Comparative kinetic characterization of the activity of glycosylated and non-glycosylated trypsin-like serine protease isolated from adults of *Rhyzopertha dominica* (Coleoptera: Bostrichidae) reared on the grain of three different cultivars of wheat

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Abstract. *Rhyzopertha dominica* is a pest that uses trypsin-like serine protease enzymes to hydrolyse the proteins in the cereal grains on which it feeds. The present study reveals for the first time that that there are both glycosylated and non-glycosylated serine proteases. The progeny of *R. dominica* reared on the grain three varieties of wheat were used to fractionate their trypsin-like serine proteases using Concanavalin A affinity chromatography. The albumin fractions from the wheat cultivars used in this study were subjected to size exclusion chromatography to fractionate the albumin inhibitors that are highly specific for the serine protease activity of *R. dominica.* Kinetic and thermodynamic assays were used to differentiate both types of enzymes. In general, the catalytic efficiency values V_{max}/K_m for glycosylated proteases were higher, indicating that glycosylation increases the affinity for the substrate. Inhibition assays using wheat albumins revealed that the glycosylated enzymes had higher K_i values, indicating a low affinity for the inhibitors than the non-glycosylated enzymes. Thermodynamic analysis indicates that glycosylation increases the activation energy *E*a improving the serine proteases' catalysis. Thus it is likely that *R. dominica* uses glycosylated proteases in order to optimize the hydrolysis of cereal proteins and nullify the action of wheat grain protease inhibitors and increase its chances of survival.

INTRODUCTION

Insects obtain essential amino acids from their food by efficient digestive hydrolysis of plant proteins (Gholamzadeh et al., 2013; Zhu-Salzman & Zeng, 2015). Their digestive proteases catalyse the hydrolysis of proteins important for growth and development. The proteases are classified according to the chemical nature of the groups responsible for catalysis, i.e., serine, threonine, cysteine, aspartic or metalloproteases (López-Otín & Bond, 2008; Barrett et al., 2012). In the digestive tract of some species of insects such as *Mayetiola destructor* (Diptera: Cecidomyiidae) (Chen et al., 2013), *Oulema melanopus* L. (Coleoptera: Chrysomelidae) (Wielkopolan et al., 2015) and *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Oppert et al., 2010) the predominate proteases are cysteine and serine proteases that digest proteins. The coleopteran *Rhyzopertha dominica* uses serine-proteases to digest protein, specifically trypsin (Konarev, 1996; Zhu & Baker, 1999; Osuna-Amarillas et al., 2012). *R. dominica* is a destructive pest of stored wheat (Cinco-Moroyoqui et al., 2008; Priya et al., 2010), which

is resistant to pyrethroids and organophosphates insecticides, making it difficult to control (Edde, 2012; Oppert & Morgan, 2013). It has been suggested that plant inhibitors might induce the regulation of expressed digestive proteases by activating specific genes that are usually not active (Silva et al., 2001; Chen et al., 2013). The enzymatic activity is dependent on several factors such as temperature (Sotelo-Mundo et al., 2007; Mehrabadi et al., 2011), plant inhibitors (Mahbobe et al., 2012), pH and posttranslational modifications such as glycosylation and phosphorylation (Wei et al., 2006, Vandenborre et al., 2011).

Glycosylation is a biochemical process that modifies the structure and function of proteins by attaching carbohydrates to the side groups of the amino acids asparagine (Asn), serine (Ser) and threonine (Thr). This modification of the protein molecules affects their thermodynamic, kinetic and structural properties (Nagai et al., 1997; Zhang et al., 2008; Lam et al., 2013). Studies on the glycosylation of the enzymes in insects, such as, *Tribolium castaneum, Bombyx mori, Apis mellifera, Drosophila melanogaster* and *Acyrthosiphon pisum* indicate that the effect is species specific (Vandenborre et al., 2011). Of these insects, *A. pisum, D. melanogaster* and *T. castaneum* use glycosylated proteases to digest proteins. Similarly, Wei et al. (2006) report that the enzymatic activity of fungal cellulases depends on N-linked glycols. As these posttranslational modifications alter the enzyme molecules' polarity and activity, insects might modulate that property by glycosylating their enzymes to overcome molecule solubility constraints that would limit their activity or alter the affinity for a particular substrate or inhibitor. Therefore, the identification and characterization of glycosylated digestive proteases could help in determining the critical relationship between the ability of insects to feed and reproduce on stored grains.

There are two types of glycosylation, *N*- and *O*-glycosylation. In the first, the glycan is attached to the amide group of an Asn residue located in the tripeptide consensus sequon Asn-X-Ser/Thr (where X can be any amino acid except Pro). In contrast, in the second, the glycan is attached to the hydroxyl group of Ser or Thr residues (Spiro, 2002; Réndic et al., 2008; Roth et al., 2012). However, *N*-glycosylation, which is the most common glycosidic linkage in proteins, depends on whether Ser or Thr is found in the sequon, as previously experimentally demonstrated by Bause (1984), using synthetic hexapeptides containing Asn-Xaa-Thr and Asn-Xaa-Ser (asparagine sequon), which revealed that the presence of aspartic (Asp) as the X amino acid in the sequon, in addition to Ser, reduces the carbohydrate-binding properties of asparagine.

Three trypsinogen-like cDNAs of *R. dominica* have been cloned and sequenced (Zhu & Baker, 1999). A visual analysis of the amino acid sequence of these three trypsinogens revealed that *Rdo*T1 contains one sequon susceptible to *N*-glycosylation (Ser^{103}-Asp^{104}-Asn^{105}), *Rdo*T2 one (Asn^{45}-Tyr^{46}-Thr^{47}), *Rdo*T3 three (Asn^{42}-Tyr^{43}-Thr^{44}, Asn^{148}-Ser^{149}-Thr^{150} and Asn^{251}-Ile^{252}-Thr^{253}). According to Gavel & von Heijne (1990) and Bause (1984), *Rdo*T1 is much less likely to be glycosylated than *Rdo*T2 and *Rdo*T3 as the incorpora-

tion of aspartic acid as Xaa results in a significant reduction in the properties of the glycosyl acceptor. Therefore, based on the differences in the number of potential glycosylation sites, the possibility of separating them according to their degree of glycosylation, it was decided to obtain further insights into the differences in their kinetic properties.

Concanavalin A is a lectin found in the legume *Canavalia ensiformis* (jack bean) that binds to mannose residues of glycoproteins and is used in lectin affinity chromatography procedures to fractionate glycosylated from non-glycosylated proteins. The present study aimed to isolate the glycosylated and non-glycosylated trypsin-like serine proteases from *R. dominica* reared on the grain three different cultivars of wheat in order to determine and compare their kinetic and thermodynamic properties and obtain insights into the characteristics of the serine proteases of weevil and a better understanding of their properties.

MATERIALS AND METHODS

Wheat samples

Samples of several hexaploid varieties of wheat were donated by the Wellhausen-Anderson Plant Genetic Resource Centre at the International Maize and Wheat Improvement Centre (CIMMYT, El Batán, México) and cultivated at the INIFAP (National Institute of Forestry, Agriculture and Livestock Research), Dr. Norman E. Borlaug Experiment Station, Cd. Obregón, Son., México, located in the Yaqui Valley. Samples of mature wheat grains of the selected varieties were stored at 4°C before analysis.

Insects

Samples of 500 g of grains of the wheat cultivars Villa Juarez, Borlaug and Kronstad were infested, each with 100 unsexed adults of *R. dominica* and placed in a rearing chamber at 27°C and 70% R.H. for 15 days for oviposition. Then insects were removed by sifting infested grains using a 7/64" sieve. The infested grains were incubated under the same conditions for 30 days until insects emerged. Those that emerged over a period of 45 days were used to prepare the proteolytic extracts.

Preparation of the proteolytic extract

The proteolytic extracts were obtained according to Osuna-Amarillas et al. (2012) with some modifications. The intestinal tracts were macerated and homogenized in 20 mM Tris-HCl buffer, pH 8, containing 20 mM NaCl and 10 mM CaCl$_2$. The macerates were centrifuged at 10 000 *g* for 10 min and 4°C. The supernatants were filtered through a 0.45 μm nylon filter and stored at 4°C for subsequent analyses.

Lectin affinity chromatography of the proteolytic extracts

Three millilitres of the proteolytic extracts from each group of *R. dominica* (approximately 250 units of trypsin activity) were loaded onto a 0.8 × 15 cm Concanavalin A lectin column (AffiGel Con-A, BioRad Laboratories, Richmond, CA, USA) and equilibrated with 20 mM Tris-HCl, pH 7.4, containing 5 mM MnCl$_2$ and 10 mM MgCl$_2$. Proteins that did not bind and flowed through the column were eluted with equilibration buffer and designated as non-glycosylated trypsin. Elution of bound protein was done by washing the column with 20 mM Tris-HCl, pH 7.4, containing 5 mM MnCl$_2$, 10 mM MgCl$_2$, and 0.05 M mannopyranose, and designated as glycosylated trypsin. One mL fractions were collected at a flow rate of 18 mL/h. Protein detection was carried out at 280 nm using a Bio-Rad SmartSpec 3000 UV/Vis spectrophotometer (Bio-Rad, Richmond, CA, USA).
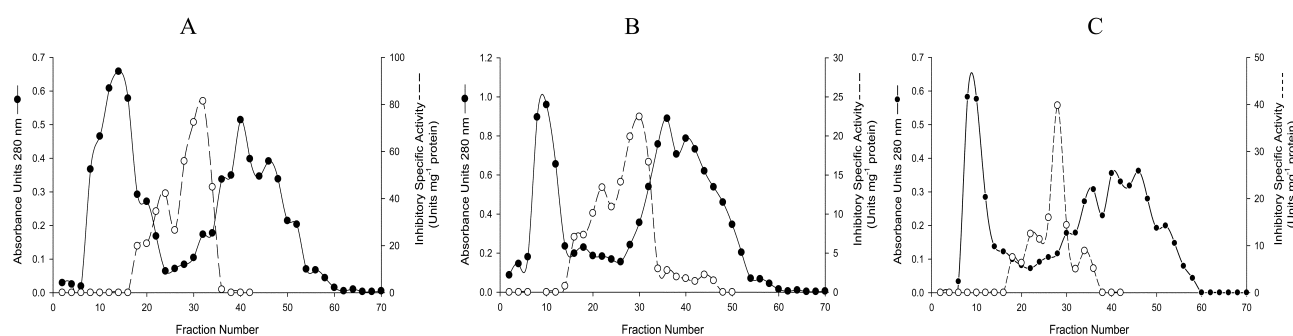
Fig. 1. Chromatographic profiles of wheat albumin fractions with inhibitory activity against the trypsin-like activity of *Rhyzopertha dominica*. Grain of wheat used for rearing this weevil was that of the cultivars (A) Borlaug, (B) Kronstad and (C) Villa Juárez. Chromatographic separations were done using a 2.5 × 100 cm column packed with Bio-Gel P-150 (Bio-Rad, Richmond, CA, USA). The elution buffer was 0.01 M sodium phosphate, pH 8.

Size exclusion chromatography of wheat albumins

Extracts of the grain of wheat varieties Bourlag, Kronstad and Villa Juarez were finely ground and passed through a 60-mesh sieve to obtain uniform particle size and optimize protein extraction. One gram of the resulting flour was combined with 5 mL of 0.01 M sodium phosphate buffer, pH 8, in order to extract the albumin fraction that contained the trypsin inhibitors. Protein extraction was carried out for 1 h at room temperature under mechanical agitation. Samples were centrifuged at 10 000 *g*, and the supernatant was recovered and filtered through 0.45 µm Cameo 17N nylon filters (Osmonics Laboratory Products, Minnetonka, MN).

Size exclusion chromatography was initiated by loading, in separate runs, 4 mL of wheat extracts (approximately 80 mg of protein of each wheat albumin extract) onto a 2.5 diameter, 100 cm long column packed with Bio-Gel P-150 (Bio-Rad, Richmond, CA, USA) previously equilibrated with 0.01 M phosphate buffer, pH 8. Protein was eluted from the column with the equilibration buffer and 3 mL fractions were collected. Protein detection in the fractions was carried out at 280 nm using a Bio-Rad SmartSpec 3000 UV/Vis spectrophotometer (Bio-Rad, Richmond, CA, USA). The chromatographic fractions were analysed for their capacity to inhibit the glycosylated and non-glycosylated like trypsin extracted from *R. dominica*. Those fractions with the highest activity in inhibiting trypsin were used in assays to determine the constant inhibition, K_i. Fig. 1 shows the chromatographic profiles of the wheat albumins, including the inhibitory profiles of their activity against the trypsin like activity of *R. dominica*.

Determination of protein content

The protein content of the proteolytic extracts of the adults and the pooled chromatographic fractions from the Concanavalin A affinity column was determined using the Bradford dye-binding assay (Bradford, 1976) with bovine serum albumin as standard.

Detection of protease activity by zymography

The crude extracts obtained from adult *R. dominica* and the chromatographic fractions containing the protein bound to the Concanavalin A affinity column were subjected to electrophoretic analysis under non-reducing conditions. The enzyme extracts were diluted 1:1 with non-denaturing Laemmli's sample buffer (0.125 M Tris-HCl, pH 6.8, containing 20% glycerol and 0.004% bromophenol blue). Aliquots of 25 µl of the resulting dilutions were loaded onto a polyacrylamide gel system consisting of a 12% acrylamide separation gel and a 3.83% acrylamide stacking gel (Laemmli, 1970). The proteins were separated at a constant voltage of 200 *V* for 45 min. After electrophoresis, the gel containing the separated proteases was laid onto another polyacryla-

mide gel containing co-polymerized 0.27% (w/v) casein (Heussen & Dowdle, 1980). Both gels were placed in a plastic container for 2 h in a water bath at 37°C to allow the separated proteases to hydrolyse the casein. The gel with co-polymerized casein was recovered and stained with 0.1% amido black in 7% acetic acid and 30% methanol. Protein bands were visualized by de-staining carried out by washing the gel with 7% acetic acid until clear bands of hydrolysed casein were visible against a dark background.

### Molecular weights of the glycosylated proteases

Molecular weights of the glycosylated proteases were determined using the electrophoretic procedure of Laemmli (1970), described by Osuna-Amarillas et al. (2012). To construct a standard curve, the following molecular weight markers were used: myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). The molecular weight values of glycosylated serine proteases were estimated using the regression equation derived from plotting the relative mobility R_m versus the log molecular weight of the protein standards.

Protease assay

Determination of trypsin-like activity in the crude extract and the chromatographic fractions was carried out according to Osuna-Amarillas et al. (2012) using N-α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) as a substrate. One hundred microliters of the enzyme preparations were combined with 300 µL of 1 mM phosphate buffer, pH 8 and incubated at 37°C. The reaction was started by adding 200 µL of 1 mM BA*p*NA and stopped after 10 min by adding 150 µL of 30% (v/v) acetic acid. One unit of trypsin-like activity was defined as 1 nmol of *p*-nitroaniline released under the conditions of the assay using a molar extinction coefficient of 8800 cm^{-1} M^{-1} at 410 nm.

Determination of kinetic parameters

The glycosylated and non-glycosylated proteins eluted from the Concanavalin A affinity column of each enzymatic preparation from adult *R. dominica* were used to determine their kinetic parameters K_m and V_{max} using BA*p*NA in the range of 0.1 to 0.5 M and combined with two units of BA*p*NAase activity in 100 mM phosphate buffer, pH 8.0. The reaction was carried out as described previously for proteolytic activity. The estimates of K_m and V_{max} were obtained by fitting the observed values of V_0 versus the substrate concentration to the Michaelis-Menten equation using non-linear correlation. The V_{max}/K_m ratio was used to compare the hydrolytic efficiency of the glycosylated and non-glycosylated enzymes. The constant inhibition K_i for the glycosylated and non-glycosylated enzymes was determined using Dixon plots
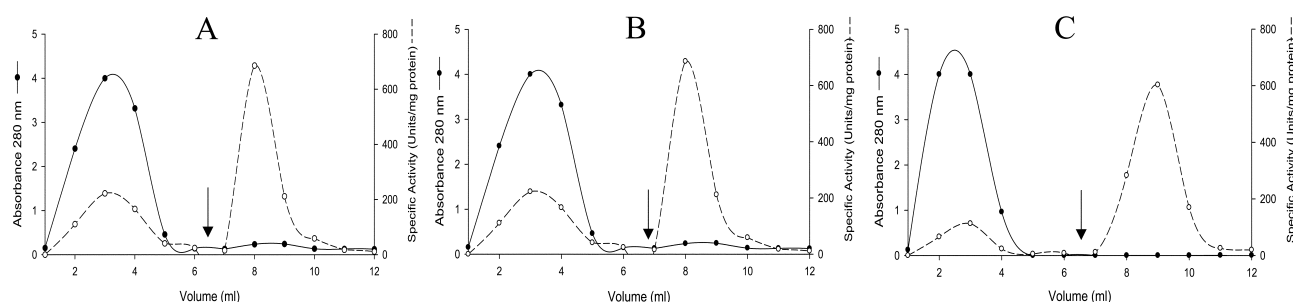
Fig. 2. Affinity chromatography using Concanavalin A Sepharose™ 4B for fractionation of glycosylated and non-glycosylated trypsin-like serine protease activity in *Rhyzopertha dominica* reared on the grain of the wheat cultivars (A) Borlaug, (B) Kronstad and (C) Villa Juárez. The glycoproteins were eluted with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.05 M mannopyranose. Arrows indicate the beginning of the elution of glycosylated proteins bound to the Concanavalin A column.

(Burlingham & Widlanski, 2003) and two fixed substrate concentrations (0.07 M and 0.115 M Ba*p*Na) combined with wheat albumin concentrations in the range of 0.2×10^{-3} to 1.5×10^{-3} mg. The inhibitory wheat albumin was prepared by size exclusion chromatography described by González-Ruiz et al. (2021). Dixon plots were prepared by plotting the reciprocal velocity $1/V_0$ against the albumin concentrations. The intersection of the lines obtained was used to calculate the dissociation equilibrium constant of the enzyme-inhibitor complex, K_i.

Determination of activation energy E_a

The activation energy (E_a) of an enzymatic reaction was determined by measuring the slope of the Arrhenius plot over the temperature range 30°C to 55°C, at intervals of 5°C, by plotting ln V_0 versus T^{-1} and using the equation:

$$\ln V_0 = \ln A - E_a / RT \qquad (1)$$

where E_a is the activation energy, T (°K) the corresponding absolute temperature and R the gas constant (8.314 J mol^{-1} °K^{-1}).

Data analysis

All analyses were carried out in triplicate. Results were expressed as mean values ± standard deviation. Data were subjected to an analysis of variance following general model procedures (SAS Institute, 2005). The resulting mean values were compared using Tukey's multiple range test with significance at $P \le 0.05$.

RESULTS

### Purification of proteases by affinity chromatography with Concanavalin A

Concanavalin A is frequently used to fractionate glycosylated from non-glycosylated protein molecules. There are a vast number of reports that demonstrate that Concanavalin A is useful for that purpose. In the present study, affinity chromatography using the lectin Concanavalin A was used to separate two types of trypsin-like proteases from each of the *R. dominica* that developed and emerged from the grain of the wheat cultivars Borlaug, Kronstad and Villa Juarez. Fig. 2 shows the chromatographic profiles of the separation of the two types of proteases, glycosylated and non-glycosylated. This chromatographic procedure proved to be very efficient in separating the two groups of trypsin-like serine proteases based on their affinity for lectin. Likewise, the amount of protein not bound to Concanavalin A was significantly higher in the extracts of three groups of beetles. In contrast, the bound protein corresponding to the glycosylated protein fraction was relative-

ly low. Taking into consideration the amounts of protein and the proteolytic activity of the glycosylated fractions, the specific proteolytic activity represented 77, 76 and 88% of the total proteolytic activity of the beetles reared on the grain of Bourlag, Kronstad and Villa Juarez, respectively.

Detection of protease activity using zymography

The trypsin-type serine-protease activity was assayed using the synthetic substrate Ba*p*NA (α-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride). Therefore, the combined use of affinity chromatography using Concanavalin A with the substrate Ba*p*NA resulted in the detection of the two fractions, glycosylated and non-glycosylated. Fig. 3 shows a zymogram gel containing the protein bands of the crude extracts and the proteins with trypsin-like activity eluted from the Concanavalin A affinity column. The zymograms of the crude extracts indicated they all had approximately the same protease composition (Fig. 3, A1, B1, and C1), but that of the beetles reared on grain of Villa contained one additional band of around 30 kDa (Fig. 3, A1), which was not present in those reared on that of Kronstad and Borlaug. The proteolytic activity selectively eluted from the column with 0.05 M mannopyranose differed in protein band composition for the different groups of beetles. Those reared on Villa Juarez contained three strong bands of glycosylated proteases of MW around 10, 34 and 58 kDa and traces of several others (Fig. 3, A2). In contrast, the proteases of those reared on the grain of Kronstad and Borlaug that bound to the affinity column were similar, indicating each had a strong area of caseinolytic activity around 10 kDa and traces of activity of an undefined number of proteases in the MW range of 10 to 30 kDa (Fig. 3, B2 and C2).

The serine proteases *Rdo*T1, *Rdo*T2 and *Rdo*T3 of Zhu & Baker (1999) are isoenzymes with very close molecular weights because of their similar and highly conserved amino acid composition. However, not all of the groups of *R. dominica* express the synthesis of these isoenzymes. The results presented indicate the presence of a serine protease with an estimated molecular weight of 34 kDa (a value very close to those corresponding to *Rdo*T1-T3) in the beetles reared on grain of Villa Juarez, which is not present in the other two groups of beetles. Zhu & Baker (1999) possibly studied isoforms of this serine protease
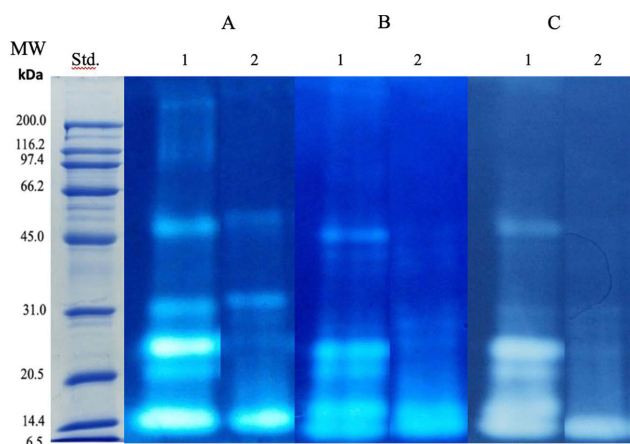
Fig. 3. Zymogram of trypsin-like serine proteases in *Rhyzopertha dominica* reared on grain of the wheat cultivars (A) Villa Juárez, (B) Kronstad and (C) Borlaug. (1) Crude extract and (2) Glycosylated proteases eluted from the Concanavalin A affinity chromatography column.

with very similar molecular weights. To conclude, it would be necessary and interesting to characterize the cDNAs of the trypsin-type serine proteases we recorded, as Zhu & Baker (1999) did with their *Rdo*T1, *Rdo*T2 and *Rdo*T3.

Determination of the inhibition constant K_i

Determination of the equilibrium dissociation constant of the enzyme-inhibitor complex K_i using the graphical procedure of Dixon (Burlingham & Widlanski, 2003) revealed that the glycosylated serine protease fraction of all the groups of beetles had higher K_i values than those of the non-glycosylated forms (Table 1). The higher the K_i, the weaker the binding of the inhibitor to the enzyme, causing the complex to separate readily, leaving the enzyme's active site available and vice versa. The K_i values of the glycosylated proteases of those reared on grain of Bourlag, Kronstad and Villa Juarez were 1.9-, 2.8- and 7.4-fold higher than their non-glycosylated counterparts. In addition, among the glycosylated preparations of the three groups of beetles the K_i values differed, with those reared on grain of Bourlag 46.0- and 7.2-fold higher than those reared on that of Kronstad and Villa Juarez. These results also indicate that the glycosylated and non-glycosylated proteases of those reared on grain of Bourlag are the least susceptible to inhibition by serine protease inhibitors in wheat grains. On the other hand the inhibitory activity of the albumins of the three wheats differ, with that from Bourlag significantly lower than that of Villa Juarez and Kronstad (P < 0.05), based on the corresponding high K_i values of the glycosylated and non-glycosylated serine-protease. The fact that the glycosylated serine proteases have higher K_i values than the non-glycosylated ones might indicate the existence of a mechanism for evading the action of the inhibitors since their interaction is weak according to the highest K_i values obtained. In contrast, the non-glycosylated serine proteases are more susceptible to being inhibited by albumins because of their lower K_i values, which indicates a strong enzyme-inhibitor interaction. Fig. 4 shows the Dixon plots for the glycosylated and non-glycosylated serine proteases.

Table 1. Inhibition constant K_i values of wheat albumins that inhibit the activity of glycosylated and non-glycosylated trypsin-like serine protease isolated from *Rhyzopertha dominica* reared on the grain of three cultivars of wheat.

Wheat albumin[a]	Trypsin-like activity	
	Glycosylated	Non-glycosylated
Bourlag	64.6 ± 0.7[A,a]	33.7 ± 0.04[B,a]
Kronstad	1.4 ± 0.3[A,c]	0.5 ± 0.02[B,c]
Villa Juarez	8.9 ± 0.4[A,b]	1.2 ± 0.03[B,b]

[a] Mean values are expressed in terms of nanograms of inhibitory albumins. Each value is the average of three replicates ± SE. Values followed by different capital letter in the same row are significantly different ($P \leq 0.05$, n = 3). Values followed by different lowercase letter in the same column are significantly different ($P \leq 0.05$, n = 3).

Determination of kinetic parameters

The kinetic parameters of the glycosylated and non-glycosylated fractions from each group of *R. dominica* are listed in Table 2. The substrate concentration K_m is a kinetic parameter that indicates the enzyme affinity for a substrate; that is, lower values indicate a high affinity for the substrate and vice versa. The K_m values of the glycosylated and non-glycosylated forms were not clearly different, i.e., the affinities of the different types of proteases for the BA*p*NA substrate were not be different. In contrast, the parameter V_{max} differed significantly for the glycosylated and non-glycosylated proteolytic activity for all four groups. V_{max} is an enzyme kinetic constant defined as the maximum velocity of an enzymatic reaction. Differences in V_{max} values for the same type of enzymes, as in the case of the glycosylated and non-glycosylated serine proteases from *R. dominica*, indicate differences in the molecular structure of the enzyme. V_{max} of the glycosylated forms was higher in all cases, being 22.3-, 5.6- and 1.5-fold (for Bourlag, Kronstad and Villa Juarez, respectively) more active than their respective non-glycosylated forms. Similarly, the catalytic efficiency estimated as V_{max}/K_m, a rate constant that differentiates the catalytic efficiency between the two types of proteolytic activity, was higher for the glycosylated than the non-glycosylated fractions, independent of the group of beetles. Among the groups, the glycosylated V_{max}/K_m

Table 2. Kinetic parameter values of glycosylated and non-glycosylated trypsin-like serine protease activity recorded for *Rhyzopertha dominica* reared on the grain of three cultivars of wheat.

Wheat	Trypsin-like activity	K_m (nM)	V_{max} (nM min⁻¹)	Catalytic efficiency (V_{max}/K_m)
Bourlag	Glycosylated	0.015[d,A]	67.1[a,A]	4473.3[a,A]
	Non-glycosylated	0.016[d,A]	3.0[e,B]	187.5[e,B]
Kronstad	Glycosylated	0.056[a,A]	65.7[a,A]	1173.2[b,A]
	Non-glycosylated	0.014[d,B]	11.7[d,B]	835.7[c,B]
Villa Juárez	Glycosylated	0.022[c,B]	25.7[b,A]	1168.2[b,A]
	Non-glycosylated	0.038[b,A]	17.0[c,A]	447.4[d,B]

Values are the mean of three replicates. Values followed by a different lower-case letter in the same column for all groups are significantly different ($P \leq 0.05$). Values followed by a different capital letter in the same column between glycosylated and non-glycosylated trypsin-like activity for each group are significantly different ($P \leq 0.05$).

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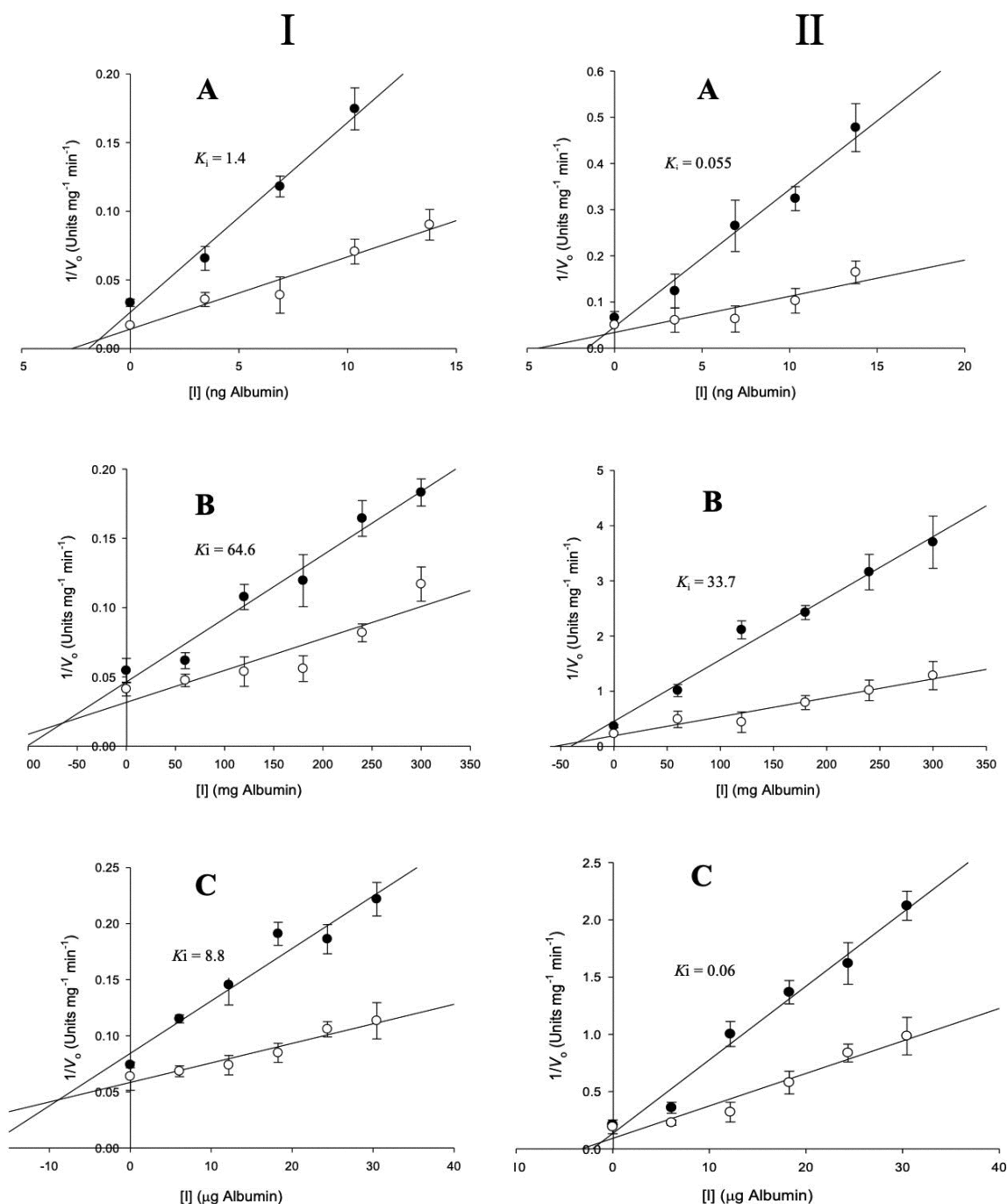
Fig. 4. Dixon diagrams used for the determination of the dissociation constant K_i of the inhibitory extracts of the wheat cultivars (A) Borlaug, (B) Kronstad and (C) Villa Juarez bound to (I) glycosylated and (II) non-glycosylated trypsin-like serine protease isoforms of *Rhyzopertha dominica* reared on the same cultivars of wheat. Two concentrations of N-α-benzoyl-DL-arginine *p*-nitroanilide (Ba*p*Na) were used as substrates: (●) 0.5 mM; (○) 1 mM. Each point is the mean of three replicates ± standard deviation.

values were higher (23.8-, 1.4-, and 2.6-fold for Bourlag, Kronstad and Villa Juarez, respectively) than the non-glycosylated fraction. These results indicate that *R. dominica* uses the cost effective mechanism of enzyme catalytic efficiency in terms of the glycosylation of the serine proteases. The carbohydrate moiety attached to the molecular structures of the glycosylated enzymes makes them less sensitive to enzyme inhibitors and enable them to perform their catalytic enzyme reactions more efficiently. Fig. 5 depicts

the Lineweaver-Burk double reciprocal plots of the glycosylated and non-glycosylated serine proteases extracted.

Determination of activation energy *Ea*

The activation energy E_a, determined by the Arrhenius equation, is the minimum amount of energy required for a chemical reaction and its magnitude depends on the temperature. E_a is a parameter that provides information about the influence of temperature on the reaction rate, in this case, of enzymes. Table 3 lists the E_a of the glycosylat-
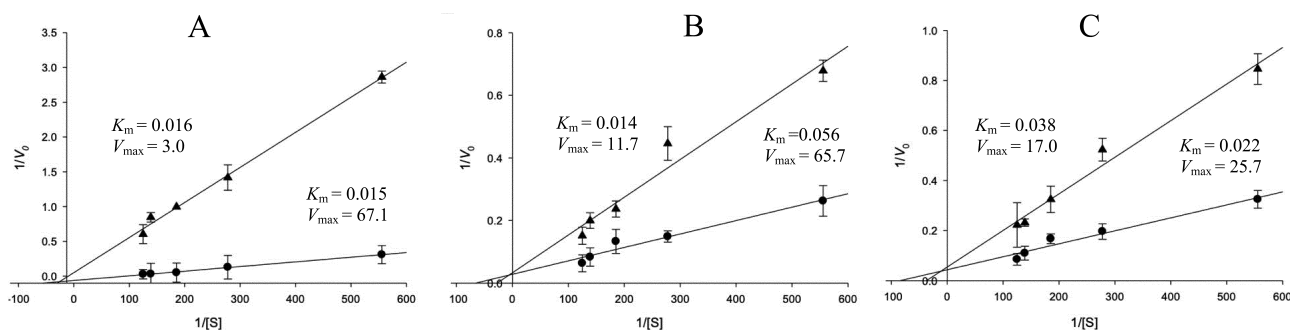
Fig. 5. Lineweaver-Burk double reciprocal plots of the trypsin-like proteolytic activity (●) of glycosylated and (▲) non-glycosylated *Rhyzopertha dominica* reared on the grain of the cultivars of wheat (A) Borlaug, (B) Kronstad, and (C) Villa Juarez.

Table 3. Arrhenius activation energy E_a values for the glycosylated and non-glycosylated trypsin-like serine protease activity recorded for *Rhyzopertha dominica* reared on the grain of three cultivars of wheat.

Reared on grain of	Activation Energy E_a (kJ mol^{-1})	
	Glycosylated	Non-Glycosylated
Bourlag	$28.6 \pm 1.2^{c,A}$	$24.2 \pm 0.5^{a,B}$
Kronstad	$38.3 \pm 0.3^{a,A}$	$20.6 \pm 0.3^{b,B}$
Villa Juarez	$33.6 \pm 0.6^{b,A}$	$11.9 \pm 0.4^{b,B}$

Each value is the average of three replicates ± SE. Values followed by different capital letter in the same row are significantly different ($P \leq 0.05$). Values followed by different lower-case letter in the same column are not significantly different ($P \leq 0.05$).
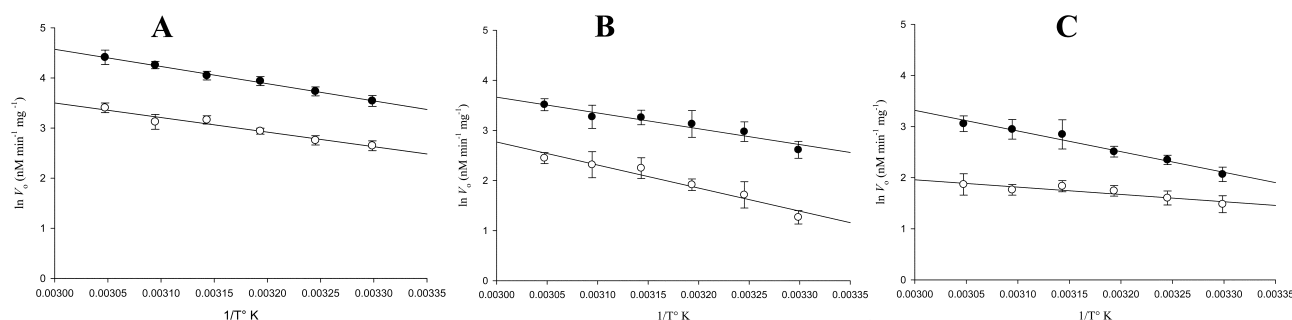
ed trypsin-like activities of Bourlag, Kronstad and Villa Juarez as 15.4, 46.2 and 64.6% higher ($P < 0.05$) than the non-glycosylated, respectively. These values indicate that the glycosylated serine proteases require more energy to generate products in enzymatic reactions than their non-glycosylated counterparts. Furthermore, marginal but significant differences in E_a of the glycosylated proteolytic activity were recorded for the three groups of beetles as well as the non-glycosylated ones ($P < 0.05$), except that for the non-glycosylated fraction of the beetles reared on grain of Villa Juarez, which was nearly half of the E_a values of the non-glycosylated trypsin-like activity of those reared on Bourlag and Kronstad. Despite this, the similarity of the E_a values for the glycosylated and non-glycosylated fractions, indicate that for all three groups of beetles there is a common pattern in activation energy needed for activating their glycosylated serine proteases. Increasing the temperature increases the rate of the enzymatic reaction and in the case of the serine proteases of *R. dominica*, all of them were activated as temperature increased. However, it is speculated that the carbohydrate moiety attached to the molecular structures of the enzyme make it less reactive, requiring more energy to perform the reaction. The Arrhenius plots for the glycosylated and non-glycosylated enzymes are depicted in Fig. 6.

DISCUSSION

This research aimed to isolate the two kinds of serine proteases, glycosylated and non-glycosylated, employing lectin affinity chromatography with Concanavalin A, as it is reported in the literature (Zhu & Baker, 1999) that the amino acid sequences of trypsin-like serine proteases from *R. dominica* contain the consensus sequon Asn-X-Ser/Thr susceptible to *N*-glycosylation. The existence of susceptible *N*-glycosylation sequons was confirmed by searching the amino acid sequences of the proteases of Zhu & Baker (1999) in the peptidase database MEROPS (https://www.ebi.ac.uk/merops/) using the taxonomy database identifier 92692 for *R. dominica* (Rawlings et al., 2018).

One of the proteins' most common post-translational modifications is glycosylation, which makes up at least 50% and as high as 70% of glycosylated proteins in organisms (An et al., 2009). Goettig (2016), citing UniProtKB (https://www.uniprot.org/uniprot/), report that serine proteases form a large group, about 73%, with about 106 glycosylated members out of 144. The isolated protein fraction from the groups of *R. dominica* that bound to the affinity column made up approximately 75–80% of the total serine protease activity detected in crude extracts. Therefore,

Fig. 6. Graphical representation of Arrhenius kinetics of (●) glycosylated and (○) non-glycosylated trypsin-like serine protease activity in *Rhyzopertha dominica* reared on the grain of the wheat cultivars: (A) Bourlag, (B) Kronstad and (C) Villa Juarez.

there was a high probability that *R. dominica* proteases are glycosylated, for which there are to our knowledge no previous reports.

Detection of protease activity using zymography revealed that, despite having almost the same profile of protease bands, the groups of *R. dominica* differed in the number of bands bound to the Concanavalin A column. The zymograms revealed the presence of three main bands for those reared on grain of Villa Juarez and one main band for those reared on that of Kronstad and Bourlag, as previously reported by Zhu & Baker (1999) and confirmed the hypothesis proposed here that *R. dominica* uses mostly glycosylated trypsin-like serine proteases for digesting protein. Based on rearing assays, González-Ruiz et al. (2021) report that the grain of the wheat cultivars Villa Juarez and Bourlag are the most and least infested by *R. dominica*, respectively, while the cultivar Kronstad is moderately infested. Therefore, taking into consideration the studies of Zhu & Baker (1999) and Gonzalez-Ruiz et al. (2021), it might appear that the synthesis and expression of serine proteases with higher *N*-glycosylation sequons is related to the concentration of inhibitory proteins in these cultivars of wheat, such as α-amylase/serine protease inhibitors. However, due to the limited number of samples of wheat cultivars used in this study, this remains to be demonstrated in the future.

Wheat grains contain different protein fractions, among which the non-gluten albumins and globulins comprise a heterogeneous group of the proteins. Albumins are water-soluble monomeric proteins mainly composed of metabolic enzymes and enzyme inhibitors implicated in plant defence, particularly the trypsin/alpha-amylase inhibitors (Cuccioloni et al., 2016). Osuna-Amarillas et al. (2012) report that wheat albumins inhibit the trypsin-like serine protease activity of *R. dominica*. These potent enzyme inhibitors are in the grains that this insect feeds on. The present study was based on the observation of Osuna-Amarillas et al. (2012) that different wheat cultivars differ in serine protease inhibitors. Separating the serine protease inhibitors from wheat grains using size exclusion chromatography allowed a more accurate evaluation of the enzyme: inhibitor i. Because inhibitory activity was highest in these fractions, they were useful for analysing the effect of their inhibitory activity against serine proteases of the three groups of *R. dominica*. Thus, the inhibition recorded was close to that recorded for the proteolytic enzymes of *R. dominica* exposed to the inhibitor-rich fractions of the wheat samples used in this study. This point is interesting because the number of glycosylated isoforms might result from induced expression of genes that overcome the inhibitory activity of trypsin-type serine protease inhibitors in grains of wheat, which compete for the active site of the proteases.

Based on the inhibition constant K_i, there is a mechanism that regulates the kinetic properties of the different forms of proteases in *R. dominica* in the presence of inhibitors. This hypothetical situation would allow the proteases to remain active, which is indicated by the higher K_i values

of the glycosylated forms. This inference is supported by other studies describing the degree of difference between enzymatic activity and inhibitory effect on glutathione s-transferase allozymes and esterase's (Goodrich & Basu, 2012; Julio et al., 2017). The existence of isoforms of serine protease is relevant because they differ in their susceptibility to inhibition by plant inhibitors. The results of this study on the differential inhibition of glycosylated and non-glycosylated serine proteases from *R. dominica* in that the isoforms are susceptible to varying degrees of inhibition by wheat inhibitors, are similar to those of other studies, such as, amylase isoenzymes in *Ephestia kuehniella* (Pytelková et al., 2009) and isoamylases in *R. dominica* (González-Ruiz et al., 2021), which also differ in their susceptibility to inhibition by wheat inhibitors.

Structural changes may affect the interaction of wheat inhibitors with *R. dominica* proteases, but their effects on these relationships remain to be determined. The Dixon graphical method indicated that the inhibition of all of the albumin fractions used in the assay was of the competitive type, as the straight lines intersect to the left of the vertical axis (Burlingham & Widlanski, 2003). However, excess substrate increases the chances of enzyme and substrate binding and overcomes competitive inhibition by allowing proteases with higher K_i values to remain active. A high K_i value means a low affinity between the enzyme and the inhibitor and vice versa.

The kinetic parameter K_m did not differentiate between glycosylated and non-glycosylated enzyme activity. However, V_{max} and the V_{max}/K_m ratio were better indicators for differentiating between the two forms of enzyme activity. It would seem that the number of glycosylated proteases is related to the catalytic efficiency V_{max}/K_m, which was higher for the group reared on grain of Bourlag, which in zymograms had the lowest number of proteases, whereas those reared on that of Villa Juarez and Kronstad had the highest number of proteases. However, no conclusions can be drawn until further studies are carried out, e.g., using genetic engineering to modify the number of glycosylated proteases or the number of *N*-glycosylation sequons to determine whether it alters their catalytic efficiency. These results might indicate that glycosylation of serine proteases in *R. dominica* avoids the tight binding of serine protease inhibitors, which is reflected in the higher K_i and V_{max}/K_m values. The significance of glycosylation in modulating and enhancing catalytic efficiency is well documented for antibacterial glycopeptides (Talat et al., 2011), a highly glycosylated metalloprotease (Chavaroche et al., 2014) and alkaline proteases in *Bacillus stearothermophilus* (Abdel-Naby et al., 2017). Bonzom et al. (2019) report that glycosylated feruloyl esterase in *Myceliophthora thermophila* is 10 times more catalytically active than the non-glycosylated form. This feature of glycosylated protein molecules seems to be a metabolic strategy for increasing their substrate affinity or their biological function.

There is growing evidence that organisms use glycosylation to increase the catalytic efficiency of their enzymes (Taylor et al., 2012; Liebminger et al., 2013). Taylor et

al. (2012) used glycan to engineer glycosylate glycoside hydrolases and report that its enzyme activity was greater than that of the non-glycosylated form. Liebminger et al. (2013) report that the glycoprotein enzyme KOR1, essential for plant cellulose biosynthesis, is less active after de-glycosylation. Therefore, N-glycosylation increases enzymatic activity. This strategy has been used as a model to improve the properties of proteins used as therapeutic agents and the catalytic efficiency of industrial enzymes by glycoengineering (Guan et al., 2018; Ma et al., 2020). Glycosylation, however, does not always increase catalytic efficiency but can have an inhibitory effect or reduce catalytic efficiency (Goettig, 2016), as it decreases glycosylation sites in *Trichoderma reesei* cellobiohydrolase I expressed in *Pichia pastoris* (Siadat et al., 2016) or optimizes glycosylation sites in β-1,4-endoglucanase CTendo45 from *Chaetomium thermophilum* (Han et al., 2020). These reports support the suggestion that glycosylation improves enzymes' catalytic efficiency and, accordingly, it is not unlikely that *R. dominica* uses this strategy to improve its trypsin-like serine proteases used to digest dietary proteins, but it also remains to be determined whether it has an additional role in the metabolism of this insect.

The steep slope in the Arrhenius representation shows that the reaction rate is sensitive to temperature and vice versa (Javed et al., 2008). E_a shift is both physiologically and ecologically relevant for organisms. Activation energy E_a refers to the temperature dependence of physiological traits. Enzyme reactions require energy for their activation and in order to understand the metabolism of organisms one needs to study enzyme kinetics. Activation energy E_a is the minimum energy required for a reaction to occur. That energy comes from food and the environment in which they live influences their metabolism. Glycosylation affects the catalytic properties of certain enzymes by either increasing or decreasing E_a. In the present study, the E_a of glycosylated serine proteases in all groups of *R. dominica* was higher than that of the non-glycosylated isoforms, indicating that enzymatic reactions are very dependent on temperature. Thus, *R. dominica* benefits as temperature increases because the catalytic efficiency of its glycosylated serine proteases increases. Delanghe et al. (1989) report higher E_a values for the enzyme gamma-glutamyltransferase due to the presence of sialic acid in its structure. Also, increased E_a values are reported when synthetic sucrose polymers are attached by site-directed mutagenesis to a thermostable α-amylase from *B. licheniformis* and mesophilic amylase from *B. amyloliquefaciens*, which have similar catalytic efficiencies and higher thermostability than the native enzymes (Srimathi & Jayaraman, 2005). The presence of glycans in the structure of flavonoids confers higher heat stability with higher E_a values than the non-glycosylated ones (Chaaban et al., 2017). In contrast, although glycosylation improves thermal stability and enzyme catalysis it decreases the thermodynamic parameters of α-chymotrypsin (Solá & Griebenow, 2006), the catalytic activity of the cyclodextrin glycosyltransferase from *Bacillus cereus*, decreasing E_a to 3.3, compared to that of the non-glycosylated native

form of 5.62 kcal mol^{-1} (Abdel-Naby et al., 2015). Similarly, the glycosylated keratinase from *Bacillus pumilus* has an E_a of 5.04 compared to the native form of 6.13 kcal mol^{-1} (Abdel-Naby et al., 2016).

CONCLUSIONS

In this study, the activity of the trypsin-like serine protease in the beetle *R. dominica* was dependent on whether it was glycosylated or non-glycosylated according to its affinity for the lectin Concanavalin A. Zymography revealed that the weevils reared on the grain of different wheat cultivars contained a variable number of glycosylated proteases that had higher catalytic efficiencies and higher K_i values, which means they are less likely to form complexes with wheat grain protease inhibitors, and have higher activation energy E_a values than the non-glycosylated serine proteases. Interestingly, glycosylation improves the kinetic performance of *R. dominica* serine proteases. The total number of proteases was similar in all groups of weevils. However, the number of glycosylated proteases that were selectively separated by affinity chromatography using Concanavalin A differed. In addition, the non-glycosylated proteases are not the same as the glycosylated ones, which leads to the conclusion that *R. dominica* has the ability to activate genes that express the synthesis of glycosylated proteases that improve their ability to digest dietary proteins and neutralize the protease inhibitors present in their diet.

REFERENCES

Abdel-Naby M.A., Fouad A.A. & El-Refai H.A. 2015: Catalytic and thermodynamic properties of glycosylated *Bacillus*

cereus cyclodextrin glycosyltransferase. — *Int. J. Biol. Macromol.* **76**: 132–137.

ABDEL-NABY M.A., IBRAHIM M.H. & EL-REFAI H.A. 2016: Catalytic, kinetic and thermodynamic properties of *Bacillus pumilus* FH9 keratinase conjugated with activated pectin. — *Int. J. Biol. Macromol.* **85**: 238–245.

ABDEL-NABY M.A., AHMED S.A., WEHAIDY H.R. & EL-MAHDY S.A. 2017: Catalytic, kinetic, and thermodynamic properties of stabilized *Bacillus stearothermophilus* alkaline protease. — *Int. J. Biol. Macromol.* **96**: 265–271.

AN H.J., FROEHLICH J.W. & LEBRILLA C.B. 2009: Determination of glycosylation sites and site-specific heterogeneity in glycoproteins. — *Curr. Opin. Chem. Biol.* **13**: 421–426.

BARRETT A.J., RAWLINGS N.D. & WOESSNER J.F. 2012: *Handbook of Proteolytic Enzymes. 3rd ed.* Elsevier Academic Press, San Diego, CA, 984 pp.

BAUSE E. 1984: Model studies on N-glycosylation of proteins. — *Biochem. Soc. Trans.* **12**: 514–517.

BONZOM C., HÜTTNER S., MIRGORODSKAYA E., CHONG S.-L., UTHOFF S., STEINBÜCHEL A., VERHAERT R.M.D. & OLSSON L. 2019: Glycosylation influences activity, stability, and immobilization of the feruloyl esterase 1a from *Myceliophthora thermophila*. — *AMB Express* **9**(126): 13 pp.

BRADFORD M. 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. — *Anal. Biochem.* **72**: 248–254.

BURLINGHAM B.T. & WIDLANSKI T.S. 2003: An intuitive look at the relationship of Ki and IC50: a more general use for the Dixon plot. — *J. Chem. Educ.* **80**: 214–218.

CHAABAN H., IOANNOU I., CHEBIL L., SLIMANE M., GÉRARDIN C., PARIS C., CHARBONNEL C., CHEKIR L. & GHOUL M. 2017: Effect of heat processing on thermal stability and antioxidant activity of six flavonoids. — *J. Food Process. Preserv.* **41**: e13203, 12 pp.

CHAVAROCHE A., CUDI M., GIULIANOTTI M., HOUGHTEN R.A., FIELDS G.B. & MINOND D. 2014: Glycosylation of a disintegrin and metalloprotease 17 (ADAM17) affects its activity and inhibition. — *Anal. Biochem.* **449**: 68–75.

CHEN H., ZHU Y.C., WHITWORTH R.J., REESE J.C. & CHEN M.S. 2013: Serine and cysteine protease-like genes in the genome of a gall midge and their interactions with host plant genotypes. — *Insect Biochem. Mol. Biol.* **43**: 701–711.

CINCO-MOROYOQUI F.J., DÍAZ-MALVÁEZ F.I., ALANÍS-VILLA A., BARRÓN-HOYOS J.M., CÁRDENAS-LÓPEZ J.L., CORTEZ-ROCHA M.O. & WONG-CORRAL F.J. 2008: Isolation and partial characterization of three isoamylases of *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae). — *Comp. Biochem. Physiol. (B)* **150**: 153–160.

CUCCIOLONI M., MOZZICAFREDDO M., ALI I., BONFILI L., CECARINI V., ELEUTERI A.M. & ANGELETTI M. 2016: Interaction between wheat alpha-amylase/trypsin bi-functional inhibitor and mammalian digestive enzymes: Kinetic, equilibrium and structural characterization of binding. — *Food Chem.* **213**: 571–578.

DELANGHE J.R., DE BUYZERE M.L., DE SCHEERDER I.K., FAUST U., ROGER J. & WIEME R.J. 1989: Activation energy and lectin affinity chromatography of gamma-glutamyltransferase as a marker for enzyme heterogeneity. — *Clin. Biochem.* **22**: 115–119.

EDDE P. 2012: A review of the biology and control of *Rhyzopertha dominica* (F.), the lesser grain borer. — *J. Stored Prod. Res.* **48**: 1–18.

GAVEL Y. & VON HEIJNE G. 1990: Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. — *Protein Eng.* **3**: 433–442.

GHOLAMZADEH C.M., GHADAMYARI M. & SHARAFI M. 2013: Identification and characterization of gut proteases in the fig tree skeletonizer moth, *Choreutis memoranda* Hübner (Lepidoptera: Choreutidae). — *Plant Protect. Sci.* **46**: 19–26.

GOETTIG P. 2016: Effects of glycosylation on the enzymatic activity and mechanisms of proteases. — *Int. J. Mol. Sci.* **17**(12): 1969, 24 pp.

GONZÁLEZ-RUIZ C.R., DEL-TORO-SÁNCHEZ C.L., CORNEJO-RAMÍREZ Y.I., RODRÍGUEZ-FÉLIX F., WONG-CORRAL F.J., MÁRQUEZ-RÍOS R., CÁRDENAS-LÓPEZ J.L. & FRANCISCO J. CINCO-MOROYOQUI 2021: Differential biochemical and kinetic properties of α-amylases from *Rhyzopertha dominica* (F.) progenies reared on wheat varieties differing in α-amylase inhibitory activity. — *J. Stored Prod. Res.* **90**: 101748, 9 pp.

GOODRICH J.M. & BASU N. 2012: Variants of glutathione s-transferase pi 1 exhibit differential enzymatic activity and inhibition by heavy metals. — *Toxicol. In Vitro* **26**: 630–635.

GUAN X., CHAFFEY P.K., WEI X., GULBRANSON D.R., RUAN Y., WANG X., LI Y., OUYANG Y., CHEN L., ZENG C. & ET. AL. 2018: Chemically precise glycoengineering improves human insulin. — *ACS Chem. Biol.* **13**: 73–81.

HAN C., WANG Q., SUN Y., RUIRUI Y., LIU M., WANG S., LIU Y., ZHOU L. & LI D. 2020: Improvement of the catalytic activity and thermostability of a hyperthermostable endoglucanase by optimizing N-glycosylation sites. — *Biotechnol. Biofuels* **13**: 30, 11 pp.

HEUSSEN C. & DOWDLE E.B. 1980: Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. — *Anal. Biochem.* **102**: 196–202.

JAVED M.R., RASHID M.H., NADEEM H., RIAZ M. & PERVEEN R. 2008: Catalytic and thermodynamic characterization of endoglucanase (CMCase) from *Aspergillus oryzae* cmc-1. — *Appl. Biochem. Biotechnol.* **157**: 483–497.

JULIO A.H.F., GIGLIOLLI A.A.S., CARDOSO K.A.K., DROSDOSKI S.D., KULZA R.A., SEIXAS F.A.V., RUVOLO-TAKASUSUKI M.C.C., DE SOUZA C.G.M. & LAPENTA A.S. 2017: Multiple resistance to pirimiphos-methyl and bifenthrin in *Tribolium castaneum* involves the activity of lipases, esterases, and laccase2. — *Comp. Biochem. Physiol. (C)* **195**: 7–43.

KONAREV A. 1996: Interaction of insect digestive enzymes with plant protein inhibitors and host-parasite coevolution. — *Euphytica* **92**: 89–94.

LAEMMLI U.K. 1970: Cleavage of structural proteins during the assembly of the head bacteriophage T4. — *Nature* **22**: 77–80.

LAM P.V.N., GOLDMAN R., KARAGIANNIS K., NARSULE T., SIMONYSN V., DOIKS V. & MAZUMDER R. 2013: Structured-based comparative analysis and prediction of N-linked glycosylation sites in evolutionarily distant eukaryotes. — *Genom. Proteom. Bioinf.* **11**: 96–104.

LIEBMINGER E., GRASS J., ALTMANN F., MACH L. & STRASSER R. 2013: Characterizing the link between glycosylation state and enzymatic activity of the endo-b1,4-glucanase KORRIGAN1 from *Arabidopsis thaliana*. — *J. Biol. Chem.* **288**: 22270–22280.

LÓPEZ-OTÍN C. & BOND J. 2008: Proteases: multifunctional enzymes in life and disease. — *J. Biol. Chem.* **283**: 30433–30437.

MA B., GUAN X., LI Y., SHANG S., LI J. & TAN Z. 2020: Protein glycoengineering: An approach for improving protein properties. — *Front. Chem.* **8**: 622, 14 pp.

MAHBOBE S., MOLOUD G., MOHAMMAD G. & MARYAM A. 2012: Identification and characterization of midgut digestive proteases from the rosaceous branch borer, *Osphranteria coerule-*

scens Redtenbacher (Coleoptera: Cerambycidae). — *Rom. J. Biochem.* **49**: 33–47.

Mehrabadi M., Bandani A.R., Saadati F. & Mahmudvand M. 2011: α-Amylase activity of stored products insects and its inhibition by medicinal plant extracts. — *J. Agr. Sci. Tech.* **13**: 173–182.

Nagai K., Ihara Y., Wada Y. & Taniguchi N. 1997: N-glycosylation is requisite for enzyme activity and Golgi retention of N-acetylglucosaminyltransferase III. — *Glycobiology* **7**: 767–776.

Oppert B. & Morgan T.D. 2013: Improved high-throughput bioassay for *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae). — *J. Stored Prod. Res.* **52**: 68–73.

Oppert B., Elpidina E.N., Toutges M. & Mazumdar-Leighton S. 2010: Microarray analysis reveals strategies of *Tribolium castaneum* larvae to compensate for cysteine and serine protease inhibitors. — *Comp. Biochem. Physiol. (D)* **5**: 280–287.

Osuna-Amarillas P.S., Cinco-Moroyoqui F.J., Cárdenas-López J.L., Ezquerra-Brauer J.M., Sotelo-Mundo R., Cortez-Rocha M.O., Barrón-Hoyos J.M., Rouzaud-Sández O. & Borboa-Flores J. 2012: Biochemical and kinetic characterization of the digestive trypsin-like activity of the lesser grain borer *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae). — *J. Stored Prod. Res.* **51**: 41–48.

Priya S., Kaur N., Anil K. & Gupta A.K. 2010: Purification, characterization, and inhibition studies of α-amylase of *Rhyzopertha dominica*. — *Pestic. Biochem. Physiol.* **98**: 231–237.

Pytelková J., Hubert J., Lepšík M., Šobotník J., Šindelka R., Křížková I., Horn M. & Mareš M. 2009: Digestive alpha-amylases of the flour moth *Ephestia kuehniella* – adaptation to alkaline environment and plant inhibitors. — *FEBS J.* **276**: 3531–3546.

Rawlings N.D., Barrett A.J., Thomas P.D., Huang X., Bateman A. & Finn R.D. 2018: The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. — *Nucl. Acids Res.* **46**: D624–D632.

Rendić D., Wilson I.B.H. & Paschinger K. 2008: The glycosylation capacity of cells. — *Croat. Chem. Acta* **82**: 7–21.

Roth Z., Yehezkel G. & Khalaila I. 2012: Identification and quantification of protein glycosylation. — *Int. J. Carbohydr. Chem.* **2012**: 640923, 11 pp.

SAS Institute 2005: *PROC User's Manual, 8th Ver.* SAS Institute, Cary, NC.

Siadat S.O.R., Mollasalehi H. & Heydarzadeh N. 2016: Substrate affinity and catalytic efficiency are improved by decreasing glycosylation sites in *Trichoderma reesei* cellobiohydrolase I expressed in *Pichia pastoris*. — *Biotechnol. Lett.* **38**: 483–488.

Silva C.P., Terra W.R., de Sá M.F.G., Samuels R.I., Isejima E.M., Bifano T.D. & Almeida J.S. 2001: Induction of digestive α-amylases in larvae of *Zabrotes subfasciatus* (Coleoptera:

Bruchidae) in response to ingestion of common bean α-amylase inhibitor 1. — *J. Insect Physiol.* **47**: 1283–1290.

Solá R.J. & Griebenow K. 2006: Chemical glycosylation: New insights on the interrelation between protein structural mobility, thermodynamic stability, and catalysis. — *FEBS Lett.* **580**: 1685–1690.

Sotelo-Mundo R.R., López-Zavala A.A., García-Orozco K.D., Arvizu-Flores A.A, Velázquez-Contreras E.F., Valenzuela-Soto E.M., Rojo-Domínguez A. & Kanost M.R. 2007: The lysozyme from insect (*Manduca sexta*) is a cold-adapted enzyme. — *Protein Pept. Lett.* **14**: 774–778.

Spiro R. 2002: Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptides bonds. — *Glycobiology* **12**: 43R–56R.

Srimathi S. & Jayaraman G. 2005: Effect of glycosylation on the catalytic and conformational stability of homologous α-amylases. — *Protein J.* **24**: 79–88.

Talat S., Thiruvikraman M., Kumari S. & Kaur K.J. 2011: Glycosylated analogs of formaecin I and drosocin exhibit differential pattern of antibacterial activity. — *Glycoconj. J.* **28**: 537–555.

Taylor C.B., Talib M.F., McCabe C., Bu L., Adney W.S., Himmel M.E., Crowley M.F. & Beckham G.T. 2012: Computational investigation of glycosylation effects on a family 1 carbohydrate-binding module. — *J. Biol. Chem.* **287**: 3147–3155.

Vandenborre G., Smagghe G., Ghesquière B., Menschaert G., Nagender R., Gevaert K. & Van Damm J. 2011: Diversity in protein glycosylation among insect species. — *PLoS ONE* **6**(2): e16682, 9 pp.

Wei Y.D., Lee K.S., Gui Z.Z., Yoon H.J., Kim I., Je Y.H., Lee S.M., Zhang G.Z., Guo X., Sohn H.D. & Jin B.R. 2006: N-linked glycosylation of a beetle (*Apriona germani*) cellulose Ag-EGase II is necessary for enzymatic activity. — *Insect Biochem. Mol. Biol.* **36**: 435–441.

Wielkopolan B., Walczak F., Podlesny A., Nawrot R. & Obrepalska-Steplowska A. 2015: Identification and partial characterization of proteases in the cereal leaf beetle (*Oulema melanopus*, Chrysomelidae, Coleoptera). — *Arch. Insect Biochem. Physiol.* **88**: 192–202.

Zhang Z., Chen Y., Tang Y. & Sheng Z. 2008: Prediction of mucin-type *O*-glycosylation sites in mammalian proteins using the composition of *k*-spaced amino acids pairs. — *BCM Bioinform.* **9**: 101, 12 pp.

Zhu Y.-C. & Baker J. 1999: Characterization of midgut trypsin-like enzymes and three trypsinogen cDNAs from the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). — *Insect Biochem. Mol. Biol.* **29**: 1053–1063.

Zhu-Salzman K. & Zeng R. 2015: Insect response to plant defensive protease inhibitors. — *Annu. Rev. Entomol.* **60**: 233–252.