The Glycolytic Enzymes Activity in the Midgut of Diabrotica virgifera virgifera (Coleoptera: Chrysomelidae) adult and their Seasonal Changes

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The western corn rootworm, Diabrotica virgifera virgifera LeConte (Coleoptera: Chrysomelidae) is an important pest of maize. The diet of the D. virgifera imago is rich in starch and other polysaccharides present in cereals such as maize. Therefore, knowledge about enzymes involved in digestion of such specific food of this pest seems to be important. The paper shows, for the first time, the activities of main glycolytic enzymes in the midgut of D. virgifera imago: endoglycosidases (α-amylase, cellulase, chinatase, licheninase, laminarainase); exoglycosidases (α- and β-glucosidases, α- and β-galactosidases) and disaccharidases (maltase, isomaltase, sucrase, trehalase, lactase, and cellobiase). Activities of α-amylase, α-glucosidase, and maltase were the highest among assayed endoglycosidases, exoglycosidases, and disaccharidases, respectively. This indicates that in the midgut of D. virgifera imago α-amylase, α-glucosidase and maltase are important enzymes in starch hydrolysis and products of its digestion. These results lead to conclusion that inhibition of most active glycolytic enzymes of D. virgifera imago may be another promising method for chemical control of this pest of maize.

Key Words: α-amylase, glycogenidase, midgut, western corn rootworm, Diabrotica virgifera
β-1,3-glycosidic bonds and licheninase (EC 3.2.1.73)—split β-1,3 and 1,4-glycosidic bonds of lichenin and β-1-glucans, which occur abundant in the grass (Terra and Ferreira 1994, 2003, Ferreira 2003, Terra and Ferreira 2005).

Among exoglycosidases activity of: α-glucosidase (EC 3.2.1.20)—hydrolysis of terminal, nonreducing α-1,4-linked glucose residues of dextrins, saccharides, oligosaccharides with releasing α-D-glucose was determined. This activity is responsible for the final starch digestion. Then: β-glucosidase(EC 3.2.1.21)—cleaves of celllobiose, oligosaccharides, glycoproteins, glycolipids, and glycosidic derivatives to β-D-glucose, α-D-galactosidase (EC 3.2.1.22)—hydrolysis terminal, non-reducing α-1,4-linked glucose residues from oligosaccharides (melibiose, raffinose, stachyose widely present in plants, mainly in grains rich in lipids), galactomannans, galactolipids (mainly in the leaves); β-galactosidase (EC 3.2.1.23)—cuts β-D-galactose from oligosaccharides, hemicelluloses, glycoproteins. As opposed to α-amylase these enzymes act on an ectoperitrophic space of midgut (Terra and Ferreira 1994, Ferreira et al. 1998, Grossmann and Terra 2001, Ferreira 2003, Terra and Ferreira 2005).

There was also tested the specific saccharidases activity: maltase (EC 3.2.1.20), isomaltase (EC 3.2.1.10), sucrase (EC 3.2.1.48), trehalase (3.2.1.23), celllobiose (3.2.1.21) hydrolyze glycosidic bonds, respectively: α-1,4- of maltose, α-1,6- of isomaltose, α,β-1,2- of sucrose, α-1,1- of trehalose, β-1,4- of lactose, β-1,4- of celllobiose (BRENDA, The Comprehensive Enzyme Information System 2012).

For analyses of the pH optimum for enzymes activity 50 mM buffers were used as follows—the α-amylase: citrate-phosphate (pH 2.6–5.8), phosphate (pH 5.8–7.8), Tris-HCl (pH 7.8–9.0); the α-glucosidase: citrate-phosphate (pH 2.6–7.0); the β-glucosidase: citrate-phosphate (pH 2.6–5.8), phosphate (pH 5.8–7.8); the α-galactosidase: citrate-phosphate (pH 3.4–6.2); the β-galactosidase: citrate-phosphate (pH 2.6–5.8), phosphate (pH 5.8–7.8); the isomaltase: citrate-phosphate (pH 2.6–5.8), phosphate (pH 5.8–7.8), Tris-HCl (pH 7.8–9.0). The linear dependence of enzymes activity and incubation time in the range 0–360 min for the maltase and trehalase was also examined. All analyses were performed using a modified standard methods cited below (also summarized in Nakonieczny et al. 2006).

Endoglycosidases activity assay was based on Noelting and Berndfeld method (1948) that allows quantitative indication of reducing groups of disaccharides resulting from enzymatic hydrolysis of substrates, using a standard curve prepared for maltose. The following substrates were used: α-amylase—soluble starch, cellulase—sodium carboxymethylcellulose, chitinase—colloidal chitin (prepared according to Hsu and Lockwood 1975), laminarinase—laminarin from Laminaria digitata, licheninase—lichenin from Cladophora asparagodes. Samples were incubated (KS 4000) control incubator, IKA Laboratory Technology, Staufen, Germany) at 30°C (± 1°C) for 24 h, except the α-amylase, which was incubated for 40 min. The activities were measured colorimetrically at 540 nm, with a Helios Aquamate Ultraviolet-visible spectrophotometer (UV/VIS) spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) in the 620 µl of reaction mixture: 50 mM citrate-phosphate buffer (pH 4.2), 20 µl of homogenate (10 µl for α-amylase), 200 µl of 3,5-dinitrosalicilic acid reagent used for reaction termination and 300 µl H2O. The unit activity was expressed as the µmol maltose/mg protein·min⁻¹.

The analyses of the glycosidases hydrolyzing particular mp-glycopyranosidases (α- and β-glucosidase, α- and β-galactosidase) were performed according to Terra et al. (1979) method description. The yellow product (p-nitrophenol) is released from the substrate, which consists the appropriate monosaccharide and p-nitrophenol linked with α- or β-glycosidic bond. The amount of released product was measured colorimetrically as above and calculated on the basis of standard curve prepared for the different concentration of p-nitrophenol. The following substrates were used: α-glucosidase—p-nitrophenyl α-glucopyranosidase, β-glucosidase—p-nitrophenyl β-glucopyranosidase, α-galactosidase—p-nitrophenyl α-galactopyranosidase, β-galactosidase—p-nitrophenyl β-galactopyranosidase. Samples were incubated at 30°C (± 1°C) for 40 min, except for the α-glucosidase which was incubated 20 min. The reaction mixture contained 540 µl of 50 mM citrate-phosphate buffer (pH 5.0) (for β-galactosidase: pH 4.2) and 45 µl of homogenate. After incubation 450 µl of 1% sodium dodecyl sulfate (SDS) in 100 mM bicarbonate buffer (pH 10.4) was added. The activities of the glycosidases hydrolyzing mp-glycopyranosidases were expressed as µmol of p-nitrophenol·mg⁻¹ protein·min⁻¹.

The analyses of disaccharidases activity were performed according to the modified method of Dahlqvist (1968), which quantifies glucose released from particular disaccharide. Glucose concentration was determined in the reaction mixture using a diagnostic kit “Liquick Cor-Glucose” (PZ CORMAY S.A., Lomianki, Poland) according to Barham and Trinder (1972) method. Absorbance was measured at 500 nm with a Tecan Infinite F200 Microplate Reader (Tecan Group Ltd., Männedorf, Switzerland). The amount of released glucose was calculated from the standard curve. The following substrates were used: maltose, isomaltose, sucrose, lactose, trehalose, and celllobiose (0.056 M). The reaction mixture (370 µl) contained: 10 µl of 50 mM citrate-phosphate buffer (pH 5.0) with the appropriate substrate, except for isomaltase and celllobiose: citrate-phosphate buffer (pH 5.4 and 3.8, respectively) and 10 µl of homogenate. Samples were incubated for 120 min at 30°C (± 1°C), (maltase—30 min). After incubation (KS 4000) control incubator, IKA Laboratory Technology, Staufen.
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Germany) 200 μl of 5% trichloroacetic acid (TCA) was added. From this mixture 20 μl was collected and added to 350 μl of working solution of the glucose kit and incubated for 15 min at 37°C. The activities of disaccharidases were calculated per 1 molecule of glucose released from hydrolysis of particular disaccharide and expressed as μmol glucose·mg⁻¹ protein·min⁻¹.

All enzymatic assays from the same stock subsamples were performed. The protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as standard.

Enzymes Activity Versus Maize Growing Season. To clarify whether the activity of glycolytic enzymes of the D. virgifera imago depend on maize growing season the assays of the most active enzymes, according to obtained results were chosen—α-amylase, maltase, isomaltase, and sucrase. The investigations were performed in the two following years: 2010 and 2011. The insects for these determinations were collected in each maize growing season, at three chosen periods of maize development in July, August, September:

- 2010—I. BBCH 69–71 (the phase from the end of flowering to kernel’s development with the consistency of watery), II. BBCH 83 (the phase from the beginning of kernels wax maturity), III. BBCH 85 (the phase of full kernels wax maturity);
- 2011—I. BBCH 67 (the phase of full flowering), II. BBCH 75–83 (the phase from the full kernels milky maturity to the beginning of kernels wax maturity), III. BBCH 85–87 (the phase from the full kernels wax maturity to physiological maturity, the kernels contain 60% dry matter).

Statistical Analysis. Statistical analysis of the obtained data of α-amylase and disaccharidases activities of D. virgifera imago versus maize growing season (2010 and 2011 year) was performed using the analysis of variance (ANOVA), post hoc Least Significant Difference (LSD) test (P < 0.05) (STATISTICA, data analysis software system), version 10 (StatSoft Inc. 2011).

Results

Glycolytic Activities. The highest α-amylase activity in D. virgifera imago midgut was stated in the acidic pH (citrate-phosphate buffer at pH 4.2) and it was relatively high up to pH 6.2 (Fig. 1). The α-amylase activity was the highest among all polysaccharidases assayed in this study. Specific activities of the other—laminarinase, licheninase, chitinase, and cellulase were at least about 270 times less.

For exoglycosidases (α- and β-glucosidase, α- and β-galactosidase) also optimal activity at low pH were stated. The highest activity of α-glucosidase, α-galactosidase, and β-glucosidase was stated at pH 5.0, and of β-galactosidase at pH 3.8 in citrate-phosphate buffer. All glycosidases activity, with the exception of β-galactosidase, showed broad pH optima (Fig. 2). The enzymes ordered according to decreasing activities are as follows: α-glucosidase, β-galactosidase, α-galactosidase, and β-glucosidase (Table 1).

Among the enzymes activity hydrolyzing particular disaccharides as the substrate the highest for maltase was stated. For other disaccharidases: lactase, trehalase, isomaltase, cellobiase lower activities were found (Table 1). For isomaltase activity the pH optimum at 5.4 was determined (Fig. 3). Figure 4 shows an effect of incubation time on maltase and trehalase activities in the midgut of D. virgifera imago. The activity of maltase increased rapidly with incubation time 0–360 min, in contrast to the trehalase activity.

The seasonal changes in selected glycolytic enzymes activity. The dependence of enzymes activity determined with corn growing season, in the two following years (2010, 2011) showed significant statistical differences for activities of α-amylase and maltase in 2010, as well as maltase and sucrase in 2011 year (Figs 5 and 6). Within disaccharidases the maltase was the most active enzyme regardless of year or the maize growing season. Among these enzymes in both years only for isomaltase the same pattern of enzymes activity was stated.

Discussion

Acidic pH occurs in midgut of the majority of Coleoptera larvae and adults (Wolfson and Murdock 1990, Terra and Ferreira 1994, Silva et al. 1999, Hosseinzinahve et al. 2007, Lopes et al. 2010). This optimal pH is characteristic both for α-amylase and cysteine proteases (the main group of proteolytic enzymes) of D. virgifera larvae (Kaiser-Alexnat 2009). A relatively wide range of α-amylase activity at acidic pH for D. virgifera imago was stated. It is similar to those obtained for other herbivorous insects such as the maize weevil (Sitophilus zeamais) imago and two members of the family Chrysomelidae Chrysomela pardinina, Chrysomela herbacea with a pH range of 5.0 to 7.0 (Nakonieczny 2007, Lopes et al. 2010). Wide pH optimum of α-amylase activity is probably a result of the occurrence of several isoforms of the enzyme. Probably it is an evolutionary adaptation to the presence of secondary plant metabolites in their diet, including enzymes inhibitors and toxins (Titarenko and Chrispeels 2000, Hosseinzinahve et al. 2007).

![Fig. 1. The effect of pH on α-amylase activity in the midgut of Diabrotica virgifera imago.](https://academic.oup.com/jinsectscience/article-abstract/15/1/56/2583149)
Different profiles of glycolytic activities of insect’s species express the adaptation of digestive processes to their diet. Imago of *D. virgifera* are polyphagous—initially, they feed on maize pollen, rich in digestible nitrogen and carbon compounds including free amino acids (mainly proline, serine, alanine), proteins, but also carbohydrates such as glucose, maltose, sucrose or stachyose. *Z. mays* has pollen rich in starch (starchy pollen) (Barker 1977, Baker and Baker 1979, Mullin et al. 1997, Hollister and Mullin 1999, Zona 2001, Bennetzen and Hake 2003, Terra and Ferreira 2005, Genta et al. 2006).

The analyses of glycolytic activities in the midgut of *D. virgifera* imago showed that the most active enzyme was α-amylase, followed by α-glucosidase and maltase, among polysaccharidases and disaccharidases, respectively. These enzymes belong to the starch metabolism pathways that hydrolyze molecules of poly-, oligo- and disaccharides with α-glycosidic bonds. The diet of *D. virgifera* imago, mentioned earlier, explains this profile of the glycolytic activities. Also, the studies of carbohydrate digestion in the Mexican bean weevil (*Zabrotes subfasciatus*) and the bruchid beetle (*Callosobruchus maculatus*) feeding on seeds of legumes have shown that the most active glycolytic enzymes were α-amylase, α-glucosidase, and maltase (Silva et al. 1999).

In our study, the lower activities of other polysaccharidases may result from much less content of compounds with β-1,3-, β-1,4- and β-1,3,1,4-glycosidic bonds in the diet of adult *D. virgifera*. Usually high activity of laminarinase in insects feeding on fungi is observed. Microorganisms which possess licheninase and laminarinase, occurring in the midgut, serve rather in the detoxification processes of plant toxic aglycones than in food digestion (Scrivener et al. 1997, Azevedo et al. 2003, Terra and Ferreira 2005, Genta et al. 2006).

The glycosidases in the midgut of *D. virgifera* imago hydrolyzing α- and β-glucosidic, α- and β-galactosidic bonds showed maximum activities in acidic pH. For α- and β-glucosidases and α-galactosidase relatively wide pH optima of reaction were determined. Measurements of enzymes activities hydrolyzing pnp-glycopyranosides as a substrates in the midgut of *D. virgifera* imago have shown the highest activity of α-glucosidase against NPαGlu. This enzyme is also responsible for the final phase of starch digestion (Silva et al. 1999). An insect α-glucosidases are structurally and functionally variable. In *Musca domestica* larvae two isoforms of α-glucosidase were identified. The smaller one (Molecular Weight = 72.7 kDa) hydrolyses preferably oligosaccharides longer than maltotetrose. The larger form (230 kDa) prefers smaller substrates such as maltose, maltotriose, maltotetrose (Jordão and Terra 1991, Fonseca et al. 2010). In Z. subfasciatus and *C. maculatus* α-glucosidases which can react with at least five

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**Table 1. The glycosidases activity in the midgut of Diabrotica virgifera imago**

| Enzyme: Substrate, concentration | Activity | SD |
|----------------------------------|----------|----|
| **Endoglycosidases: maltose, 540 nm, [μmol maltose mg protein⁻¹ min⁻¹]** |          |    |
| α-amylase Soluble starch in H₂O, 0.50% | 149.275  | 38.040 |
| Laminarinase Laminarin (from Laminaria digitata), 0.25% | 0.553  | 0.139 |
| Licheninase Lichenin (from Cetraria islandica), 0.25% | 0.390  | 0.067 |
| Chitinase Colloidal chitin, 0.50% | 0.112  | 0.043 |
| Cellulase Carboxymethyl cellulose (CMC), 0.25% | 0.053  | 0.017 |
| **Disaccharidases: glucose, 500 nm, [μmol glucose mg protein⁻¹ min⁻¹]** |          |    |
| Maltase Maltose, 0.056 M | 41.397  | 8.602 |
| Sucrase Sucrose, 0.056 M | 18.639  | 4.905 |
| Lactase Lactose, 0.056 M | 11.779  | 2.839 |
| Trehalase Trehalose, 0.056 M | 7.883  | 3.091 |
| Isomaltase Isomaltose, 0.056 M | 3.746  | 2.692 |
| Cellobiase Cellobiose, 0.056 M | 0.676  | 0.357 |
| **Exoglycosidases: p-nitrophenol, 420 nm, [μmol p-nitrophenol mg protein⁻¹ min⁻¹]** |          |    |
| α-glucosidase pNPαGlu, 20 mM | 1.506  | 0.189 |
| β-glucosidase pNPβGlu, 20 mM | 0.775  | 0.223 |
| α-galactosidase pNPGal, 8 mM | 0.413  | 0.080 |
| β-galactosidase pNPβGAL, 20 mM | 0.822  | 0.085 |

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Fig. 2. The effect of pH on activities of exoglycosidases in the midgut of *D. virgifera* imago.
substrates, both for maltooligosaccharides and synthetic substrates NPβGlu were identified (Silva et al. 1999).

The activities of enzymes hydrolyzing NPβGlu as a substrate differed significantly from activities against other p-nitrophenyl substrates. In the midgut of the D. virgifera imago they were about two times higher than against NPβGlu and NPβGal. This may indicate a lower content of food components with β-glycosidic bonds in their diet or presence of toxic compounds which are not effectively digested. The β-glycosidases, occurring in the midgut of insects, may come both from plant food and midgut microflora (including symbionts). The β-glycosidases mainly hydrolyze oligosaccharides, but also disaccharides, derived from cellulose and hemicelluloses which contain β-1,3-, β-1,4- and β-1,6-glycosidic bonds (Terra and Ferreira 1994, Ferreira 2003). The low β-glycosidases activities also may result from a lower level of glycolipids in the diet, especially membranes lipids such as monogalactosyldiglycerides and digalactosyldiglycerides. The last one is hydrolyzed by β-galactosidase only after removing one residue of galactose by α-galactosidase (Grossmann and Terra 2001, Azevedo et al. 2003, Ferreira 2003). In the midgut of the D. virgifera the level of α-galactosidase activity among all tested glycosidases was the lowest against the p-nitrophenyl β-galactopyranoside as substrate.

Analyses of the disaccharidases activity among β-glycosidases showed higher activity against lactose than cellobiose as a substrate. Lower activity of cellobiase may result from low levels of disaccharides and oligosaccharides with β-glucosidic bonds in the diet. But higher level of lactase activity involved in hydrolysis of β-glucosidic bonds indicate that hydrophilic substrates with β-galactosidic bonds (like oligosaccharides, hemicelluloses, and glycoproteins), common in the tissues of green plants, may be efficiently hydrolyzed by D. virgifera imago (Ferreira et al. 1998). In Tenebrio molitor β-glycosidase has two active sites. This multifunctional enzyme is proposed as an adaptation to plant glycidoses, because their hydrolysis generates toxic aglycones (Ferreira et al. 1998, Ferreira 2003).
Disaccharidases activity in the midgut of *D. virgifera* imago showed that the most active enzymes are maltase and sucrase, what seems to be obvious results related to the diet of this beetle. Maltose is a product of starch hydrolysis and occurs in large amounts in maize kernels, silk, and pollen (Shivanna and Sawhney 1997, Bennetzen and Hake 2009). The high activity of sucrase may indicate that the beetle diet is also high in sucrose and stachyose (Barker 1977). Among the examined enzymes isomaltase is the third one, involved in starch hydrolysis.

The starch digestion to glucose requires the presence of several enzymes which hydrolyze both the a-1,4- and a-1,6-glycosidic bonds and among them the isomaltase is responsible for cleavage of a-1,6-glycosidic bond (Kanehisa 1996). Its low activity in the *D. virgifera* midgut may result from the low expression of this enzyme because starch contains a few a-1,6-glycosidic bonds or due to the overall small amounts of compounds with this bond in the diet of *D. virgifera* beetle.

Trehalase is involved in the hydrolysis of a-1,1-glycosidic bond of trehalose. This disaccharide diffuse from the hemolymph into the midgut and trehalase helps to keep a proper gradient of glucose in the epithelium of the gut. But it does not play a significant role as a digestive enzyme in the midgut of *D. virgifera* imago (Applebaum 1985, Terra and Ferreira 1994, Nation 2002).

The diverse enzymatic profile of *D. virgifera* imago in the 2 yr and three periods of maize development may result from different weather conditions (air temperature, rainfall) and thereby the different availability of food in each year. For *D. virgifera* imago, maize is the main host plant, but after its senescence, the insects also feed on other plants. It was documented that the imago can also feed on plant species belonging to at least 29 families (e.g., Poaceae, Asteraceae, Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Malvaceae) (Hollister and Mullin 1998, Vidal et al. 2005). In particular, the fertilized females for the normal development of eggs need the food rich in amino acids. The changes in enzymes activity during the maize phenology reflect beetle adaptation to plant age or feeding on other plants (Elliot et al. 1990, O’Neal et al. 2002). The results may indicate that the weather conditions may significantly influence the daily activity of *D. virgifera* imago (Isard et al. 1999), and thus the intensity of feeding, which consequently can affect the digestive processes. Perhaps a further correlation of obtained results with weather data prove the changes of particular enzymes activity (inhibition or stimulation).

In summary, analyses of glycolytic enzymes activity in the midgut of *D. virgifera* imago showed that the most active enzymes are maltase and sucrase, what seems to be obvious results related to the diet of this beetle. Maltose is a product of starch hydrolysis and occurs in large amount in maize kernels, silk, and pollen (Shivanna and Sawhney 1997, Bennetzen and Hake 2009). The high activity of sucrase may indicate that the beetle diet is also high in sucrose and stachyose (Barker 1977). Among the examined enzymes isomaltase is the third one, involved in starch hydrolysis.

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