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

**FERNANDA S. ZAVALA-IBARRA**¹, **ALDO A. ARVIZU-FLORES**²*, **OLIVIERT MARTÍNEZ-CRUZ**⁴, **PABLO S. OSUNA-AMARILLAS**³, **JOSÉ L. CÁRDENAS-LÓPEZ**⁴, **CARMEN L. DEL-TORO-SÁNCHEZ**¹, **CARLOS R. GONZÁLEZ-RUIZ**⁴, **JOSÉ A. TAPIA-HERNÁNDEZ**³, **REY D. ITURRALDE-GARCÍA**¹ and **FRANCISCO J. CINCO-MOROYOQUI**¹*, *

¹ Departamento de Investigación y Posgrado en Alimentos, Grupo de Investigación en Bioquímica, Química Agrícola y Manejo Postcosecha (BioQAMPO), Universidad de Sonora, 83000 Hermosillo, Sonora, México; e-mails: a219230159@unison.mx, oliviert.martinez@unison.mx, joseluis.cardenas@unison.mx, carmen.deltoro@unison.mx, joseaugustin.tapia@unison.mx, rey.iturralde@unison.mx, javier.cinco@unison.mx

² Departamento de Ciencias Químico-Biológicas, Universidad de Sonora, Blvd. Luis Encinas y Blvd. Rosales, Hermosillo, Sonora 83000, México; e-mail: aldo.arvizu@unison.mx

³ Universidad Estatal de Sonora, Carretera Navojoa-Huatabampo km 5, Navojoa, Sonora 85874, México; e-mail: pablo.osuna@ues.mx

⁴ Instituto Potosino de Investigación Científica y Tecnológica A.C., Camino a la Presa de San José 2055, Lomas 4ta Sección, 78216 San Luis, S.L.P., México; e-mail: carlos.gonzalez@ipicyt.edu.mx

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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 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 Acrithosiphon pismum indicate that the effect is species specific (Vandenborre et al., 2011). Of these insects, A. pismum, D. melanogaster and T. castaneum use glycosylated proteases to digest proteins. Similarly, Wei et al. (2006) report that the enzymatic activity of fungal proteases 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; Rendic 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 RdoT1 contains one sequon susceptible to N-glycosylation (Ser103-Asp104,Asn105), RdoT2 one (Asn196-Tyr206, Thr207), and RdoT3 three (Asn251-Asp252-Asn253, Thr254). According to Gavel & von Heijne (1990) and Bause (1984), RdoT1 is much less likely to be glycosylated than RdoT2 and RdoT3 as the incorporation 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, Cjd. Obregon, Son., Mexico, 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 CaCl2. 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 (Affi Gel Con-A, BioRad Laboratories, Richmond, CA, USA) and equilibrated with 20 mM Tris-HCl, pH 7.4, containing 5 mM MnCl2 and 10 mM MgCl2. 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 MnCl2, 10 mM MgCl2 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).
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 × 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.

Determination of protein content

The protein content of the proteolytic extracts of the adults and the chromatographic fractions 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 polyacrylamide gel containing co-polymerized 0.27% (w/v) casein (Heusser & 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.

Determination of kinetic parameters

The glycosylated and non-glycosylated enzymes 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 $V_{	ext{max}}$ using BAPNA in the range of 0.1 to 0.5 mM and combined with two units of BAPNAase 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 $V_{	ext{max}}$ were obtained by fitting the observed values of $V_e$ versus the substrate concentration to the Michaelis-Menten equation using non-linear correlation. The $V_{	ext{max}}/K_m$ 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.

Detection of trypsin-like activity in the crude extract

The glycosylated and non-glycosylated proteases 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 $V_{	ext{max}}$ using BAPNA in the range of 0.1 to 0.5 mM and combined with two units of BAPNAase 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 $V_{	ext{max}}$ were obtained by fitting the observed values of $V_e$ versus the substrate concentration to the Michaelis-Menten equation using non-linear correlation. The $V_{	ext{max}}/K_m$ 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.
Chromatography with Concanavalin A

Purification of proteases by 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), (Burlingham & Widlanski, 2003) and two fixed substrate concentrations (0.07 M and 0.115 M Ba(p)) 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/V₀ against the albumin concentrations. The intersection of the lines obtained was used to calculate the dissociation equilibrium constant of the enzyme-inhibitor complex, Kᵢ.

**Determination of activation energy Eₐ**

The activation energy (Eₐ) 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 V₀ versus T⁻¹ and using the equation:

$$\ln V_0 = \ln A - \frac{E_a}{RT}$$

where Eₐ 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 ≤ 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 relatively 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 BapNA 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 mannopyranosid—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 *RdoT1*, *RdoT2* and *RdoT3* 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 *RdoT1-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.
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 RdoT1, RdoT2 and RdoT3.

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 those of 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.

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 BApNA 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 $V_{max}/K_m$ values are the mean of three replicates. Values followed by different lowercase letter in the same column are significantly different ($P \leq 0.05, n = 3$).
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 $E_a$**

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-
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.
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*<sub>i</sub> 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*<sub>i</sub> 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 pathogens, 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*<sub>i</sub> values to remain active. A high *K*<sub>i</sub> value means a low affinity between the enzyme and the inhibitor and vice versa.

The kinetic parameter *K*<sub>m</sub> did not differentiate between glycosylated and non-glycosylated enzyme activity. However, *V*<sub>max</sub> and the *V*<sub>max</sub>/*K*<sub>m</sub> 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*<sub>max</sub>/*K*<sub>m</sub>, 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*<sub>i</sub> and *V*<sub>max</sub>/*K*<sub>m</sub> 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; Liebninger 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 cellulohydrolase 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 $E_a$ reflects 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 (Chaabhan 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 cyclohexatin 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.

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