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Original article

Serine-like proteolytic enzymes from common carp *Cyprinus carpio* L. seminal plasma are able to degrade sperm proteins

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ABSTRACT

The application of zymography, with sperm proteins as a substrate, allowed for the first time the visualisation of two serine proteinases with a molecular weight of 76 and 163 kDa from common carp *Cyprinus carpio* L. seminal plasma. Twenty four hours of incubation in a development solution with a pH of 7.5 and incubation at 37 °C were the best conditions for the visualisation of serine proteinase; however, proteolysis was also observed at 4 °C. Our results indicate that serine proteinase from common carp seminal plasma with a molecular weight of 76 and 163 kDa may be involved in the degradative mechanism of sperm proteins. This mechanism may be responsible for the removal of damaged sperms by the digestion of native sperm proteins.

1. Introduction

The seminal plasma of fish is a unique system responsible for many biological processes connected with the maturation, storage and/or aging of sperm. Firstly, seminal plasma proteins are related to regulating spermatogenesis in the testes and sperm motility [1,2]. Secondly, species-specific proteins of seminal plasma protect sperm motility and its fertilisation ability during storage [3]. Lastly, proteins present in seminal plasma take part in remodelling the testes during and after seasonal breeding. Proteinases and their inhibitors play a crucial role in the mentioned processes [4–6].

Metalloproteinases and serine proteinases are the main proteinases whose activities were detected in the seminal plasma of teleosts using gelatine and casein as a substrate [7–9]. The activities of these enzymes were also detected in common carp *Cyprinus carpio* L. seminal plasma and testes fluid [10,11]. In freshwater fish, the physiological functions of seminal plasma and/or testes fluid proteinases have been postulated to be related to the regulation of spermatogenesis, sperm motility activation and aging processes (removal of immature or damaged sperm) [12,13].

Recently the trypsin-like proteinase and proteasome subunits were identified in common carp seminal plasma [14]. It is known that trypsin could be involved in various steps of spermatogenesis in the testis [15]. Similarly to the proteasome-ubiquitin complex [16], serine protease could be responsible for cell apoptosis and tissue turnover. However, there are no data proving the potential to degrade sperm protein by proteinases present in common carp semen.

Analysis of the proteolytic activity in biological material together with their inhibitors is usually impossible in one step. In fish, protease inhibitors are dominant proteins in seminal plasma [6]. For that reason, it is impossible to measure any proteolytic activity using simple kinetic methods. However, using the substrate polyacrylamide gel, it is possible to visualise and evaluate proteolytic activities in simple one step [7,8,10]. Therefore, the use of substrate electrophoresis is the most effective option for evaluation of the fish seminal plasma proteolytic enzymes.

In order to identify proteolytic activities in the seminal plasma of common carp, different substrates (gelatine, casein, albumin and haemoglobin) have been used [11]. It has been shown that gelatine is a suitable substrate for the identification of metalloproteinases and serine proteinases; however, when using casein, only serine proteinases were detected in common carp seminal plasma. On the other hand, other substrates, like sperm proteins, have never been used for the detection of enzymatic activity in seminal plasma. Incorporating the sperm proteins into polyacrylamide gel as a substrate may help to answer the question regarding the involvement of seminal plasma proteinases in sperm degradation.

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The objective of this study was to examine whether seminal plasma proteinases are able to degrade sperm proteins. Moreover, the activity of proteolytic enzymes with regard to degrading sperm proteins was measured in different *in vitro* conditions such as temperature and pH. This allows us to discuss their potential physiological role in common carp reproductive tracts.

2. Materials and methods

2.1. Semen collection and sperm extraction

Semen samples were collected through gentle abdominal massage of adult males of common carp (n = 10) originating from the Knieja Fishery Farm (50°49′31.6″N 19°29′34.6″E) in May. Before semen collection, the males were anaesthetised with 2-phenoxyethanol (Sigma-Aldrich, St. Louis, MO, USA) given at a dose of 0.5 mL L^{-1}. Special care was taken during semen collection to avoid contamination with urine, faeces, blood or mucus.

The seminal plasma was separated from the sperms by centrifugation at 10,000 *g* for 10 min. Seminal plasma and sperms pellets were stored at −80 °C. The sperm was thawed in extraction buffer (50 mM Tris-HCl at pH 7.6), stored for 1 h at 4 °C and centrifuged twice (10,000 *g* for 10 min). The supernatant was considered as sperm extract and was used as a source of sperm proteins. The supernatant was stored at −80 °C.

2.2. Electrophoretic detection of the proteolytic activity of common carp seminal plasma using sperm containing polyacrylamide gel

The protein content in the sperm extract was determined using the methods of Lowry et al. [17]. Representative samples of seminal plasma from common carp (n = 3) were subjected to electrophoresis in sperm protein-containing (0.1% of sperm proteins) polyacrylamide (10% acrylamide) gels in the presence of SDS and under non-reduction conditions [18]. Seminal plasma in the amount of 27 μL (10 μL seminal plasma diluted one-to-one with 0.7% NaCl and 7 μL of the double-concentrated stain with SDS) was loaded into each well.

Electrophoresis was conducted at 200 V and 40 mA (for two gels) for 85 min, in the presence of an electrode buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS (pH 8.3) in an Mini-Format Vertical electrophoresis system (Bio-Rad, Hoefer, San Francisco, CA, USA). After electrophoresis, the gels were washed twice at room temperature with 2.5% Triton X-100 (Sigma-Aldrich) for 30 min and then incubated in developing solution, 50 mM Tris-HCl buffer containing 200 mM NaCl, 0.02% Triton X-100 with and without 5 mM $CaCl_2$. The following conditions were tested to optimise the method: length of time of gel incubation in the development solution (4, 8, 12 and 24 h), pH of the development solution (5.5, 6.5, 7.5, 8.5 and 9.5) and incubation temperature (4, 22 and 37 °C). The development solution contained 10 mM benzamidine, 5 mM EDTA or 20 μM E-64 (Sigma-Aldrich) which act against serine-like proteinases, metalloproteinases and cysteine proteinases, respectively. After incubation, the gels were stained in 0.025% Coomassie Brilliant Blue (Sigma-Aldrich) for 24 h and stored in 2% acetic acid. The gels were documented with a digital camera (Canon Inc., Ōta, Tokyo, Japan).

Molecular weight was estimated using pre-stained SDS-PAGE standards (Bio-Rad) such as myosin (Mr 198.6 kDa), ß-galactosidase (Mr 110.8 kDa), bovine serum albumin (Mr 64.1 kDa), ovalbumin (Mr 44.7 kDa), carbonic anhydrase (Mr 31.5 kDa), soybean trypsin inhibitor (Mr 28.6 kDa), lysozyme (Mr 19.3 kDa) and aprotinin (Mr 6.6 kDa). The molecular weight of proteinase bands were estimated with the use of the Kodak1D program (Eastman Kodak Company, New Haven, USA). In addition, relative optical density (ROD) was measured.

2.3. Gel filtration of the sperm extract

Gel filtration (GF) was performed on a Superdex 200 X/K HiLoad 16/60 column equilibrated with 50 mM Tris-HCl at pH 7.6 and 50 mM Tris-HCl, 150 mM NaCl; 0.1% SDS at a flow rate of 1.0 mL/min

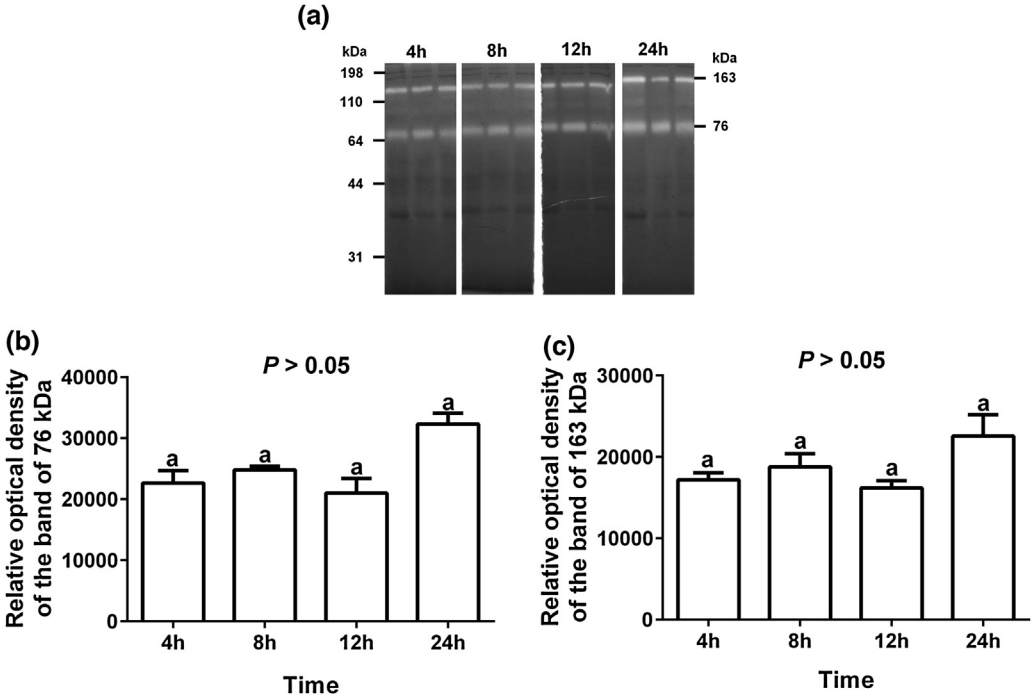

Fig. 1. Zymograms of seminal plasma using sperm proteins as a substrate (a) at different lengths of incubation (4 h, 8 h, 12 h and 24 h). An ROD band of 76 kDa (b) and 163 kDa (c) was measured using the Kodak 1D program. Data represent means ± SEM. Means with the same letters indicate no statistical differences.