



Identification of parvalbumin-like protein as a major protein of common carp (*Cyprinus carpio* L) spermatozoa which appears during final stage of spermatogenesis

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ABSTRACT

Parvalbumin is well known as the major fish allergen that is typically present in high amounts in muscles, where it functions in calcium buffering and is involved in the relaxation process in fast-twitch muscles. We show in our current study that parvalbumin-like protein is present in high amounts in carp spermatozoa. It is the first report to demonstrate the presence of parvalbumin-like protein in fish spermatozoa. Using antibodies produced against purified carp parvalbumin-like protein, we localized parvalbumin-like protein to spermatids and spermatozoa. Our results indicate that parvalbumin-like protein appeared during the final stage of spermatogenesis. We also detected high amounts of parvalbumin-like protein in carp seminal plasma but not in blood plasma which suggests that its function may be specific for the male reproductive tract. The activation mechanism of carp sperm movement is not fully understood, but in carp, Ca^{2+} influx is the prerequisite for the initiation of sperm motility. The appearance of parvalbumin-like protein in high amounts in mature spermatozoa coincides with their acquiring the ability to move. The presence of parvalbumin-like protein in spermatozoa and seminal plasma strongly suggests that parvalbumin-like protein is an important part of the Ca^{2+} -mediated mechanism of sperm activation in carp.

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1. Introduction

Parvalbumins are small, acidic calcium-binding proteins. Parvalbumin was discovered in carp muscle (Henrotte, 1952) and became the first protein to be recognized to possess the EF-hand motif (Kretsinger and Nockolds, 1973). This motif is the most common calcium-binding motif found in proteins (Lewit-Bentley and Réty, 2000). The EF-hand family of Ca^{2+} -binding proteins includes the well known calmodulin, troponin C and S100 proteins (Kawasaki et al., 1998).

Parvalbumins, intracellular proteins, exist in fairly high amounts in the white muscle of lower vertebrates (Pechère, 1977), including fish, or in a lower quantity in the fast-twitch muscle of higher vertebrates (Lehky et al., 1974). Intensive studies on cod (*Gadus callarias*) identified parvalbumins as a fish allergen (Elsayed and Aas, 1971). Later they were proven to be the major fish allergen in a variety of fish species including carp (Bugajska-Schretter et al., 1998; Swoboda et al., 2002; Hildebrandt, 2010). Besides the muscle, parvalbumin is also present in the central nervous system and in lower levels in the kidney, adipose tissue, ovary and testis (Schneeberger and Heizmann, 1986; Berchtold et al., 1984).

Information concerning the possible role of parvalbumin in the male reproductive tract is limited and restricted to mammals. In the rat testis, parvalbumin may be involved in the production of testosterone in Leydig cells and in Ca^{2+} -regulated events during spermatogenesis (Kägi et al., 1987). Recently, Cui et al. (2008) have found that parvalbumin is a key protein that may be an early molecular target responsible for spermatogenesis suppression induced by hormone treatment. There are no data concerning the presence of parvalbumin and its role in the male reproductive system of fish.

Extracellular Ca^{2+} is a prerequisite for the initiation of sperm motility in carp (Alavi and Cosson, 2006). Sperm motility cannot be initiated in Ca^{2+} -free hyposmotic solutions and the initiation of motility is accompanied with an increase in the intracellular Ca^{2+} levels (Krasznai et al., 2000; 2003). The latter indicated that Ca^{2+} channel blockers reversibly inhibit the initiation of sperm motility which suggests that Ca^{2+} influx is the prerequisite for the initiation of carp sperm motility. It is very likely that calcium-binding proteins are involved in the regulation of Ca^{2+} movement across carp sperm membranes.

During our previous work (Wojtczak et al., 2007a) we observed on PAGE gels after electrophoresis of common carp (*Cyprinus carpio* L) seminal plasma small acidic protein (Fig. 1). This protein can be considered as one of the major for carp seminal plasma (as they can be already visualized by Commassie Brilliant Blue staining, see left lane in

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Fig. 1) and seemed to be specific for semen, because it was not detected in blood plasma. Moreover, during native-PAGE we observed a similar protein in carp sperm extract. Identification of specific proteins can be a prerequisite for a better understanding of reproduction physiology of male fish. Herein we describe the procedure for purification and preliminary characteristics of one of the major proteins from carp spermatozoa and seminal plasma. These proteins were identified as parvalbumin-like proteins. The presence of parvalbumin-like protein, a calcium-binding protein, in carp semen suggests that this protein may be involved in the Ca^{2+} -mediated mechanism of the activation of sperm motility. These results may be fundamentally important for a better understanding of the mechanism for controlling fish sperm motility.

2. Material and methods

2.1. Source of milt and muscle

European common carp (*C. carpio* L.) was maintained under ambient temperature in ponds of the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gołysz, Poland. Before hormonal stimulation the fish were transferred from ponds to tanks with a water temperature of 20 °C reached after seven days. The common carp was injected intradorsally 24 h before collection of sperm with Ovopel (one pellet containing of 18–20 µg of GnRH analog and 8–10 mg of metoclopramide per one kg of fish bw; Interfish Ltd, Hungary). Milt was obtained from 5–7 years old carp with an average body weight of 5–7 kg ($n=50$). The milt samples were pooled and used for the isolation of parvalbumin. Milt samples were centrifuged twice at 8000 g for 10 min and seminal plasma and sperm pellets were collected and stored at –80 °C. Muscle was obtained from common carp raised in hatchery condition in the Department of Ichthyology (University of Warmia nad Mazury, Poland). Carp skeletal muscle (5.5 g) was homogenized on ice in 1 volume of muscle with 3 vol. of 20 mM Tris–HCl (pH 7.6) containing 5 mM EDTA using a homogenizer (UltraTurrax T25, IKA-Labortechnik, Poland). The extract was centrifuged at 8000 g for 30 min at 4 °C. The supernatant was centrifuged at 14,000 g for 30 min.

Approval by the Animal Experiments Committee in Olsztyn, Poland was gained before starting any experiments.

2.2. Purification of parvalbumin-like protein from carp spermatozoa and seminal plasma

Spermatozoa (from 30 mL of carp semen) were thawed at room temperature for 1 h with 0.02 M Tris–HCl buffer, pH 7.6 at 4 °C,

centrifuged and the supernatant obtained was applied to a Q-Sepharose X/K 16/10 column (GE Healthcare, Uppsala, Sweden) previously equilibrated with 20 mM Tris–HCl buffer, pH 7.6 (buffer A). After elution of unbound proteins with buffer A, bound parvalbumin-like protein was eluted using an increasing linear gradient from 0.00 to 0.35 M of NaCl in the same buffer over 135 min at a flow rate of 1 mL min^{–1}. Fractions of 1 mL each were collected. Parvalbumin-like protein was eluted at the NaCl concentration range of 18.8–21.5%. The fractions containing parvalbumin-like protein were pooled separately, dialyzed 24 h against 10 mM Tris–HCl, pH 7.6 (with two changes), lyophilized and subjected to preparative electrophoresis. Preparative native-PAGE was run according to the discontinuous buffer system of Laemmli (1970). The lyophilizate was diluted with 200 µL 10 mM Tris–HCl, pH 7.6 and mixed with an equal volume of sample buffer (0.0625 M Tris–HCl, pH 6.8, 25% glycerol, 0.012% bromophenol blue) and loaded on stacking gel. Electrophoresis was run in Mini Prep Cell (Bio-Rad, Hoefer, San Francisco CA, USA) at 500 V, 5 mA (4 °C). The elution flow rate was 0.25 mL min^{–1}. Fractions of 250 µL were collected. Those showing parvalbumin-like protein were pooled, dialyzed and concentrated by lyophilization for further studies. Purification efficiency was monitored by Coomassie Brilliant Blue stain of PAGE (Laemmli, 1970).

Seminal plasma (16.5 mL) was dialyzed for 24 h against 20 mM Tris–HCl buffer, pH 7.6 at 4 °C. The dialysate was subjected to ion-exchange chromatography which was carried out as described above. Parvalbumin-like protein was eluted at the NaCl concentration range of 18.5–20.3%. The fractions containing parvalbumin-like protein were pooled separately, dialyzed 24 h against 10 mM Tris–HCl, pH 7.6 (with two changes) and lyophilized. The lyophilizates from two ion-exchange chromatographies were dissolved in 50 mM buffer Tris–HCl, 0.15 M NaCl (pH 7.6) and applied on HiLoad 16/60 Sephadex 75 column (GE Healthcare) equilibrated with the mentioned buffer. Purification efficiency was monitored by the Coomassie Brilliant Blue stain of PAGE. After gel filtration, fractions containing parvalbumin-like protein were lyophilized. Final purification of parvalbumin-like protein was achieved by preparative electrophoresis as described above.

2.3. Determination of internal sequence of parvalbumin-like protein

Because the parvalbumin-like protein of carp spermatozoa and seminal plasma had a blocked N-terminus, cyanogen bromide (CNBr) cleavage was performed to produce fragments for internal sequencing. Parvalbumin-like proteins (100 µg) were lyophilized and redissolved in 0.05% HCl to a final concentration of 2 µg µL^{–1}. CNBr was added to the sample at 40:1 (w/w) ratio of parvalbumin-like protein to CNBr. After 24 h of incubation at room temperature in the dark, samples were

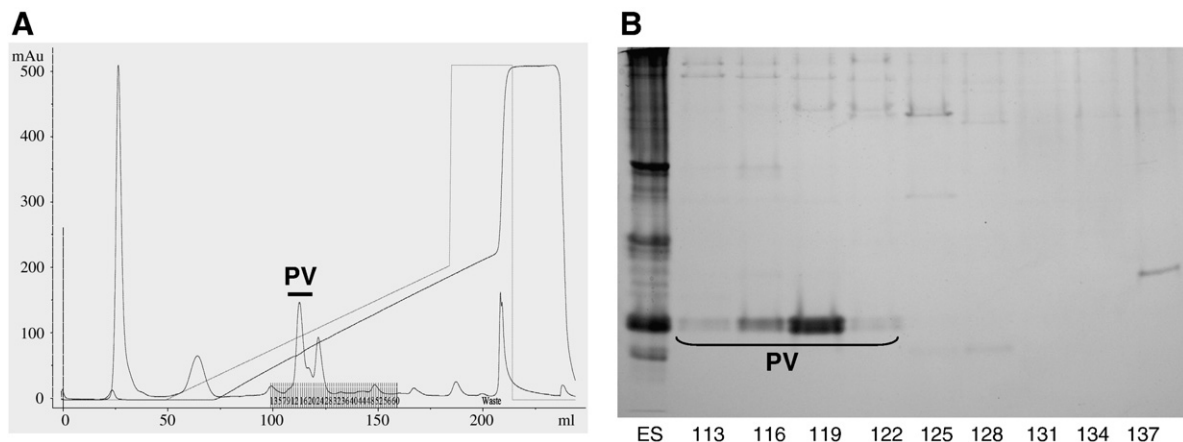


Fig. 1. Ion-exchange chromatography of the proteins of carp spermatozoa extract on a X/K 16/10 Q-Sepharose column. A — elution profile of carp spermatozoa extract. B) Electropherogram (PAGE) of fractions of extract from carp spermatozoa obtained after ion-exchange chromatography. Proteins were stained with Coomassie Brilliant Blue. ES — extract from carp spermatozoa, 113–122 — fractions which contained parvalbumin-like protein (PV).

diluted with water, frozen and lyophilized. The resultant peptide mixtures were diluted with Tris–HCl, pH 8.0, separated in Tris–Tricine SDS-PAGE (Schagger and Von Jagow, 1987) and transferred onto 0.22 µm PVDF membrane by wet electroblotting in 0.1 M CAPS buffer (pH 11.0) containing 10% methanol at 30 V for 16 h at 4 °C for N-terminal amino acid sequencing. The amino acid sequences were determined by automated Edman degradation performed on a gas-phase sequencer at the BioCentre (Crakow, Poland). The BLAST (Basic Local Alignment Search Tool <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to perform the sequences alignment.

2.4. Determination of molecular mass and isoelectric point

The molecular mass of carp spermatozoa and seminal plasma parvalbumin-like protein was estimated by mass spectrometry which was performed on a Bruker Esquire 3000 Plus Electrospray Ionization (ESI) in a trap mass spectrometer at the Regional Laboratory of Physicochemical Analyses and Structural Researches (Cracow, Poland). The isoelectric point was determined in ready IEF gels (Bio-Rad, Hoefer, San Francisco CA, USA) with a pH range of 3–10 according to the manufacturer's protocol.

2.5. Production of anti-carp seminal plasma parvalbumin-like protein polyclonal antibodies

Polyclonal antibodies against the purified parvalbumin-like protein of carp seminal plasma were raised in rabbits. The rabbits were intradermally immunized with parvalbumin-like protein (100 µg) three times as described by Wojtczak et al. (2007b). The polyclonal anti-carp parvalbumin IgG were purified from rabbit serum using a 1 mL HiTrap Protein A HP (GE Healthcare) affinity column (Wojtczak et al., 2007b).

2.6. Immunologic cross-reactivity

Aliquots (50 µg protein) of carp seminal plasma, carp sperm extract, blood plasma and 2 µg of purified carp seminal plasma parvalbumin-like protein were applied onto 12.5% SDS-PAGE gel. Western blot was performed as described by Wojtczak et al., 2007b. Briefly, proteins were electrotransferred onto nitrocellulose membrane followed by blocking in 5% nonfat dry milk in TBS-T (20 mM Tris–HCl, pH 8.0 containing 0.145 M NaCl and 0.1% Tween 20) for 90 min. After washing with TBS-T the membrane was incubated with affinity purified polyclonal anti-parvalbumin-like protein (1:5000 dilution) overnight at 4 °C. After washing with TBS-T, membrane was incubated with alkaline phosphatase-goat anti-rabbit IgG antibody (1:30,000 dilution) for 1 h. Antibodies bound to the membrane were visualized after immersion in alkaline phosphatase buffer containing BCIP and NBT in dark until color development.

Aliquots (75 µg protein) of carp seminal plasma and carp muscle extract were loaded into the 12.5% native-PAGE gel. After electrophoresis, the protein samples were electrotransferred onto nitrocellulose membrane. Western blot was performed as described above.

2.7. Immunohistochemistry

Common carp testicular tissues were dissected from adult carp that had been hormonally stimulated with Ovopel (Interfish, Budapest, Hungary). Testicular tissue was fixed in Bouin's fluid (saturated picric acid, formaldehyde, glacial acetic acid at 15:5:1 proportion) for 24 h, dehydrated in an increasing gradient of ethanol and embedded in paraffin. The 5 µm paraffin sections were deparaffinized in xylene, rehydrated gradually through a series of ethanol dilutions and washed in water. To optimize immunohistochemical staining, slices were immersed for 2–5 min in 10 mM citrate buffer (pH 6.0) and heated in the microwave oven (700 W). The whole procedure has been described

in detail elsewhere (Kotula-Balak et al., 2008). In short, nonspecific staining was blocked twice, first with 3% H₂O₂ in methanol for 15 min, to inhibit endogenous peroxidase activity, and second with 5% normal goat serum for 30 min at room temperature to block nonspecific binding sites. Thereafter, sections were incubated overnight at 4 °C in a humidified chamber in the presence of carp seminal plasma antibody against parvalbumin-like protein (dilution, 1:2000). Next, a biotinylated secondary antibody, goat anti-rabbit IgG (dilution, 1:400; Vector, Burlingame, CA USA) was applied for 1 h. Finally, avidin-biotinylated horseradish peroxidase complex (ABC/HRP; dilution, 1:100; Dako/AS, Glostrup, Denmark) was used for a further 30 min. After each step in these procedures, sections were carefully rinsed with Tris-buffered saline (TBS; 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.6). Bound antibody was visualized with TBS containing 0.05% 3,3'-diaminobenzidine and 0.07% imidazole for 3–4 min. The slides were processed immunohistochemically at the same time with the same treatment so that the staining intensities among testicular cells could be compared; at least 20 serial sections were evaluated. The cells were considered immunopositive if brown reaction product was present and appeared as a signal in testicular cells; the cells without any specific immunostaining were considered immunonegative (Dietrich et al., 2010). Control sections included the omission of primary antibody and substitution by irrelevant IgG. The sections were examined with a Leica DMR microscope (Leica, Microsystems GmbH Wetzlar, Wetzlar, Germany) using Nomarski interference contrast.

2.8. Replicates and statistical analysis

Isoelectric points of carp semen parvalbumins were estimated with the use of the Kodak 1D program version 3.5.4 (Eastman Kodak Company, New Haven, USA) in triplicates. Molecular masses were measured in duplicate. The immunohistochemical experiments were repeated three times.

3. Results

3.1. Isolation of parvalbumin-like protein from carp spermatozoa and seminal plasma

Parvalbumin-like protein from carp spermatozoa extract was purified to homogeneity by the application of two purification steps. Ion-exchange chromatography led to partial separation of parvalbumin-like protein from proteins of a lower migration rate (Fig. 1). The final purification was achieved by preparative electrophoresis.

The applied three-step isolation procedure allowed efficient purification of parvalbumin-like protein from carp seminal plasma. Parvalbumin-like protein was purified to homogeneity by successive ion-exchange chromatography, Sephadex 200 gel filtration and preparative electrophoresis. During ion-exchange chromatography, the parvalbumin-like protein was eluted before main carp seminal plasma proteins (Fig. 2). The gel filtration allowed the separation of parvalbumin-like protein from the proteins with high molecular mass (Fig. 3). Although fractions after gel filtration produced a single band under PAGE condition, after Tris–Tricine SDS-PAGE we observed additional protein bands with low molecular weight. The final purification was achieved by preparative electrophoresis which allowed the separation of parvalbumin-like protein from proteins with a low molecular mass.

3.2. Determination of internal sequence of parvalbumin-like protein

N-terminal sequencing of parvalbumin-like proteins from carp spermatozoa and seminal plasma was unsuccessful due to the blockage of N-terminal amino acid. CNBr cleavage of parvalbumin-like proteins yielded one 8 kDa peptide during Tris–tricine electrophoresis and also the remains of uncleaved parvalbumin-like proteins (Fig. 4). The

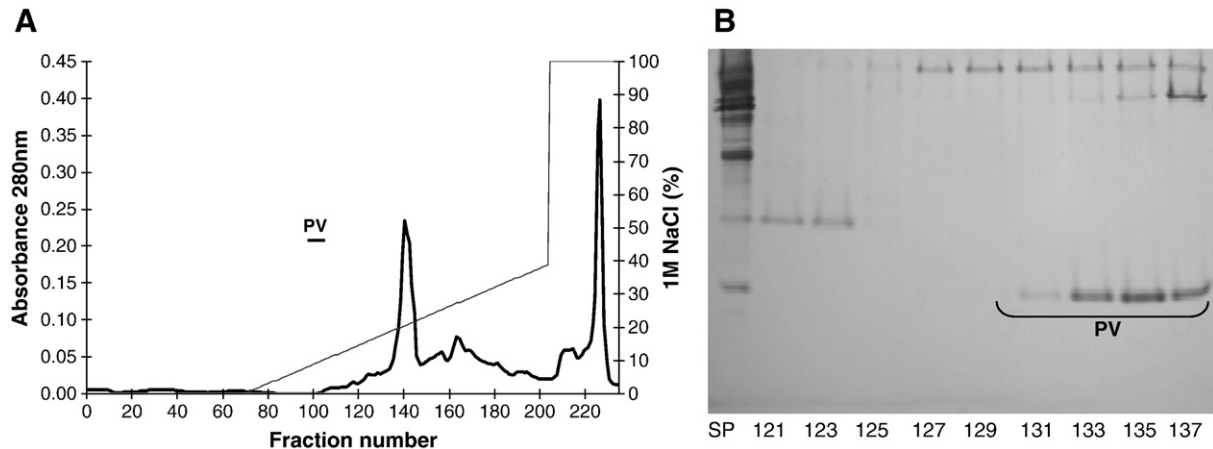


Fig. 2. Ion-exchange chromatography of the carp seminal plasma proteins on a X/K 16/10 Q-Sepharose column. A – elution profile of carp seminal plasma. B) Electropherogram (PAGE) of fractions of carp seminal plasma obtained after ion-exchange chromatography. Proteins were stained with Coomassie Brilliant Blue. SP – carp seminal plasma, 130–137 – fractions which contained parvalbumin-like protein (PV).

sequence analysis of peptide obtained after CNBr cleavage of parvalbumin-like protein from spermatozoa allowed the identification of 41 amino acids VGLKAKSAEDVKKAFHILDADSSGFIEEEELKFVKHFATD and the same 28 amino acids for seminal plasma parvalbumin-like protein VGLKAKSAEDVKKAFHILDADSSGFIE.

3.3. Determination of molecular mass and isoelectric point

The molecular mass estimated by mass spectrometry was 12,774 Da and 12,098 Da for parvalbumin-like protein of spermatozoa and seminal plasma, respectively. The isoelectric point was determined to be 4.06 and 4.1 for parvalbumin-like protein of spermatozoa and seminal plasma, respectively (Fig. 5).

3.4. Immunologic cross-reactivity

The anti-parvalbumin-like protein antibodies cross-reacted with purified parvalbumin from carp seminal plasma and parvalbumin-like protein from seminal plasma and spermatozoa in Western blot analysis under the condition of SDS-PAGE (Fig. 6A). No reaction in carp blood plasma was observed.

Under the condition of native-PAGE, muscle parvalbumin was resolved into 3–4 bands (Fig. 6B). The carp seminal plasma parvalbumin-like protein was characterized by faster migration rate

in comparison to parvalbumin from carp muscle. At the same time, band of moderate migration rate were recognized in carp seminal plasma.

3.5. Immunohistochemical localization of parvalbumin-like protein in carp testis

The immunohistochemical study showed that in carp testis a positive, strong to very strong staining for parvalbumin-like protein was localized mainly to sperms within the lumina and spermatids (Fig. 7). Numerous cysts containing spermatogonia, primary and secondary spermatocytes as well as Sertoli cells and the interstitial Leydig cells were immunonegative for the staining.

In the control sections, in which the primary antibodies were omitted, no immunopositive staining for parvalbumin-like protein was observed.

4. Discussion

The present study is the first to report the presence of parvalbumin-like protein, a calcium-binding protein, in high amounts in fish semen. The efficient isolation procedures of parvalbumin-like proteins from carp spermatozoa and seminal plasma have been established. Molecular masses and isoelectric point have been

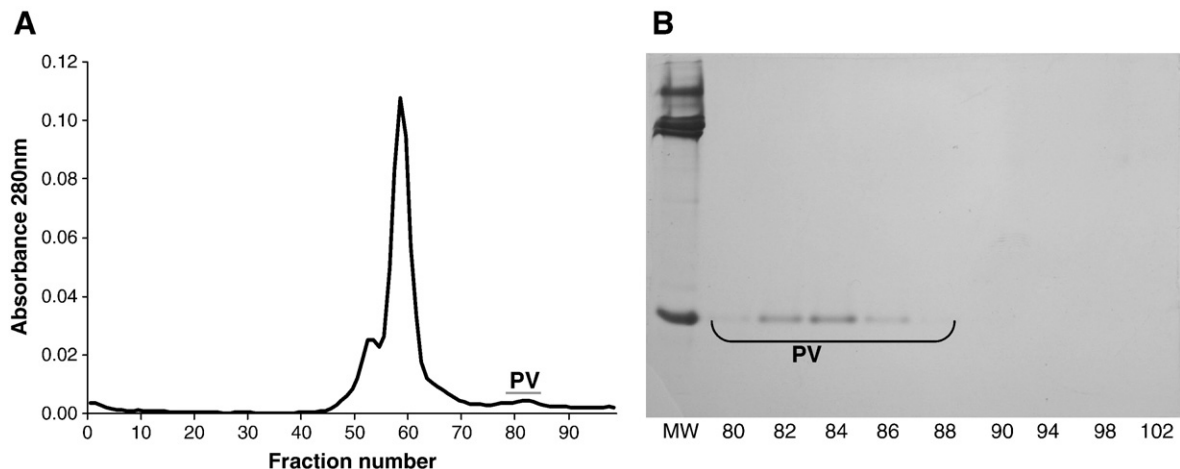


Fig. 3. Elution profile of carp seminal plasma proteins obtained after gel filtration on HiLoad 16/60 (A). Electropherogram (PAGE) of carp seminal plasma protein fractions obtained after gel filtration (B). Proteins were stained with Coomassie Brilliant Blue. MW – parvalbumin-containing fractions after ion-exchange chromatography. 80–88 – fractions which contained parvalbumin-like protein (PV).

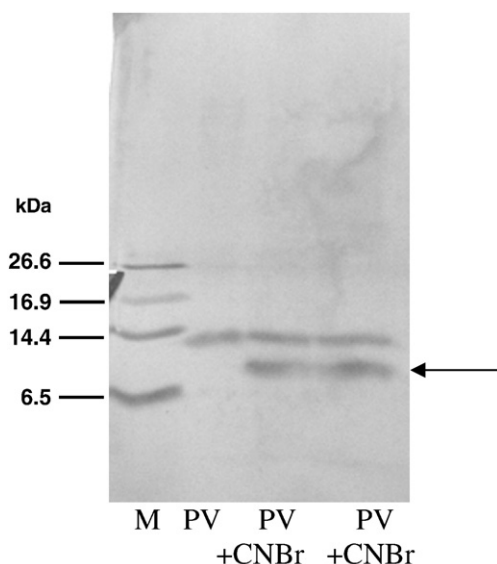


Fig. 4. Separation of peptide fragments of parvalbumin-like protein (PV) after CNBr cleavage. Arrow indicates sequenced fragment. M-molecular weight marker. Tris-Tricine SDS-PAGE.

determined and the internal amino acid sequences have been obtained. Immunohistochemical analysis indicated the localization of parvalbumin-like protein in spermatids and spermatozoa.

We have developed a fast and efficient two-step procedure for isolating parvalbumin-like protein from carp spermatozoa, consisting of ion-exchange chromatography and preparative electrophoresis. Additionally, an efficient three-step procedure consisting of ion-exchange chromatography, gel filtration and preparative electrophoresis was established for the purification of parvalbumin-like protein from carp seminal plasma. Moreover, the later method allows the isolation of transferrin and serine proteinase inhibitors from carp seminal plasma simultaneously (Wojtczak et al., 2005). The described procedures are the first isolation method of parvalbumin-like protein from fish semen. So far, only procedures for the isolation of parvalbumin from carp muscle were established (Bugajska-Schretter et al., 2000; Koppelman et al., 2010; Liu et al., 2010). Generally, heating at 85 °C and the removal of heat-labile proteins by centrifugation is used for the preparation and separation of parvalbumin. This approach should be tested in the future to evaluate its usefulness for the improvement of our purification protocol and to determine if other isoforms of parvalbumin-like protein do exist in carp semen.

Parvalbumins represent low molecular mass (~10–12 kDa), acidic (pI 3.90–5.50), water-soluble calcium-binding proteins with a remarkable resistance to heat and proteolytic enzymes (Bugajska-Schretter et al., 1998). The molecular masses and isoelectric points of carp semen parvalbumin-like proteins fell within the range described for vertebrate parvalbumin (Berchtold et al., 1984). The molecular masses of parvalbumin-like protein from carp spermatozoa extract (12,777 Da) and seminal plasma (12,098 Da) were higher than the molecular mass of parvalbumin from carp muscle calculated from the amino acid sequence (11,569 Da) and mass spectroscopic analysis (11,373 Da; Bugajska-Schretter et al., 2000; Liu et al., 2010). The isoelectric point of parvalbumin-like protein from the extract of carp spermatozoa and seminal plasma estimated by isoelectric focusing (pI 4.06 and 4.10) was lower than pI calculated for a major isoform of parvalbumin from carp and other fish muscle (4.70–4.80; Bugajska-Schretter et al., 2000; Schoenman et al., 2010). Nevertheless, the pI value of carp semen parvalbumin-like protein was similar to pI 4.20 estimated for a minor isoform of parvalbumin from carp muscle (Bugajska-Schretter et al., 2000). Summing up, the carp semen

parvalbumin-like proteins are characterized by a higher molecular mass and lower isoelectric point.

The two main types of parvalbumins, α and β , are distinguished by numerous characteristics including their different isoelectric points (α parvalbumin display pI values above 5.0, while β parvalbumin typically have pI below 4.5–5.0) and characteristic features of their amino acid sequences (Föhr et al., 1993; Jia and Pérez, 2009). It is assumed that there are at least eleven residues characteristically different between α and β forms of human parvalbumin (Föhr et al., 1993). Based on low pI value (4.1), our results suggest that the parvalbumin-like protein of carp semen can be classified as β parvalbumin. This principle is true for known carp muscle parvalbumins isoforms, and there are identified seven β parvalbumins with pI below 5.0 and one α parvalbumin with pI above 5.0 (Brownridge et al., 2009). However, the sequence analysis strongly suggests that the parvalbumin-like protein of carp semen belongs to α -lineage of parvalbumin because the following amino acids assumed to be characteristic for α -parvalbumin (Föhr et al., 1993) can be identified: Met at pos. 33, His at 49, and Lys at 69. However, there is one discrepancy for pos. 53, where Ala is present instead of Lys (Fig. 7). Moreover, in the sequence of carp semen parvalbumin-like protein we did not identify Phe at pos. 67 and Gln at 69 which are characteristic for β -type parvalbumin. Recently, Friedberg (2005) suggested that the classification of parvalbumin isoforms from zebrafish as simply α and β is no longer sustainable. Our results are in agreement with this suggestion and indicated that, unambiguous classification of carp semen parvalbumin-like proteins as α or β -type is not possible using the criteria described for mammalian parvalbumin (Föhr et al., 1993).

N-terminal amino acid sequencing revealed that both parvalbumin-like proteins from carp sperm extract and seminal plasma were blocked. The amino acid sequences of carp parvalbumin-like protein isoforms confirmed the presence of methionine as the first amino acid, which indicated that the isoforms were naturally N-acetylated (Brownridge et al., 2009). The identified internal amino acid sequences of semen parvalbumin-like proteins were identical. Despite a lack of direct evidence this strongly suggests that both parvalbumin-like proteins from seminal plasma and spermatozoa are very similar or identical.

The amino acid alignment of carp semen parvalbumin-like protein with other parvalbumins from different organisms (Fig. 8) revealed

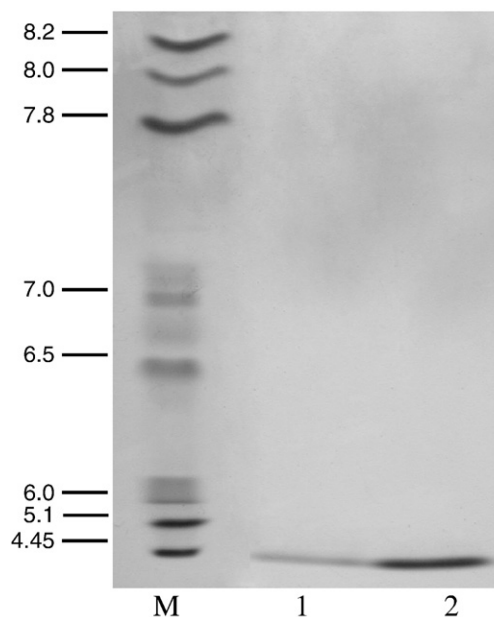


Fig. 5. Isoelectrofocusing of purified parvalbumin-like protein in IEF acrylamide gel. 1 – carp seminal plasma parvalbumin-like protein, 2 – parvalbumin-like protein from carp sperm extract, M – IEF standards, IEF range 4.45–9.6.

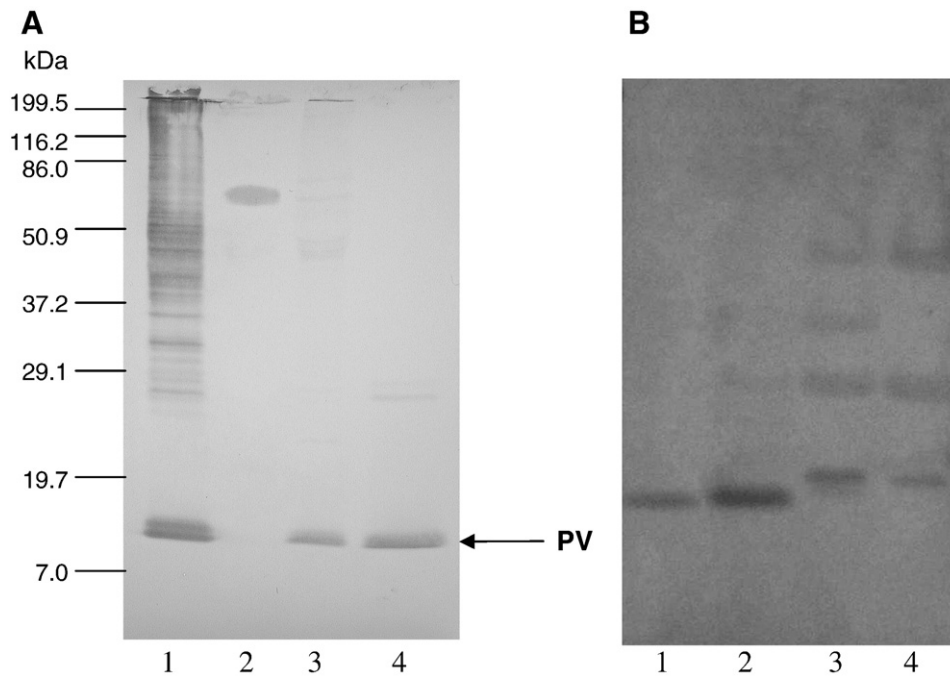


Fig. 6. (A) Cross-reactivity between anti-parvalbumin-like protein antibodies and carp sperm extract (1), carp blood plasma (2), carp seminal plasma (3) and purified parvalbumin-like protein (PV) from carp seminal plasma (4) under the condition of SDS-PAGE. (B) Cross-reactivity between anti-parvalbumin-like protein antibodies and carp seminal plasma (1,2) and carp muscle (3,4) under the condition of native-PAGE.

very high homology within 33–74 residues to α parvalbumin from *Salmo salar* (85% identity) and parvalbumin 6 from *Danio rerio* (83% identity). In contrast, carp semen parvalbumin-like protein exhibits a lower degree of homology with parvalbumin isoforms from carp muscle (63% identity). Based on lower sequence homology between parvalbumin-like protein from carp semen and α -type parvalbumin from muscle, different molecular mass and pI values, we can suggest that parvalbumin-like protein from carp spermatozoa and seminal plasma could be distinct from carp muscle parvalbumins.

We demonstrated that polyclonal anti-parvalbumin antibodies raised against parvalbumin-like protein from carp seminal plasma recognized parvalbumin-like protein from carp sperm extract. We did not detect parvalbumin-like protein in carp blood plasma which is in agreement with the absence of parvalbumin in blood, shown by Berchtold et al. (1984). The presence of parvalbumin-like protein in seminal plasma but not in blood plasma suggests that this protein is specific for the male reproductive tract. Moreover, to our knowledge it is the first report to demonstrate the presence of considerable amounts of parvalbumin in body fluid: until now parvalbumins were recognized as intracellular proteins present mainly in muscle and nervous system.

In this study we demonstrated the presence of parvalbumin-like protein in carp spermatids and spermatozoa. Information concerning the presence of parvalbumin in the male reproductive tract is limited and restricted to mammals. Berchtold et al. (1984) identified parvalbumin in rat Leydig cells. The latter demonstrated that besides Leydig cells, parvalbumin is also present in the acrosome region of maturing spermatids. Parvalbumin was not detected in spermatogonia, spermatocytes and spermatozoa (Kägi et al., 1987). In carp testis, similarly to the rat, we localized parvalbumin-like protein in spermatids. In contrast to the rat, we detected parvalbumin-like protein in spermatozoa but not in Leydig cells. Our results suggest that parvalbumin of higher and lower vertebrates may differ in testis localization.

The presence of parvalbumin-like protein in carp semen coincides with the important role of calcium ions in the regulation of the sperm motility. The activation mechanism of carp sperm movement is not

fully understood. However, it is clear that extracellular Ca^{2+} is necessary for the initiation of sperm motility in carp (Alavi and Cosson, 2006). An influx of extracellular Ca^{2+} into carp spermatozoa induces the release of Ca^{2+} from intracellular stores. The increase in intracellular Ca^{2+} level is prerequisite for the initiation of carp sperm motility (Krasznai et al., 2000, 2003). Since parvalbumin acts as a Ca^{2+} buffer (Gillis et al., 1982), it is reasonable to hypothesize that parvalbumin-like protein present in carp seminal plasma plays a role in the control of Ca^{2+} gradient, which is important for the initiation of sperm motility. Moreover, the presence of a high amount of parvalbumin-like protein in carp spermatozoa strongly suggests that parvalbumin may be involved in the mobilization of intracellular calcium. The appearance of parvalbumin-like protein in high amounts in mature spermatozoa coincides with their acquiring the ability to move. In conclusion, the presence of much parvalbumin-like protein in both carp seminal plasma and spermatozoa suggests that parvalbumin is an important part of the Ca^{2+} -mediated mechanism of sperm activation in carp.

Krasznai et al. (2003) obtained indirect evidence suggesting that calmodulin, bound close to the sperm plasma membrane of carp participates in the initiation of sperm motility. The calmodulin is the EF-hand calcium sensor, which changes its conformation upon binding Ca^{2+} and mediates the Ca^{2+} signal by interaction with other proteins. In contrast, parvalbumin does not change conformation upon Ca^{2+} binding and works as Ca^{2+} -buffer. Summing up, results of this study and data of Krasznai et al. (2003) suggest that at least two calcium-binding proteins which differ in Ca^{2+} -binding mechanism are involved in the regulation of carp sperm motility.

An influx of Ca^{2+} is implicated in programmed cell death, and therefore it is possible that parvalbumin-like protein present in carp seminal plasma may also play a possible role in the protection of carp spermatozoa against apoptosis. Recently Cui et al. (2008) showed that human parvalbumin could protect Leydig cells as well as some germ cells against apoptosis and hence promote cell survival. Spermatozoa of most teleost fish, including carp, are stored for prolong time in the male reproductive tract, therefore, the protection of sperm fertilizing ability, viability and motility is necessary during this period. An optimal

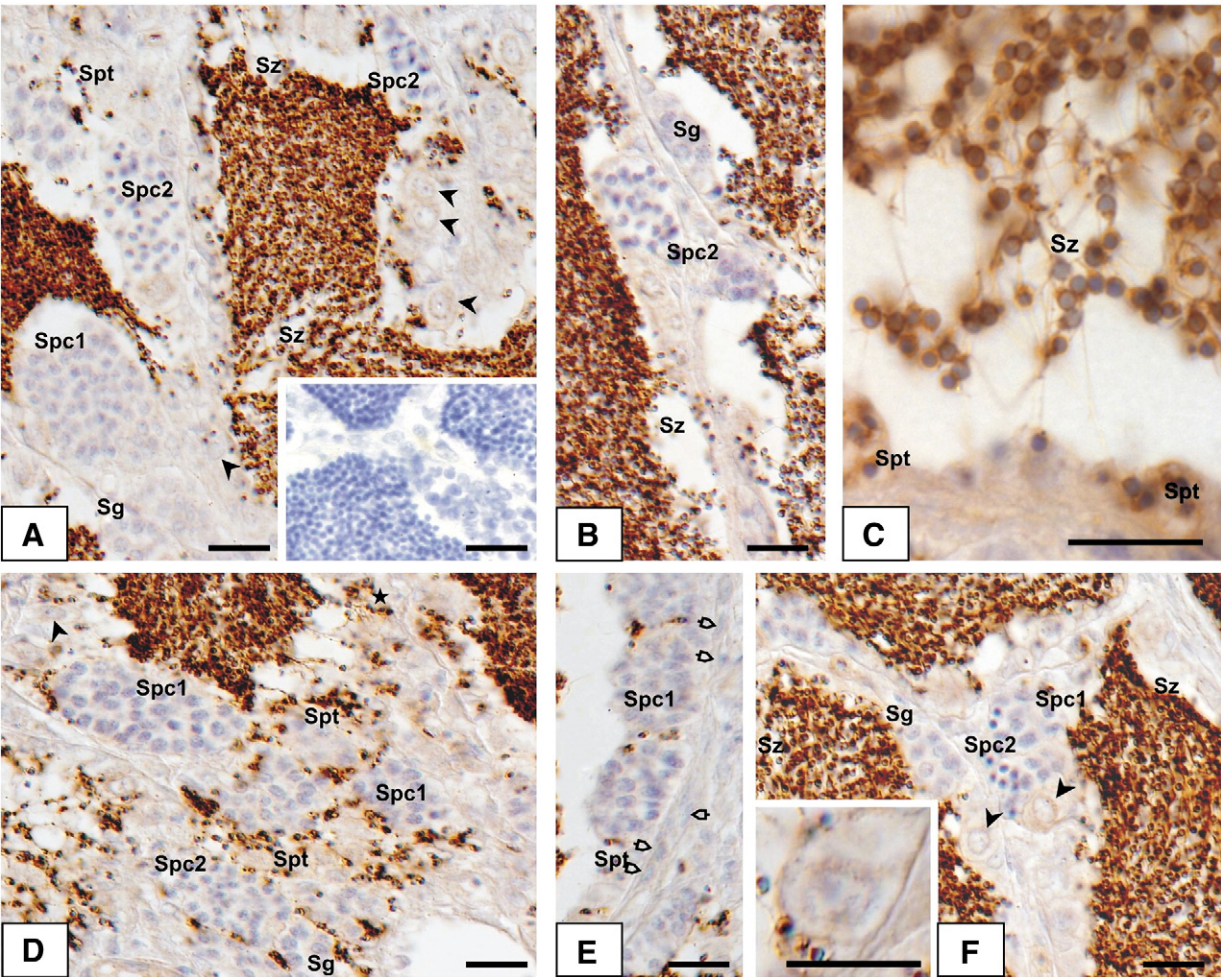


Fig. 7. A–F. Immunohistochemical localization of parvalbumin-like protein in carp testis. Immunostaining was performed using carp seminal plasma antibody against parvalbumin-like protein followed by anti-rabbit IgG, and ABC/HRP visualized by DAB. Nomarski interference contrast. Bars = 10 μm. (A–B, D, F). Photomicrographs depict strong staining for parvalbumin-like protein localized to sperms (Sz) and spermatids (Spt). At higher magnification a cytoplasmic pattern of the staining is visible (C). Note immunonegative cysts consisted of spermatogonia (Sg), primary spermatocytes (Spc1), and secondary spermatocytes (Spc2). Immunonegative Sertoli cells are clearly visible (arrowheads; at higher magnification, insert in F). (E) Immunonegative Leydig cells are visible (open arrowheads). No immunopositive staining for parvalbumin-like protein is observed when the primary antibody is omitted (insert in A).

environment for spermatozoa is secured by seminal plasma. The role of seminal fluid proteins in sperm protection is not well understood. Recent data suggest that transferrin and proteinase inhibitors can protect spermatozoa against microbial attack, the former may also protect spermatozoa against oxidative damage (Magnadóttir, 2006; Wojtczak et al., 2005, 2007b). The presence of parvalbumin-like protein in carp seminal plasma suggests that it may act as a Ca^{2+} buffer that protects spermatozoa from cytotoxic Ca^{2+} overload and consequently death by apoptosis.

In conclusion, we have demonstrated that contrary to mammalian parvalbumins, carp semen parvalbumin-like proteins possess characteristic features for both α and β parvalbumin. Parvalbumin-like protein seems to be specific for the male reproductive tract because it was not detected in carp blood plasma. The presence of parvalbumin-like protein in high amounts both in carp seminal plasma and spermatozoa suggests that parvalbumin is an important part of the Ca^{2+} -mediated mechanism of sperm activation in carp. Another possible function of parvalbumin is to protect spermatozoa against apoptosis. Further

<i>Cyprinus carpio</i> (spermatozoa)	1	MVGLKAKSAEDVKKAFHILDADSSGFIEEEELKFVFKHFATD ₄₁	Identity
<i>Cyprinus carpio</i> (seminal plasma)	1	MVGLKAKSAEDVKKAFHILDADSSGFIEE ₂₈	(100%)
<i>Salmo salar</i>	33	MVGLKAKSAEDVKKAFHVLDAASGFIEEEELKFVLKGFASD ₇₄	(85%)
<i>Danio rerio</i>	33	MVGLKAKASDDVKKAFHLLDADNSGFIEEEELKFVLKAFATD ₇₄	(82%)
<i>Cyprinus carpio</i>	34	KVGLTSKSADDVKKAFHIIIDQKSGFIEEDELKFLQNFKAD ₇₄	(63%)
<i>Homo sapiens</i>	33	MVGLKKKSADDVKKVFHMLDKDKSGFIEEDELGFILKGFSPD ₇₄	(69%)

Fig. 8. Homology alignment of the sequence of parvalbumin-like proteins from common carp spermatozoa and seminal plasma with the sequence of parvalbumin from *Salmo salar* (NP_001167235), *Danio rerio* (NP_991136), *Cyprinus carpio* (CAC83659) and *Homo sapiens* (CAQ07296) and comparison of amino acid sequences in relation to characteristic amino acids for human parvalbumin α (bold and underlined).

studies are necessary to corroborate the specific role of parvalbumin in these processes.

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