

Acid glycosidases in the isthmus of the hen oviduct and egg shell membranes

Maria Droba¹, Bogusław Droba, Dorota Błędnik
Department of General and Physiological Chemistry,
University of Rzeszów, Poland

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SUMMARY

Specific activities of seven acid glycosidases: β -hexosaminidase, α - and β -galactosidase, α - and β -mannosidase, α -glucosidase and α -fucosidase were determined in various parts of the domestic hen oviduct (infundibulum, isthmus, shell gland and vagina). The activity of most enzymes was the highest in the isthmus. Multiple forms of all acid glycosidases from the isthmus were separated by strong anion exchange chromatography at pH 6.0. The isoelectric points of the isthmus forms of β -hexosaminidase, β -galactosidase and α - and β -mannosidase were determined by chromatofocusing. For the first time the high β -galactosidase activity was found in hen egg shell membranes. *Reproductive Biology 2006 6 Suppl. 2:55–63.*

Key words: multiple forms of acid glycosidases, oviduct, egg shell membranes, hen

INTRODUCTION

Acid glycosidases are enzymes which hydrolyse glycosidic bonds present in carbohydrates, glycoproteins and glycolipids, with optimum activity at

¹Corresponding author: Department of General and Physiological Chemistry, University of Rzeszów, 35-601 Rzeszów, Ćwiklińskiej 2, Poland; e-mail: mdroba@univ.rzeszow.pl

acidic pH. The enzymes are found intracellularly in lysosomes and acrosomes of the spermatozoon and are also present in extracellular fluids [5, 9]. Acid glycosidases are believed to play an important role during fertilization [13].

In birds, oviduct collects the ovum within 15 min after ovulation. It provides the appropriate environment for fertilization and secretes components of the albumen, shell membranes, shell, and cuticle i.e. layers which concentrically surround the ovum [4]. Only few reports concerning acid glycosidases in hen oviduct have been published so far. Nine glycosidases were found in whole hen oviduct of which α - and β -mannosidase (α - and β -MAN; [10]) and β -hexosaminidase (β -HEX; [8, 12]) were purified and characterized high activity of β -HEX has been found in the egg shell membranes of the hen [14]. It is thought that β -HEX, present in egg shell membranes and albumen, may play a role in slowing down bacterial penetration into the egg [7, 14].

Besides magnum, acid glycosidases have not been investigated in particular parts of the hen oviduct yet. Previously [3], we compared multiple forms of acid glycosidases in the magnum part of the oviduct and egg albumen. In the present study, we investigated specific activities of acid glycosidases from the remaining parts of the hen oviduct i.e. infundibulum, isthmus, shell gland, vagina and multiple forms of these enzymes from the isthmus part of the oviduct. Additionally, we also described specific activities of β -hexosaminidase and β -galactosidase in egg shell membranes.

MATERIALS AND METHODS

Oviducts and eggs from 12- to 15-month-old Greenleg Partridge (Z-11) hens were used in the study. The hens represented native, laying and general-purpose breed, kept at the Experimental Station of the National Research Institute of Animal Production in Chorzelów, Poland. Birds were kept in a flock under 14L:10D and fed *ad libitum* a proprietary standard breeder's ration. Additionally, eggs from high producing layer crosses of Rhode Island White and Red (MESSA-45 and Hy-line) from the same source were used in the study.

Hens were decapitated during the last stage of the ovulatory cycle. The oviducts were removed, fragmented into parts and stored at -20°C until required. Tissue extracts (20% w/v) of infundibulum, isthmus, shell gland and vagina of the oviducts were prepared by homogenization in 1% NaCl (Vir-Tis homogenizer) at 0°C . The eggs were frozen at -20°C on day they were laid. Inner and outer shell membranes were separated after egg thawing, rinsed with distilled water, dried on filter paper and cut into small pieces.

Tissue homogenate was centrifuged at $16\,000\times g$ for 30 min and 50 μl of clear supernatant (or about 5 mg of wet egg shell membranes) were used to determine enzyme activity. The activities of β -HEX, α - and β -galactosidase (α - and β -GAL), α -mannosidase (α -MAN), β -MAN, α -glucosidase (α -GLU) as well as α -fucosidase (α -FUC) were determined spectrophotometrically according to Barrett and Heath [1] with slight modifications. Supernatants (50 μl) were added to 100 μl of 1 mM proper p-nitrophenol substrate (Sigma, St. Louis, MO, USA) in 0.2 M citrate buffer at optimum pH (for β -HEX 4.0, for β -GAL 3.5, for α - and β -MAN 4.75, for α -GAL 4.5, for α -GLU 6.2 and for α -FUC 5.5). Reaction mixtures were incubated at 37°C for 10-30 min, and then 250 μl of 3.3% (w/v) trichloroacetic acid was added. Supernatants were obtained by centrifugation. To transparent supernatants (250 μl) 250 μl of 0.5 M carbonate buffer were added, and then absorbance at 400 nm was measured. Supernatant proteins were measured using the biuret method [6]. Protein content in egg shell membranes was calculated on the assumption that 1mg of wet shell membranes contain 0.13 mg of proteins [14]. One unit of enzyme activity (U) is defined as an amount of enzyme that hydrolyses 1 μmol substrate/1 minute under the conditions described for specific glycosidases (see above).

Samples of the isthmus supernatants containing up to 4 mg of protein, were loaded onto the Econo-Pac[®] High Capacity Q strong basic anion exchange cartridge (1 ml) connected to BioLogic LP Chromatography System (Bio-Rad Labs., Richmond, CA, USA) equilibrated with 25 mM histidine-HCl buffer, pH 6.0. Unretained proteins were eluted with the column equilibration buffer and then a linear gradient of NaCl (1.25-48 mS) was applied. Finally, the column was eluted with 1 M NaCl in the same buffer.

The multiple forms of enzymes from the isthmus were separated and their isoelectric points (pI) were determined by the chromatofocusing in PBE gel using Polybuffer 7-4 reagent (following the manufacturer's instructions: Pharmacia LKB Biotechnology Chromatofocusing with Polybuffer™ and PBET™). Supernatants from the isthmus, dialysed against imidazol-HCl buffer of 0.025 M, pH 6.0 were applied on the PBE column (0.9 x 25 cm), equilibrated with the same buffer, pH 7.4. Proteins were eluted with diluted Polybuffer at pH 4.0. Finally, the column was eluted with 1 M NaCl in the same buffer. The collected fractions (1.5 ml) were analysed for enzyme activity and pH (at 20°C). Data were analyzed using one-way analysis of variance followed by LSD-test (Statistica, StatSoft Inc., Tulsa, OK, USA).

RESULTS

The specific activities of seven acid glycosidases: β -HEX, α - and β -GAL, α - and β -MAN, α -GLU and α -FUC in various parts of the hen oviduct (infundibulum, isthmus, shell gland and vagina) are presented in Figure 1.

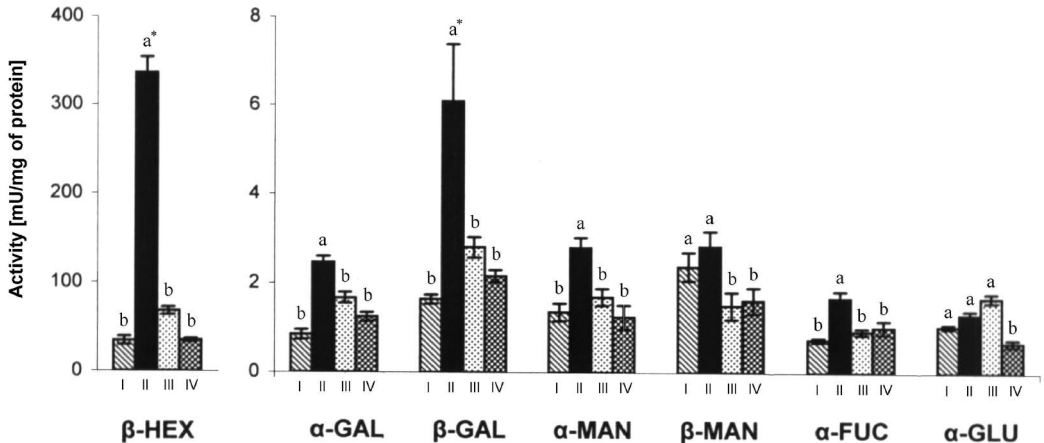


Figure 1. Specific activities (mU/mg of protein) of acid glycosidases: β -hexosaminidase (β -HEX), α -galactosidase (α -GAL), β -galactosidase (β -GAL), α -mannosidase (α -MAN), β -mannosidase (β -MAN), α -fucosidase (α -FUC) and α -glucosidase (α -GLU) in various parts of the oviduct: infundibulum (I), isthmus (II), shell gland (III) and vagina (IV). Values are given as mean \pm SEM (n=7). Bars with different letters differ significantly at $p < 0.05$ (a*: $p < 0.001$).

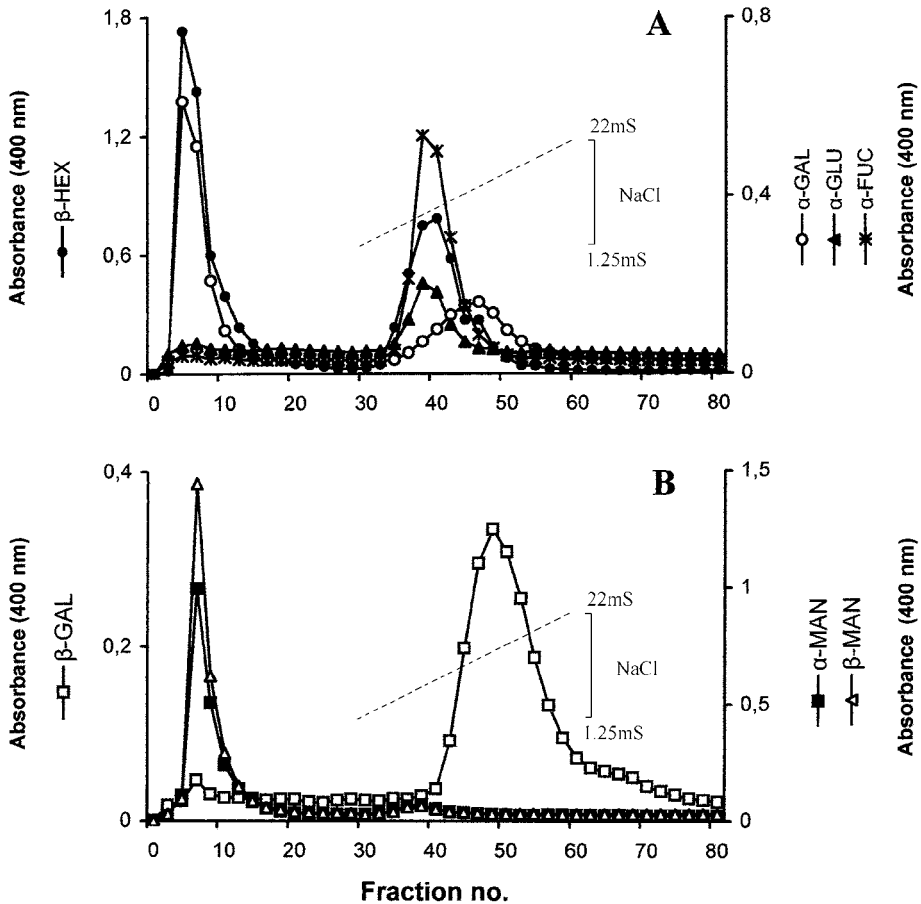


Figure 2. Separation of acid glycosidases: A/ β -hexosaminidase (β -HEX), α -galactosidase (α -GAL), α -glucosidase (α -GLU), α -fucosidase (α -FUC); B/ β -galactosidase (β -GAL), α -mannosidase (α -MAN) and β -mannosidase (β -MAN) from the isthmus on Econo-Pac[®] High Capacity Q strong basic anion exchanger. Fraction of 500 μ l were collected and assayed for enzymes activity.

The highest activities of all enzymes except α -GLU were found in the isthmus. The highest activity in the isthmus was shown for β -HEX and β -GAL, and the specific activity of β -HEX was 55 times higher than that of β -GAL.

Figure 2 shows the results of High Q anion exchange chromatography of all enzymes from the isthmus. β -HEX and α -GAL were resolved into

two peaks of activity: one peak represented unbound enzyme, and the second one was eluted at 8.7 mS (β -HEX) and 13 mS (α -GAL; fig. 2A). The entire β -GAL activity was bound to the column and eluted at 15 mS (fig. 2B). Up to 90% of total α - and β -MAN activity was not bound to the column (fig. 2B), while the entire α -GLU and α -FUC activities were bound to the column and eluted with NaCl at 7.1 mS (fig. 2A). Chromatofocusing of the supernatants resulted in the appearance of several multiple forms of acid glycosidases with pI values of 6.05, 5.85, 5.65 (major) and 5.34 (minor) for β -HEX; 4.82 (major) and 5.33, 5.24 (minor) for β -GAL; 6.67, 6.51 (major) and 6.10 (minor) for α -MAN; and 6.56 for β -MAN (data not shown).

The activities of β -HEX and β -GAL (mU/mg of protein) in the shell membranes of Greenleg Partridge (Z-11), Hy-line and MESSA-45 hens eggs are presented in Figure 3. The activities of the other acid glycosidases were negligible. β -GAL activity in the egg shell membranes did not differ among all analyzed breeds, while β -HEX activity was significantly lower ($p < 0.01$) for Hy-line hens than those of other breeds.

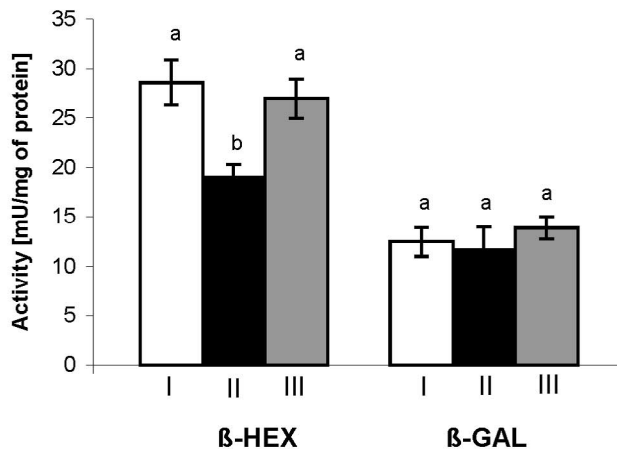


Figure 3. β -hexosaminidase (β -HEX) and β -galactosidase (β -GAL) specific activities (mU/mg of protein) in egg shell membranes of various breeds of hens: Greenleg Partridge (I), Hy-line (II), MESSA-45 (III). Values are given as mean \pm SEM (n=10). Bars with different letters differ significantly at $p < 0.01$.

DISCUSSION

The mean specific activities of acid glycosidases from particular parts of the oviduct were comparable to that reported for the whole hen oviduct by Sukeno et al. [10]. Specific activities of acid glycosidases found previously in magnum were: 100.06 ± 16.75 for β -HEX, 1.03 ± 0.39 for β -GAL, 0.63 ± 0.18 for α -MAN, 0.55 ± 0.15 for β -MAN, 0.29 ± 0.08 for α -GAL, 1.02 ± 0.32 for α -GLU and 0.42 ± 0.16 mU/mg of protein for α -FUC [3]. The specific activities of the enzymes, except α -GLU, were the highest in the isthmus (fig. 1).

In our previous paper [3] we found that β -HEX from magnum was separated by anion-exchange chromatography into two forms (I and II), which were composed of multiple forms of different pI values (6.18, 5.73, 5.55 and 5.34, 5.27, 5.16 for form I and II, respectively). Both forms of β -HEX (I and II) were also found in egg albumen. In this study, the β -HEX from isthmus was separated into two forms (fig. 2A), which were composed of multiple forms characterized by different pI values, very similar to those of form I and II from magnum and egg albumen. Because the isthmus secretes the components of egg shell membranes, it is expected that β -HEX from egg shell membranes will be present in multiple forms typical of the isthmus.

Similarly, β -GAL originated from magnum was separated into two forms (I and II), which were composed of multiple forms with pI values of 4.98, 4.84, 4.77 and 4.68 – 4.63 for form I and II, respectively [3]. In the egg albumen only form I of β -GAL was found. In the isthmus, unlike the magnum, we found only form I of the enzyme (fig. 2B), which was composed of multiple forms with pI values very similar to pI values of the β -GAL form I from egg albumen. Therefore, it appears that only those multiple forms of β -GAL which can take part in formation of the egg shell membranes are present in the isthmus. The multiple forms of β -HEX and β -GAL in shell membranes were not investigated because these membranes are highly resistant to solubilization agents [2].

To date, only high activities of β -HEX have been found in the egg shell membranes of the hen [14]. Unlike β -HEX activity in the egg albumen,

this activity does not decrease during egg storage at room temperature. It is believed, that β -HEX of the egg shell membranes and albumen play a role in slowing bacterial penetration into the egg [7, 14].

This study is the first to demonstrate high β -GAL specific activity in the egg shell membranes of the hen. In the albumen of Greenleg Partridge (Z-11) eggs, β -HEX activity is 100 times higher than β -GAL activity [3], while β -HEX activity in egg shell membranes of Greenleg Partridge hens was only twice that of β -GAL activity. Our data demonstrate that an exceptionally high β -GAL activity is concentrated in the egg shell membranes of the hen, because this activity in the magnum and albumen is only 1.03 and 0.026 mU/mg protein, respectively.

In light of the recent studies concerning the structure and changes of shell membranes that take place during embryonic development of Japanese quails [11, 15], it is suggested that in addition to slowing bacterial penetration into the egg, acid glycosidases may have a role in modifying egg shell membrane glycoproteins during embryo development.

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