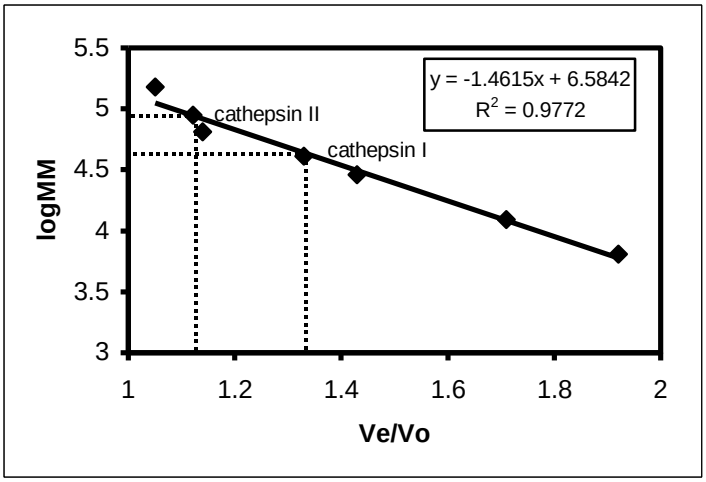

Figure 2. Molecular weights of cathepsins.

The activities of both proteolytic enzymes were higher of acid pH. The cathepsin I hydrolyzed hemoglobin most rapidly at around 2.5 and at a significantly lower rate at higher pH values such as pH 4.0 and pH 5.0. For the enzyme II the maximal proteolytic activity was at pH 3.5 (**Figure 3**).

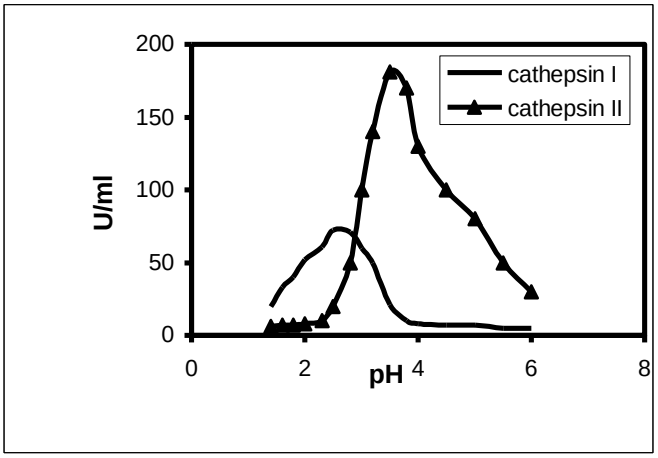

Figure 3. Hydrolytic activities of the cathepsins at different pH values.

The both proteolytic enzymes had hydrolytic activity on various protein substrates. Protein substrates such as hemoglobin, serum albumin, and casein were hydrolyzed efficiently (**Table 1**). Hemoglobin was the best protein substrate for both enzymes.

Table 1. Hydrolyses of proteins substrates by cathepsins I and II.

Substrate	Relative activity* (%)	
	Cathepsin I	Cathepsin II
Hemoglobin	100	100
Bovine serum albumin	22.3	52.3
Ovalbumin	0	2,5
γ-globulin	5.2	7.4
Casein	30.2	23.6

* Activity with hemoglobin as substrate is taken as 1005 in each case.

Potentials inhibitors of proteolytic activities were tested and the results are presented in **Table 2**. Iodoacetic acid and iodoacetic amide, compounds that irreversibly inactivate cysteine proteinases, did not significally alter the activity of both cathepsins from white skeletal muscle from *Carassius auratus gibelio.* Diisopropyl phosphofluoridate and phenylmethanesulphonyl fluoride, potent inhibitors of serine proteinases had no effects on cathepsins. The both proteolytic activities were inhibited only by pepstatin, one of the most specific inhibitor in enzymology that is highly selective for the aspartic proteinases.

Table 2. Effect of potential inhibitors on cathepsin I and II.

Compound	Final concentration	Inhibition (%)	
		Cathepsin I	Cathepsin II
Iodoacetic acid	10 mM	5.3	4.5
Iodoacetic amide	10 mM	4.1	4.6
Phenylmethanesulphonyl fluoride	1 mM	6.2	3.4
Diisopropyl phosphofluoridate	1 mM	2.5	3.5
Pepstatin	1μM	98.2	97.5

The only intracellular proteinases that may be inhibited by pepstatin are cathepsins D (EC 3.4.23.5) and cathepsins E (EC 3.4.23.34 [10]. These two cathepsins are specificity similar to pepsin. Hemoglobin is the best protein substrate for these enzymes, and its hydrolysis proceeds most rapidly at very low pH values.

The proprieties of cathepsin I, pH optimum 2.5 and molecular weight 82,000 Da, suggest that it is a cathepsin E. Catepsins E hydrolysis hemoglobin most rapidly at around pH 2.5 at a significantly lower rate at higher pH values such as pH 4.0 and pH 5.0 [11] and has a molecular weight about 76,000 – 80,000 Da [12]. The cathepsin II seems to be a cathepsin D, enzyme with a molecular weight about 38,000 – 50,000 Da and an optimum pH 3.5 [11].

The properties of cathepsin D isolated from white skeletal muscle of Carassius *auratus gibelio* are similar to those of other fish catepsins. A cathepsin D with a molecular weight of 38,000-39,000 Da, an optimum pH 2.5 and p 6.8 was purified from herring muscle (*Clupea harengus*).From herring muscle (*Clupea harengus)* was purified a cathepsin D with a molecular weight 38,000-39,000 Da, an optimum pH 2.5 and a pI 6.8. It was inhibited by pepstatin and it was able to degrade myosin, actin and tropomyosin [13]. Sex- and tissue-specific expression of aspartic proteinases was shown in zebrafish (*Dario rerio)* [14]. Antibacterial cathepsins in different types of ambicoloured Japanase flounder skin were reported [15].

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