Activity of α- and β-mannosidases in semen and reproductive organs of the drake

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SUMMARY

The activity of α- and β-mannosidase (α-MAN and β-MAN) in seminal plasma, spermatozoa and homogenates of reproductive organs has been determined in the drake. The highest specific activities of the examined mannosidases were found in epididymides. The activities of both enzymes decreased significantly during the postbreeding resting season compared to the breeding season. Elution profiles of multiple forms of mannosidases from particular organs were obtained and characterized. It was found that α- and β-mannosidases in the genital tract of the drake are similar to the enzymes described in other species of domestic fowl. The presence of highly active mannosidases in reproductive tissues and a decline of the mannosidase activities during the postbreeding season strongly suggests that the enzymes take part in reproductive processes of birds. Reproductive Biology 2009, 9, 1: 25-37.

Key words: drake, mannosidase, semen, reproductive organs

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INTRODUCTION

Acid α- and β-mannosidases (α-MAN and β-MAN) belong to the group of lysosomal glycosidases with the highest activity in the male genital tract and semen of mammals [7, 24]. These enzymes show optimum pH in the acid range of pH and catalyze the breakdown of terminal oligosaccharide units of glycoproteins and glycolipids [10]. α-Mannosidase [EC 3.2.1.24] catalyzes the cleavage of α-mannose residues, whereas β-mannosidase [EC 3.2.1.25] hydrolyzes β-mannose and N-acetylglucosamine bonds in a core of N-glycan oligosaccharides. The activity of acid glycosidases in the male gonad is associated mainly with reproductive cells and Sertoli cells [23].

It is generally accepted that acid glycosidases play a significant role in spermatozoa maturation and in the fusion of the spermatozoon and ovum during fertilization. The activity of specific neutral α-mannosidase was localized in the plasma membrane of spermatozoa, where it can serve as a receptor binding mannose oligosaccharides of the zona pellucida [3, 29]. The form found in epididymal fluid participates in modifying surface glycoproteins of sperm during maturation [28, 30]. Acid glycosidases were found to be particularly active in the mammalian epididymides compared to the other parts of the genital tract [9, 19, 20, 24]. Their accumulation in the epididymis is controlled by androgens and spermatogenesis-stage specific factors [4, 8]. The activity of acid mannosidases in the semen and reproductive tract of domestic fowl was investigated in cocks\(^1\), turkeys\(^1\) and seasonally breeding ganders [13, 17, 25]. This study is the first to explore the occurrence of α- and β-mannosidases in the semen and genital tract of the drake.

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MATERIALS AND METHODS

Animals and tissue collection

Two-year-old Pekin-type drakes from breeding flocks of the Waterfowl Genetic Resources Station in Dworzyska were used in this study. During the reproductive season 14 h of light per day (14L:10D) was applied. The birds were fed a commercial breeder diet. Semen was collected in June from ten mature drakes by the dorsoventral method [6], and individual ejaculates were combined during the collection. Freshly ejaculated semen was diluted at the ratio of 1:4 (v/v) using 0.9% NaCl and centrifuged (800×g; 10 min; 4°C). The supernatant (seminal plasma) was stored at -20°C. Spermatozoa sediment was washed three times with 0.9% NaCl and reconstituted to obtain the initial semen volume. A suspension of spermatozoa was stored at -20°C. After thawing at 20°C, supernatant I was obtained by centrifugation (800×g; 4°C). The spermatozoa sediment was resuspended in 0.5% Triton X-100, incubated at 37°C for 30 min and centrifuged (14000×g; 4°C) yielding supernatant II. Combined supernatants I and II (spermatozoa extract) were used for measuring of both mannosidases in spermatozoa.

Reproductive tissues - testes, epididymides and deferent ducts - were collected postmortem from birds in two phases of the reproductive cycle, in June (n=3) and in October (n=5). Tissues were transported to the laboratory on ice. Semen was squeezed from the deferent ducts, rinsed with 0.9% NaCl using a syringe and separated into plasma and spermatozoa as described above. Tissues were weighed and stored in -20°C. To determine the activity of enzymes, tissues were homogenized (25 000 rpm; 3×15 s; 0°C) in 0.9% NaCl at the ratio of 1:4 (w/v) using mechanical homogenizer (VirTis, Gardiner, USA). The homogenates were centrifuged at 14000×g, and the supernatants were used for the determining the enzyme activity and the testing the occurrence of multiple forms of mannosidases.
Biochemical assays

Enzymatic assays of α-D-mannosidase and β-D-mannosidase activities were performed according to the method [2] based on absorbance measurements at 400 nm of enzymatically released p-nitrophenol (p-NP) from suitable p-NP-mannosides (Sigma, St. Louis, USA). Briefly, incubation mixtures containing an appropriately diluted enzyme sample, 1 mM proper p-nitrophenol substrate and 0.1 M citrate buffer (pH 4.5) were incubated at 37°C. The reaction was terminated by an addition of 0.5 M bicarbonate-carbonate buffer (pH 10). One unit (U) is defined as the enzyme activity hydrolyzing 1 µmol of substrate per min at 37°C. Specific activities are expressed as units of enzyme activity per milligram protein (U/mg). Protein was assayed using the Lowry method [22] in tissue homogenates and the Bradford method [5] in seminal plasma and spermatozoa extract with serum bovine albumin used as a standard.

Chromatofocusing. A column of 20 cm³ with PBE 94 gel (Pharmacia LKB, Sweden) connected to the BioLogic-LP (Bio-Rad Laboratories, Hercules, USA) system was equilibrated with 25 mM imidazole-HCl buffer (pH 7.4). Samples of tissue homogenates (1-2 ml) were dialyzed for 24 h at 4°C against the 25 mM imidazole-HCl buffer (pH 6.0). Separation was carried out at room temperature with Polybuffer 74-HCl pH 4.0 according to the manufacturer’s instructions (Pharmacia LKB, Sweden).

Anion-exchange chromatography. A column of 7 cm³ with anion exchanger Macro-Prep® High Q Support (High Q) connected to the BioLogic-LP (Bio-Rad, USA) system was equilibrated with 25 mM histidine-HCl buffer (pH 6.0). Samples of tissue homogenates (0.5 ml) were dialyzed for 24 h at 4°C against the same buffer. Unbound proteins were eluted with the column buffer and then a linear gradient of NaCl (0-0.5 M) in 25 mM histidine-HCl buffer (pH 6.0) was applied. Finally the column was eluted with 1 M NaCl in the same buffer.

Statistical analysis

Enzyme activity differences for tissue samples were determined on log transformed data by one-way analysis of variance followed by LSD test.
The Bartlett test was used to test variance homogeneity. Seasonal differences in the enzyme activity were analyzed with the use of Student t-test. All analyses were done using the Statistica package (StatSoft Inc., Tulsa, USA).

**RESULTS**

The presence of β-MAN and α-MAN activities was demonstrated in seminal plasma and spermatozoa (tab. 1). In seminal plasma obtained from ejaculated semen, the activities of both enzymes were similar. The activity of β-MAN recovered from spermatozoa was higher than that of α-MAN. In seminal plasma obtained from the ductus deferens, the activities of both mannosidases were 2-4 times higher than those in seminal plasma of ejaculate. Activities of β-MAN and α-MAN in spermatozoa extract were the same for ejaculate and ductus deferens spermatozoa.

During the reproductive season, drake epididymides had significantly higher β-MAN and α-MAN activities than other reproductive tissues (tab. 2). There was no significant difference between testis and the ductus deferens in β-MAN activity, while α-MAN activity was higher in testes than in the ductus deferens. During the postbreeding resting phase, significant decreases in the activities of β-MAN and α-MAN were observed in all tissues (tab. 2). During both periods, β-MAN and α-MAN epididymal activities were higher than in testes and ductus deferens.

<table>
<thead>
<tr>
<th>Table 1. Specific activity of β- and α- mannosidases in drake semen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Semen origin</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Ejaculate</td>
</tr>
<tr>
<td>Ductus deferentes</td>
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</tbody>
</table>

Results represent average values obtained from three replications with experimental error less than 5%.
Table 2. Seasonal changes in the specific activity of acid mannosidases (mean±SD) in drake reproductive organs

<table>
<thead>
<tr>
<th>Reproductive organ</th>
<th>Breeding season (June)</th>
<th>Postbreeding resting phase (October)</th>
<th>Breeding season (June)</th>
<th>Postbreeding resting phase (October)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis</td>
<td>12.38±3.14&lt;sup&gt;a,A&lt;/sup&gt;</td>
<td>3.48±0.78&lt;sup&gt;a,B&lt;/sup&gt;</td>
<td>20.13±4.24&lt;sup&gt;a,A&lt;/sup&gt;</td>
<td>5.72±2.42&lt;sup&gt;a,B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Epididymis</td>
<td>368.20±40.03&lt;sup&gt;b,A&lt;/sup&gt;</td>
<td>43.57±12.30&lt;sup&gt;b,B&lt;/sup&gt;</td>
<td>199.23±32.30&lt;sup&gt;b,A&lt;/sup&gt;</td>
<td>28.37±2.70&lt;sup&gt;b,B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ductus deferens</td>
<td>8.16±4.12&lt;sup&gt;a,A&lt;/sup&gt;</td>
<td>1.81±1.55&lt;sup&gt;a,B&lt;/sup&gt;</td>
<td>11.41±2.71&lt;sup&gt;c,A&lt;/sup&gt;</td>
<td>3.35±1.94&lt;sup&gt;a,B&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>A,B,C</sup> means in the same row with different superscripts differ at p<0.05 (June vs. October)

<sup>a,b,c</sup> means in the same column with different superscripts differ at p<0.01

Figure 1. Elution profiles of β-MAN from drake semen and reproductive organs obtained by chromatofocusing. Samples of seminal plasma, spermatozoa extract, testes and epididymides homogenates were separated on PBE 94 column using Polybuffer 7-4. Fractions were collected and analyzed for β-MAN activity. The numbers above some peaks indicate the pI values of the forms.

Elution profiles of the multiple forms of β-MAN (fig. 1) and α-MAN (fig. 2) were obtained through chromatofocusing. For β-MAN, forms with pI of 6.30 and 5.90 were found for testes and spermatozoa, forms with pI
6.30 and 6.10 for epididymides, and forms with pI 6.10 and 5.60 for seminal plasma (fig. 1). Several multiple forms were observed for α-MAN (fig. 2), and the forms with high pI values (7.3-7.0) were specific for testes whereas forms found in seminal plasma were characterized by the low pI values (6.15-5.4).

The activity of both mannosidases was separated into two forms: bound (with isoelectric points below pH 6.0) and unbound (pI>6.0) to the anion exchanger (fig. 3). Elution profiles of testicular α-MAN obtained by anion-exchange chromatography during the reproductive season and postbreeding resting phase were very similar (fig. 3a). The major bound form was eluted at a conductivity of 6-8 mS. For β-MAN the unbound form was dominant. An additional bound form of β-MAN, specific for active testis and eluted at conductivity of 8 mS, was observed (fig. 3b).

Figure 2. Elution profiles of α-MAN from drake semen and reproductive organs obtained by chromatofocusing. Samples of seminal plasma, spermatozoa extract, testes and epididymides homogenates were separated on PBE 94 column using Polybuffer 7-4. Fractions were collected and analyzed for α-MAN activity. The numbers above some peaks indicate the pI values of the forms.
Figure 3. Seasonal changes of testicular α-MAN (a) and β-MAN (b) forms obtained by anion-exchange chromatography. Homogenates of active testes (breeding phase) and testes in regression (postbreeding phase) were separated on anion exchanger Macro-Prep® High Q Support connected to the BioLogic-LP system. Unbound proteins were eluted with the histidine-HCl buffer pH 6.0, and a linear gradient of NaCl was applied in fraction no. 40.
DISCUSSION

This is the first study describing the occurrence of acid mannosidases in the semen and the genital tract of the drake. Although α- and β-MAN were found in seminal plasma, their activities were twice as high as in the spermatozoa. In seminal plasma, the activity of α-MAN was similar to that of β-MAN, whereas in the spermatozoa, the activity of β-MAN was slightly higher than that of α-MAN. A similar relationship between α- and β-MAN activities was found for gander semen [15] and cock spermatozoa\(^1\). The seminal plasma activities of α-MAN and β-MAN were 3-4 times higher in the deferent duct than those in seminal plasma obtained from ejaculate. This relationship along with the stable level of activity in the spermatozoa ejaculated and collected from the deferent duct suggests that high volume of transparent fluid has been introduced into the semen obtained by the massage method [21].

Avian epididymides, like those in mammals, exhibit a much higher activity of lysosomal hydrolases as compared to the testes [18]. In mature drakes, a particularly high increase in both mannosidases activities was found in epididymides (about 30-fold and 10-fold for β- and α-MAN, respectively) in comparison to the testes. A similar tendency was observed for mature ganders [13], pheasants [14] and Japanese quails [12]. Moreover, Bamberg et al. [1] found that in roosters, activity of other acid glycosidase - β-galactosidase – was higher in epididymides and lower in the ductus deferens in comparison to that of testis. It appears that the distribution of acid mannosidases in the genital system of the drake, i.e. the highest activity of both mannosidases in epididymis, lower in testis and the lowest in deferent duct, closely resembles those observed in other birds and mammals.

Drakes are domestic fowl with a pronounced seasonality of reproduction. During the reproductive season, the testes weight of drakes constitutes as much as 5% of body weight whereas gander testes weight amounts to only 0.24% of body weight [17]. The reproductive season ends with a drastic

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(over 50-fold) reduction in a drake’s gonadal weight which accounts for approximately 0.1% of body weight. In ganders during the same phase testes weight is equal 0.07% of the body weight [17].

During the postbreeding phase (October), the activities of both mannosidases were significantly decreased. The decreased activity of both mannosidases was also shown in gander testes during the resting phase [17]. The activity of lysosomal hydrolases found in the reproductive tract of mammals is strictly related to sexual activity [18]. Conchie and Findlay [8] showed that the activity of acid glycosidases in mammalian epididymides during involution decreases to the level found in sexually immature birds. The high activities of tissue mannosidases in sexually active drakes followed by a post-seasonal decrease strongly suggest that these enzymes take part in spermatogenesis.

There was a similarity between elution profiles of mannosidase forms found in the semen and different parts of the genital tract of the drake. Isoelectric points of multiple forms of β-MAN from testes and spermatozoa were identical (pI 6.3-5.9), while the main form found in seminal plasma with pI of 6.1 was characteristic for the epididymides. Similar multiple forms of β-MAN were shown in gander testes and spermatozoa: 6.5, and 6.6 and 6.4, respectively [15, 16]. Forms with much lower pI (6.1 and 5.9), probably of epididymal origin, were found in seminal plasma [15]. Similar mannosidase characteristics obtained for two fowl species may suggest a different origin of β-MAN in bird semen i.e. epididymal origin for plasma enzyme and testicular origin for spermatozoa enzyme.

α-MAN from the reproductive tract of birds is characterized by a microheterogeneity as reflected by the presence of multiple forms with pI of 7.3-5.4. Five forms with pI of 7.4-4.7 are present in the seminal plasma of turkey [11]. The α-MAN present in the semen and reproductive tissue of ganders showed a similar phenomenon [15, 16]. Mannosidase forms with pI of 7.4-5.8 dominated in the drake testes and epididymides, whereas forms found in seminal plasma had much lower isoelectric points (the main form had pI of 5.6-5.4). These acidic mannosidase forms were also detectable in the elution profiles of the enzyme derived from testes, epididymides and spermatozoa. The presence of this enzyme in seminal plasma may result
not only from the secretory activity of testes and epididymides, but also from its release from spermatozoa.

The occurrence of multiple forms of acid glycosidases, resulting from different degrees of aggregation/dissociation of their subunits in environments of different pH values, may have some physiological importance, for it was shown that this phenomenon can affect the activity of glycosidases towards various substrates [26, 27]. Some acid glycosidases, such as acidic α-MAN from boar epididymal fluid, show higher activity towards natural substrates in epididymal fluid than the neutral form of α-MAN found in this fluid [20]. It is therefore assumed that the role of acid glycosidases is different in the acidic environment of lysosomes and in the extracellular environment with a higher pH value.

In summary, our results demonstrated the similarity of acid β- and α-MAN occurring in the reproductive system of drake and other domestic birds, especially gander. The presence of highly active mannosidases in the reproductive tissues and a decline in the enzyme activities during the postbreeding phase confirm a notion that β-MAN and α-MAN play a role in spermatogenesis and spermatozoa maturation. The analysis of the elution profiles of the multiple forms of acid mannosidases after chromatofocusing indicates that the epididymides contribute to the production of seminal plasma. However, the specific functions of acid mannosidases from birds’ reproductive system require further studies.

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