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CHARACTERISTICS OF ACID PHOSPHATASE FROM RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) SPERMATOZOA

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ABSTRACT. Acid phosphatase (AcP) is a commonly observed enzyme in animal semen. In this study, AcP in rainbow trout (*Oncorhynchus mykiss*) spermatozoa was partly purified and characterized. Extraction in 0.85% NaCl with 0.1% Triton X-100 enabled obtaining 95% of total AcP activity observed in sperm supernatant. Kinetic characteristics were described for the enzyme from sperm extract and for the partly purified enzyme following gel filtration. The optimum pH was 5.8 for unpurified and 5.6 for partly purified enzyme. The affinity of the substrates measured in the sperm extract for p-nitrophenylphosphate dissodium salt and β-glycerophosphate was $K_m = 1.5 \times 10^{-3}$M and $K_m = 1.9 \times 10^{-3}$M, respectively. The K_m for partly purified enzyme was similar at 1.67×10^{-3}M measured with p-nitrophenylphosphate dissodium salt. L-tartaric acid and ammonium molybdate were the inhibitors of AcP for unpurified and partly purified enzyme. SDS-PAGE electrophoresis revealed that AcP from rainbow trout had a molecular weight of about 41 kDa.

Key words: ACID PHOSPHATASE, SPERMATOZOA, RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

INTRODUCTION

The localization of acid phosphatase in spermatozoa differs in many mammal species. In boar sperm, AcP activity was commonly observed in the post-acrosomal region, but in mouse sperm it was noted in the acrosomal membrane (Mann and Lutwak-Mann 1981). Boar seminal plasma AcP activity is one of the highest in mammals, and it is usually more than 15000 U l^{-1} (Glogowski et al. 1997c). In bird semen, the main dephosphorylational enzyme is acid phosphatase, and its activity reaches 20000 U l^{-1} (Jankowski et al. 2002). The sources of AcP in mammalian semen are testicular secretions and accessory glands.

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Panara (1997) showed that activity of acid phosphatase is similar in mammalian and lower vertebrates, and demonstrated the existence of low and high molecular weight AcP in northern pike, *Esox lucius* L., tissue. Panara (1997) also found low (20 kDa) and high molecular weight (59 kDa) AcP in pike testes. The reproduction systems and fertilization processes in fish may suggest that there are many differences in the structure and biological function of AcP in comparison with mammalian enzymes. AcP in mammalian semen is linked with the sequential steps of spermatozoa production in the testes. Little is known about the enzymology of teleost fish gametes, so the biochemical and kinetic characteristics of acid phosphatase may be useful in further explaining the biological function of this enzyme in fish spermatozoa.

The acid phosphatase activity in fish semen was determined based on the method described for blood plasma (Bessey et al. 1946). Acid phosphatase is a polymorphic enzyme which might possess different properties in distinct biological material. Thus, the aim of this study was to characterize its basic kinetic parameters which might permit adapting the method to determine rainbow trout, *Oncorhynchus mykiss* (Walb.), spermatozoa acid phosphatase.

MATERIAL AND METHODS

Milt was collected from three male rainbow trout maintained at the Department of Salmonid Research, Inland Fisheries Institute in Olsztyn, Poland. The milt was stored on ice (4°C) during transport to the laboratory. The seminal plasma was separated from the spermatozoa by centrifugation (8000 × g, 10 min), and sperm pellets were stored at –79°C. The spermatozoa were thawed at room temperature (in 2 ml of 0.85% NaCl and after mixing 2 ml of 0.85% NaCl with 0.2% Triton X-100 was added) for one hour and then centrifuged.

PURIFICATION PROCEDURE

Although many electrophoretic and chromatografic methods were tested (preparative electrophoresis, gel filtration, ion exchange chromatography, hydrophobic chromatography, affinity chromatography, chromatofocusing), only gel filtration permitted obtaining enough enzymatic activity (data not shown).

The material obtained though extraction was loaded onto a Sephacryl S-100 column for gel filtration using an FPLC (Amersham Pharmacia Biotech) system. The column was equili-

brated with 0.05 M Tris HCl, pH 7.6 containing 0.15 M NaCl and 0.01% Triton X-100. The eluate was collected in 1 ml fractions at a flow rate 1 ml min^{-1}. This step permitted determining the molecular weight (M_w) of AcP using the following standard proteins: aldolase (172 kDa); bovine serum albumin (64.6 kDa); ovalbumin (43 kDa); ribonuclease A (13.7 kDa) Pharmacia-Amersham. The molecular weight was determined from the calibration curve established by plotting the logarithm of subunit M_r against the mobility of the standard proteins. The protein content in the effluent was monitored at 280 nm in each fraction.

GEL ELECTROPHORESIS

The purity of AcP was determined by electrophoresis (PAGE) on 7.5% polyacrylamide gels (Laemmli 1970). The gels used to visualize the protein bands were 0.025% Coomassie Brilliant Blue R-250 (Sigma), 20% methanol, and 7% acetic acid stained. The gels were also stained to visualize the AcP activity using the method described by Čurn (1994).

The SDS-PAGE on 12.5% polyacrylamide was used to estimate the molecular weight. Samples were denatured by suspending them in a solution containing 4% SDS and 0.2 M DTT (dithiothreitol) for 3 min at 100°C. Molecular weight was calculated from relative mobility values for standard proteins: bovine serum albumin (67 kDa); ovalbumin (43 kDa); glyceraldehyde-3-phosphate dehydrogenase; rabbit muscle (36 kDa); carbonic anhydrase (30 kDa); trypsinogen, bovine pancreas (24 kDa); trypsin inhibitor (20.1 kDa); α-lactalbumin (14.4 kDa). The molecular masses of protein bands were estimated with the Kodak 1D program (Eastman Kodak Company, New Haven, U.S.A)

ANALYTICAL PROCEDURES

Activity of acid phosphatase was determined using the method described by Glogowski et al. (1996) with 5 mM p-nitrophenylphosphate dissodium salt (pNPP) in 20 mM citrate buffer. After 30 min of incubation at 37°C, the reaction was stopped with 0.1 M NaOH and absorbance at 410 nm was measured. AcP activity is expressed in units (μM NPP hydrolyzed per min).

During the determination of AcP activity at temperatures above 40°C, pNPP was hydrolyzed without enzyme. This artefact was omitted by using a properly constructed controlled sample.

The activity of AcP was also determined with β-glycerophosphate, DL-α–glycerophosphate, adenosine-3'-monophosphate, adenosine-5'-monophosphate, adenosine-5'-triphosphate, guanosine-3'-monophosphate, guanosine-5'-monophosphate,

cytidine-5'- monophosphate, uridine-3'-monophosphate, uridine-5'-monophosphate, D-glucoso-1-phosphate, D-glucoso-6-phosphate, D-fructoso-1,6-biphosphate, D-fructoso-6-phosphate, and phospho(enol)pyruvate monosodium salt. The density of all these substrates was 40 mM. After 120 min of incubation at 37°C, the reaction was stopped with 4 ml 0.57 M sulfuric acid with 1% ammonium molybdate and ferrous sulfate with a density of 40 mg ml^{-1}, and absorbance at 700 nm was measured (Zaugg 1982). Protein was measured with the method by Lowry et al. (1951).

The effect of pH on AcP activity was tested using 0.2 M citric acid-NaOH buffers at pH 4.0-7.0 with pNPP and β-glycerophosphate as substrates. The influence of incubation temperature was determined at 20-50°C (with pNPP). The preliminary experiment revealed that AcP activity declines rapidly, so the best parameters of the stored sperm extract were determined with citric buffers (pH 5.0 and 5.8) and buffered NaCl, pH 7.6. The extracts were stored for different periods at +4 and –26°C. Affinity to the substrate was tested at 0.078, 0.156, 0.312, 0.625, 1.25, 2.5, 5.0, and 10.0 mM of pNPP (pH 5.0). K_m was calculated from the linear plots of the reciprocal of the reaction velocity versus that of the substrate concentration (Woolf's rule). The influence on AcP activity of potential inhibitors or activators (L-tartaric acid, ammonium molybdate, $ZnCl_2$ and $MgCl_2$, Sigma) was determined.

STATISTICAL ANALYSIS

The data were analyzed with the Wilcoxon paired rank test, the Mann-Whitney U-test, and the unpaired t-test. For statistical analysis, all kinetics measurements were done using AcP from Russian sturgeon, *Acipenser gueldenstaedtii* Brandt, milt obtained from three fish. The significance of differences was inferred at $P \leq 0.05$. The GraphPad PRISM package was used for these calculations.

RESULTS

Extraction with Triton X-100 enabled obtaining 95% of AcP activity as compared to the non-extraction method (sperm suspension before centrifugation). No AcP activity was detected in the sperm pellet after centrifugation. SDS-PAGE electrophoresis showed the existence of many proteins in the sperm extract (Fig. 1). The dominant proteins had molecular weights ranging from about 30-70 kDa.

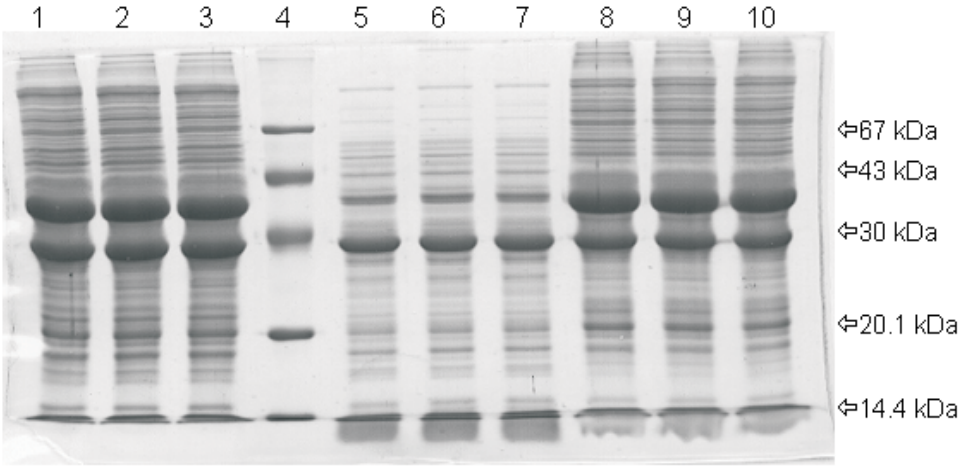

Fig. 1. Electrophoretic pattern (SDS-PAGE) of sperm extract (1, 2, 3), supernatant after extraction (5, 6, 7), sperm pellet after extraction (8, 9, 10), 4 – standard proteins.

KINETICS OF ACID PHOSPHATASE FROM SPERMATOZOA EXTRACT

AcP from sperm extract possessed different kinetic parameters according to the kind of substrates. The sampled substrates used were p-nitrophenylphosphate dissodium salt (pNPP) and β-glycerophosphate. The hydrolyzation speed was 10 times lower with β-glycerophosphate as the substrate than it was with pNPP.

The optimum pH measured with pNPP was 5.8 and was statistically different from the AcP activity at pH 5.6 and 6.0 (Fig. 2A). With β-glycerophosphate, the optimum pH was 6.0 (Fig. 2B). The temperature optimum was at 40°C (Fig. 3). It is important to note that incubation at 50°C permitted obtaining 70% AcP activity. The storage of sperm extract at –26°C and +4°C showed that the pH of the applied buffers is an important factor, and the best results were obtained at pH 5.8 and 7.6 (Fig. 4).

This observation may be useful for preparing the AcP isolation procedure. With pNPP as a substrate, $K_m = 1.5 \times 10^{-3}$M. The affinity of the enzyme to β-glycerophosphate was similar at $K_m = 1.9 \times 10^{-3}$M. Acid phosphatase from rainbow trout spermatozoa hydrolyzed purine mononucleotides, but ATP was hydrolyzed at a low degree (Table 1).

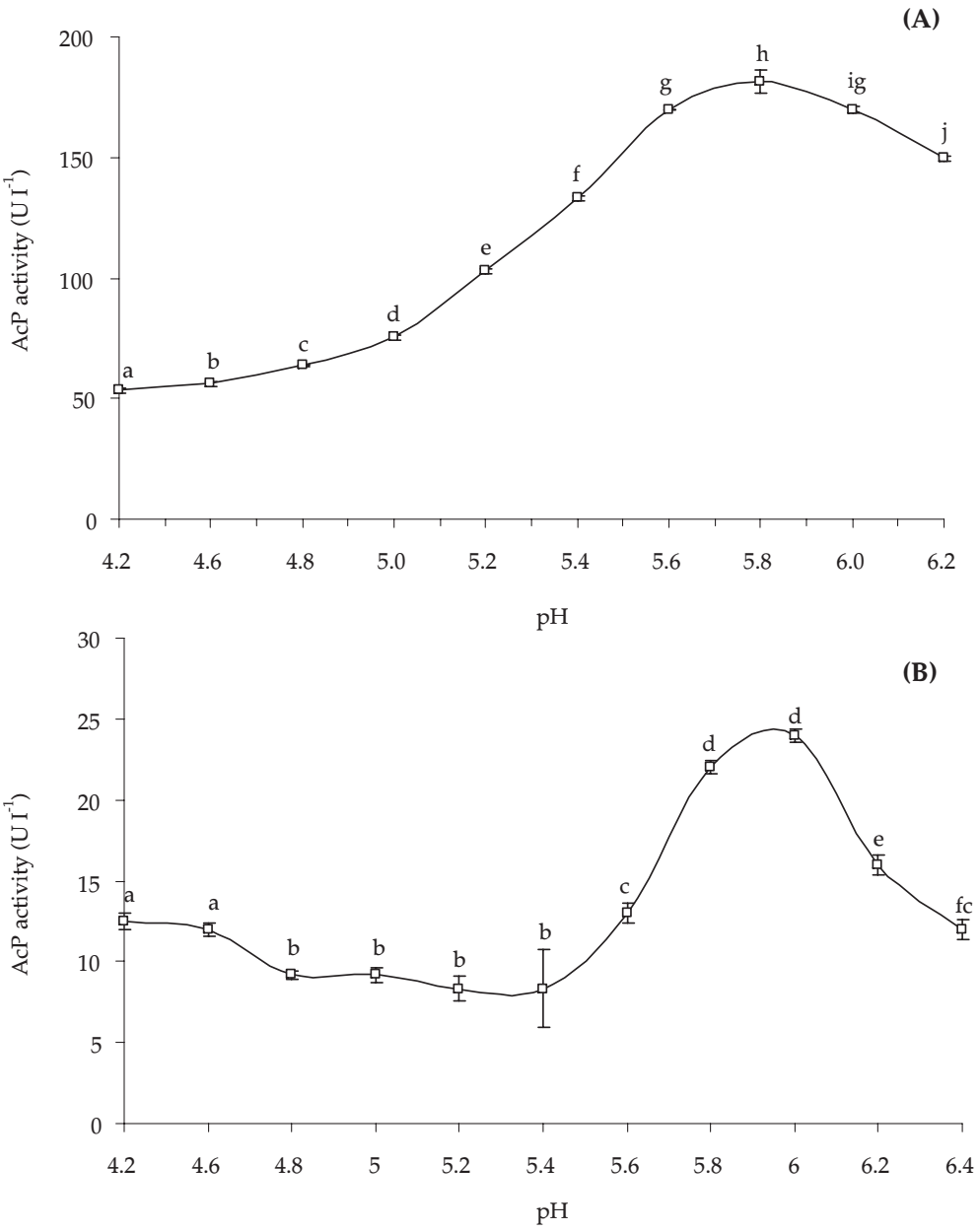

Fig. 2. Influence of pH on AcP activity from extract sperm (measured with pNPP – A, with β-glycerophosphate – B). Values with different letters are significantly different, $P \leq 0.05$; n=3.

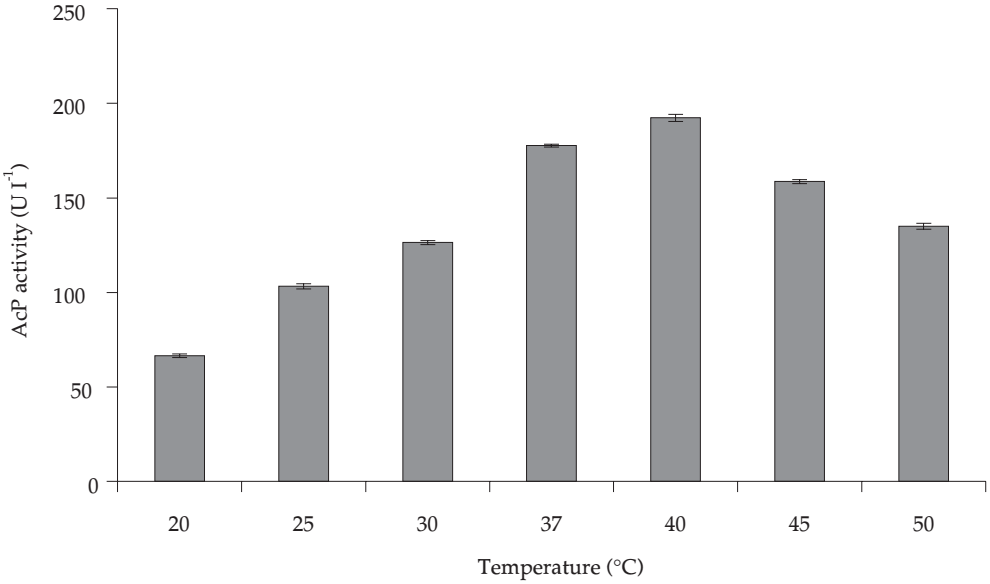

Fig. 3. Influence of incubation temperature on sperm extract AcP activity.

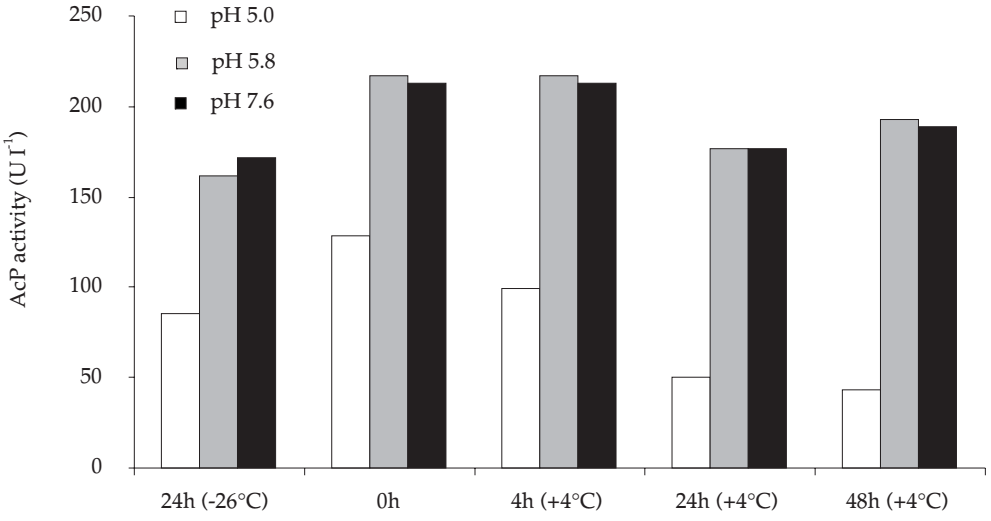

Fig. 4. Influence of pH and temperature on AcP activity from sperm extract.

TABLE 1

AcP activity with different phosphate esters (at 10 mM each). Activity measured with β-glycerphosphate was determined as 100%. Other activities are part of the above-mentioned activity

Substrates	Sperm extract			
	- Mg^{2+}		+10 mM Mg^{2+}	
	U l^{-1}	%	U l^{-1}	%
β-glycerophosphate	33.42	100	30.75	100
Adenosine-5'-monophosphate	21.80	65	21.40	69
Adenosine-3'-monophosphate	17.00	51	19.30	63
Adenosine-5'-triphosphate	2.60	8	4.60	15
Guanosine-5'-monophosphate	11.80	35	17.93	58
Guanosine-3'-monophosphate	29.41	88	22.83	74
Citidine-5'-monophosphate	11.24	34	16.36	53
Uridine-5'-monophosphate	7.67	23	19.50	63
Uridine-3'-monophosphate	5.40	16	17.60	57
α-D-glucoso-1-phosphate	1.21	3	4.33	14
α-D-glucoso-6-phosphate	4.33	13	8.12	26
α-glycerophosphate	8.68	25	9.46	31
Fructoso-1,6-bisphosphate	19.60	59	17.82	58
Fructoso-6-phosphate	8.57	25	7.67	25
Phospho(enol)pyruvate	7.00	23	11.20	36

The most effective inhibitors of AcP were ammonium molybdate and L-tartaric acid (Fig. 5A, B). With pNPP, 0.5 M L-tartaric acid decreased the AcP activity by 38% and ammonium molybdate by 82%. Magnesium and zinc ions did not influence the AcP activity measured with either substrate (data not shown).

CHARACTERISTICS OF PARTLY PURIFIED ACID PHOSPHATASE

Gel filtration permitted obtaining one peak of twice purified enzyme with a 60% yield (Fig. 6). SDS electrophoresis showed that this fraction characterized the presence of about 10 polypeptides of a molecular weight below 50 kDa with the dominant 20 kDa form (Fig. 7). AcP activity was observed in the 41 kDa fraction. PAGE electrophoresis revealed the existence of one form of AcP (Fig. 8).

Partially purified AcP had a pH optimum at 5.6 with pNPP and β-glycerophosphate as the substrate (Fig. 9 A, B). With pNPP, the K_m of partly purified AcP had a similar value to the unpurified enzyme at 1.67×10^{-3}M. Both L-tartaric acid and ammonium

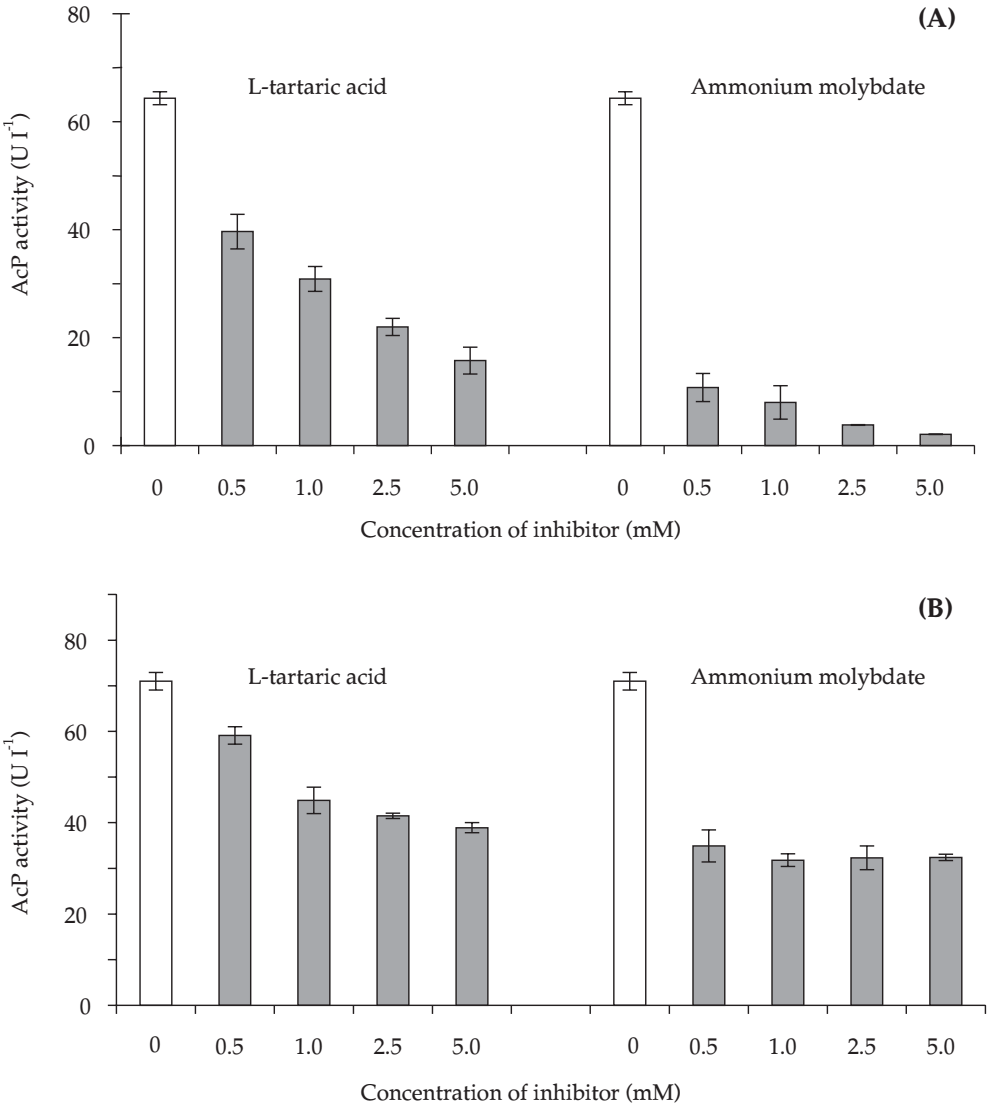

Fig. 5. Influence of inhibitors on sperm extract AcP activity with pNPP (A) or β-glycerophosphate (B) as the substrate.

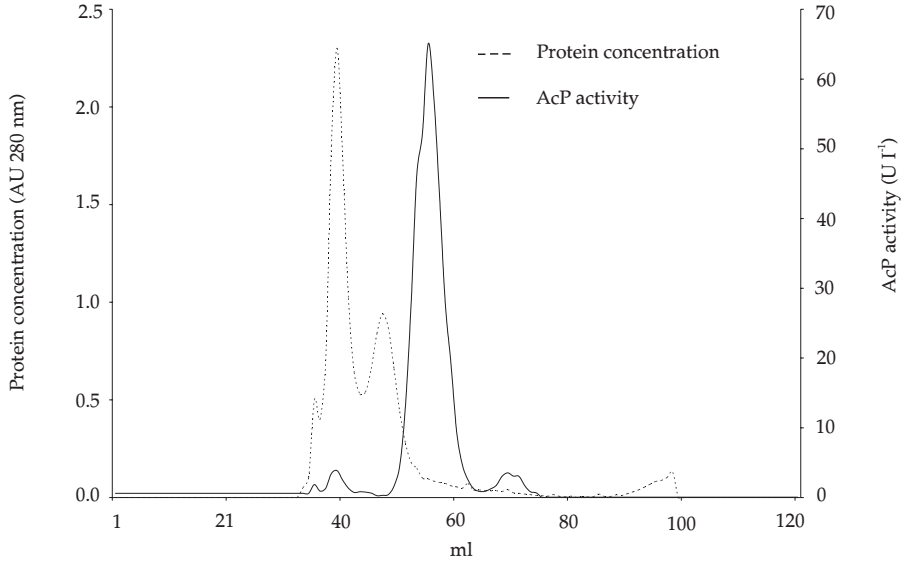

Fig. 6. Acid phosphatase profile after gel filtration.

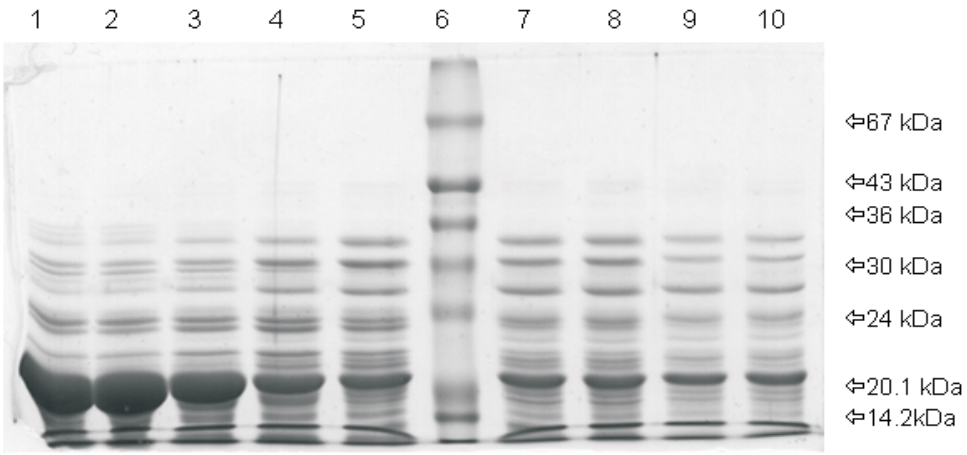

Fig. 7. Electophoretic pattern (SDS-PAGE) of sperm extract after gel filtration (1, 2, 3, 4, 5, 7, 8, 9, 10 – consecutive fractions of AcP activity ; 6 – standard proteins).

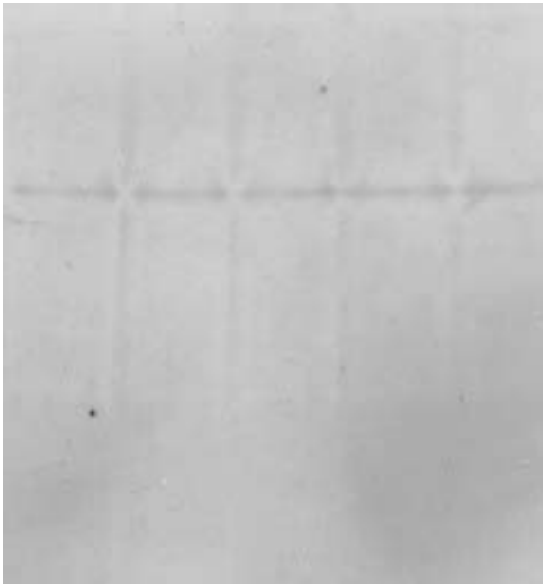

Fig. 8. Electrophoretic pattern (PAGE) of the partly purified fraction, staining on AcP activity.

molybdate reduced the AcP enzymatic activity (Fig. 10), but a 0.5 mM concentration of these inhibitors caused 11% and 47% reductions, respectively.

The affinity of the enzyme to the substrate (pNPP) was reduced by 5 mM L-tartaric acid and ammonium molybdate with values of $K_m = 2.05 \times 10^{-3}$M for the former and $K_m = 1.96 \times 10^{-3}$M for the latter.

DISCUSSION

By incubating the sperm pellet with the nonionic detergent Triton X-100 and centrifuging the extract, 95% of the AcP activity which was determined in the extract before centrifugation was obtained. No AcP activity was observed in the pellet after centrifugation. Triton X-100 is commonly used in the preparation of AcP from different sources; for example, it is used to extract acid phosphatase from mussels, *Mytilus edulis* (Pipe and da Silveira 1989) and from rat sperm (Salzberger et al. 1992).

AcP leakage was observed during the cryopreservation of rainbow trout spermatozoa (Glogowski et al. 2000). This was also described in northern pike (Glogowski et al. 1997a), bream, *Abramis brama* (L.) (Glogowski et al. 1997b), and sterlet, *Acipenser*

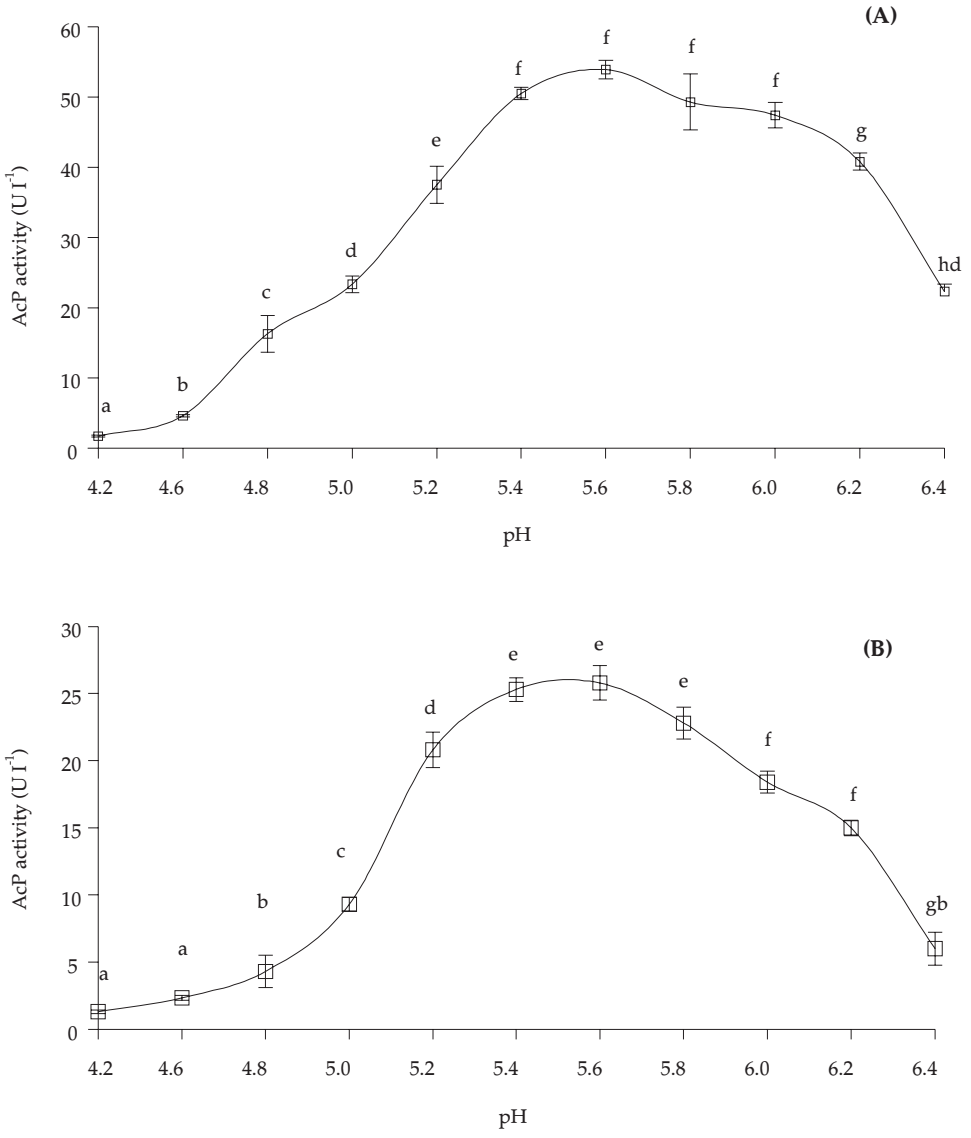

Fig. 9. Influence of pH on the activity of purified AcP with pNPP (A) and β-glycerophosphate (B) as a substrate. Values with different letters are significantly different, $P \leq 0.05$; n=3.

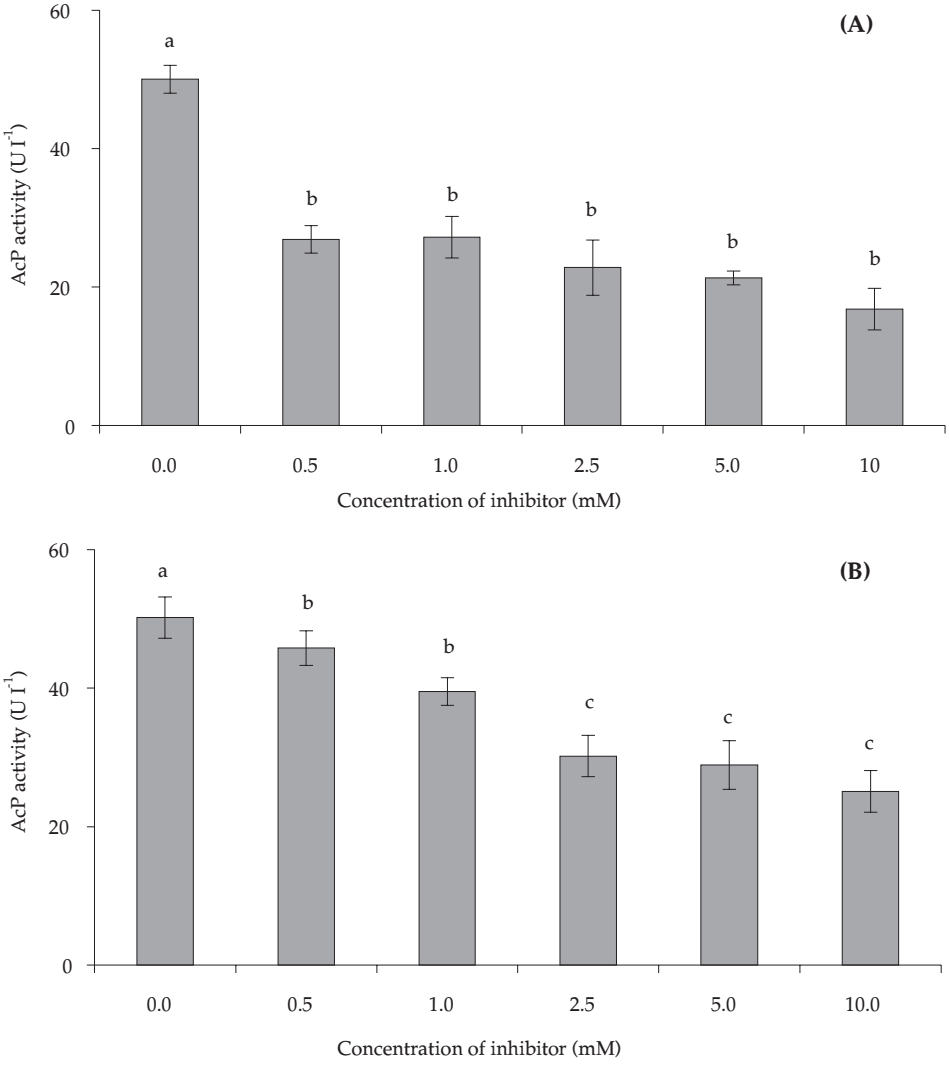

Fig. 10. Influence of ammonium molybdate (A) and L-tartaric acid (B) on the activity of purified AcP. Values with different letters are significantly different, $P \leq 0.05$; n=3.

ruthenus L., spermatozoa (Piros et al. 2002). Therefore, acid phosphatase might potentially be a good marker for the efficiency fish sperm cryopreservation.

Kinetic characteristics should be described after enzyme isolation and purification. Since no information about rainbow trout AcP was found in the scientific literature, the authors decided to isolate this enzyme. Unfortunately, most electrophoretic and chromatographic techniques caused large losses of enzymatic activity. Only gel filtration was an effective method for purifying acid phosphatase from rainbow trout spermatozoa. The loss of AcP activity might have been caused by enzyme lability and consequent denaturation, as has often been observed in the separation of the protein binding from the cell membrane (Larson and Ryden 1989).

The most effective AcP inhibitor was ammonium molybdate. The AcP activity from sperm extract was reduced by 82% (with pNPP) and 50% (with β-glycerophosphate) at a 0.5 M density of this inhibitor. The inhibition activity of L-tartaric acid and ammonium molybdate are caused by the spaced similarity of phosphate, tartare, and molybdate residue. Many experiments have confirmed the special character of L-tartaric acid affinity to high molecular weight acid phosphatase (Laron and Epstain-Halberstadt 1960, Lovelace et al. 1997), like the AcP described in this paper.

The results obtained in this work showed that in order to properly determine rainbow trout sperm AcP activity, other kinetic characteristics, especially the optimum pH of this enzyme, must be taken into account. The pH of the standard method of determining AcP activity is 5.0, but at this pH value the AcP in the current study had approximately two-fold lower activity than at pH 5.6. Therefore, measuring rainbow trout AcP should be performed at this pH value.

Experiments conducted on bird and mammalian semen indicated that the phosphorylation-dephosphorylation reaction is fundamental in the fertilization process (Urner and Sakkas 2003). In rat sperm, this enzyme may be an marker of acrosomal reaction, as was suggested by Salzberger et al. (1992). The enzymes controlling the above-mentioned reactions, kinases, phosphatases, and their natural substrates have been the subjects of intense scientific research in recent years. The functions of regulation systems and the course of the molecular process of fish fertilization differ in many aspects from mammalian fertilization and are not well understood. Knowledge of fish reproduction biology may provide valuable breeding information, and this purpose might be served by the results obtained on AcP in the current study.

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STRESZCZENIE

CHARAKTERYSTYKA FOSFATAZY KWAŚNEJ Z PLEMNIKÓW PSTRĄGA TĘCZOWEGO (*ONCORHYNCHUS MYKISS*)

Kwaśna fosfataza (AcP) jest enzymem powszechnie występującym w nasieniu wielu gatunków zwierząt. W niniejszych badaniach nasienie pobrane od pstrąga tęczowego *Oncorhynchus mykis* odwirowano, a następnie plemniki poddano ekstrakcji w 0,85% NaCl z 0,1% Tritonem X-100. Charakterystykę kinetyczną przeprowadzono dla nieoczyszczonego enzymu, a także dla częściowo oczyszczonego białka uzyskanego po filtracji żelowej. Dla enzymu z ekstraktu optimum pH wynosiło 5,8 z użyciem p-nitrofenylophosforanu jako substratu oraz 6,0 z użyciem β-glicerofosforanu (rys. 2). Dla enzymu po filtracji optimum pH wynosiło 5,6 (rys. 9). Powinowactwo do substratu określono przy użyciu p-nitrofenylophosforanu oraz β-glicerofosforanu, K_m wynosiło odpowiednio: $1{,}5 \times 10^{-3}$M i $1{,}9 \times 10^{-3}$M dla enzymu z ekstraktu plemników. K_m dla częściowo oczyszczonej AcP wynosiło $1{,}67 \times 10^{-3}$M, z użyciem p-nitrofenylophosforanu. Kwas winowy i molibdenian amonu okazały się inhibitorami badanej fosfatazy (rys. 5, 10). Elektroforeza SDS-PAGE pozwoliła na stwierdzenie, że enzym posiada masę cząsteczkową 41 kDa (rys. 7, 8).