ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Characterization of Proteinases of the Colorado Potato Beetle and Their Inhibitors From Solanaceae Plants.

Ibragimov, RI[1]*, Tsvetkov, VO[1] , Shpirnaya IA[1], Mardanshin IS[2], Valiakhmetova KI[1], and Yarullina LG[1,3].

[1]Bashkir State University, 450076, Russia, City of Ufa, Validi st., 32.
[2]Bashkir Research Institute of Agriculture of RAS, 450059, Russia, City of Ufa, Zorge st., 19
[3]Institute of Biochemistry and Genetics USC RAS, 450054, Russia, City of Ufa, Oktyabrya av., 71.

ABSTRACT

The article investigated the activity and the molecular composition of proteinases of the Colorado potato beetle (*Leptinotarsa decemlineata* Say) and their inhibitors from the leaves of solanaceous plants. In the tissues of larvae 3 low-molecular and 5 high-molecular proteins hydrolizing gelatin was identified. Inhibitors of these proteinases in the potato leaves contain 5 low-molecular-weight forms. Feeding plants of different species changes the insects' enzymes activity, but not the molecular composition.

Keywords: potato, Colorado potato beetle, necrosis, food activity, proteases, protease inhibitors.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Colorado potato beetle Leptinotarsa decemlineata Say (Coleoptera, Chrysomelidae) is one of the most dangerous pests of solanaceous crops, primarily potatoes. This kind of insects characterized by high ecological plasticity and accommodate ability that allows him successfully adapt to biotic and abiotic factors, including anthropogenic influences [1]. So the Colorado potato beetle is rapidly gaining resistance to pesticides used against him: insects have become resistant to the most used insecticides in all habitats [2, 3]. A real solution for the protection of potatoes from the Colorado potato beetle, with minimal chemical impact on the environment, is changing its food base through the using of new potato varieties that reduce the total level of adaptability and surviving of the insect.

Currently the system "phytophagon – host plant" is regarded as the result of processes of co-adaptations and co-evolution of animals-olygophagons and forage plants. As is known, the formation of the species L. decemlienata occurred without his interaction with Solanum tuberosum, as the main forage insects began to use potato plants relatively recently, since the beginning of the eighteenth century [4]. Therefore, we can assume that in the modern agro-ecosystems in populations of insects should implement intensive microevolutionary processes, primarily processes of the physiological and biochemical co-adaptations of the system "Colorado potato beetle and cultural potato" components.

In the leaves of potato discovered chemical compounds that provide toxic effect on insects and reduce the digestibility of plant foods: steroid glycoalkaloids, methyl ketones, phenols, nicotine [5-8]. Hydrolytic enzymes of phytophage and their inhibitors from plants play a major role in the successful implementation of insects feeding [9-12]. So, inhibiting the processes of proteolysis in the digestive system, the inhibitors reduce the intake of amino acids, including irreplaceable, in the body of an insect. The presence in plant foods several inhibitors of proteinases significantly reduces insect food activity [13- 15].

Evidence of the protective function of inhibitors of proteinases were first submitted in the work of S. Ryan with the co-authors [16] when the damage to the plants of potato Colorado potato beetle caused the activation or synthesis "de novo" of protective molecules, including proteins (peptides), inhibit the activity of proteinases. Moreover, the damage of even a single leaf causes activation of proteinases inhibitors throughout the plant organism [16, 17]. There was shown, that mechanical wounding of potato leaves stimulates transcription of genes of protein inhibitors of cysteine and aspartyl proteases [18]. The information obtained that in the leaves of potato activating of inhibitors of proteinases genes occurs with the participation of jasmonate signaling pathways [19].

Meanwhile, the consumption of plant foods with a high content of inhibitors evokes in the body of insect responses that lead to changes in its metabolism. Thus, the addition to food of low molecular weight inhibitor of cysteine proteases (E-64), led to a significant delay in growth of larvae of the Colorado potato beetle and other representatives of Coleoptera [20, 21]. Feeding by larvae the potato leaves with a high content of a specific inhibitors of digestive proteases stimulates the synthesis of other enzymes, non-specific to this inhibitor, in the gut of insects [22-24]. It can be assumed, that the qualitative and quantitative biochemical parameters of host plants will have an impact not only on food activity, but also to other physiological reactions of the phytophagon, in particular, on the implementation of the processes of reproduction.

The present work is devoted to the study of physico-chemical properties of the proteinases of the Colorado potato beetle and their inhibitors from potato, and influence of species and varietal specialties of plants on biochemical and parameters of insects.

EXPERIMENTAL PART

The objects of study were adults and larvae of the Colorado potato beetle (Leptinotarsa decemlineata Say), vegetative plants of potato (Solanum tuberosum L.), tomato (Solanum lycopersicum L.) and eggplant (Solanum melongena L.). Insects were collected from different local populations in the southern Urals. In experiments were used potato varieties characterized by different resistance to the Colorado beetle: Bashkirskiy is a relatively highly resistant (7 points, the damage is less than 20 % of leaf surface), Udacha – moderately resistant (5 points, damage 26-49 % of leaf surface); Nevsky – unstable (2 points, damage 50-80 %

of leaf surface). The resistance of the plants was assessed visually [25] in terms of production plantations of potatoes in northern steppe zone of the southern Urals).

An affine purification of enzymes and inhibitors

The expression of enzymes and inhibitors was performed by the method of affine chromatography with the use of the sorbent on the basis of polyacrylamide gel [26]. As a ligand in the purification of proteinases was used gelatin, in the purification of inhibitors – affine-purified on gelatin proteinases of the Colorado potato beetle.

To obtain an affine sorbent used polyacrylamide gel of high concentration with a high degree of crosslinking. Acrylamide (1.92 g) with methylene-bis-acrylamide (1.28 g) was adjusted with a buffer solution (acetate buffer, pH 5.6) to 20 ml and dissolved in heating. Separately dissolved the ligand in buffer to obtain a final concentration of 0.1%.

To a polyacrylamide solution with stirring was added TEMED and APS, thereafter added ligand solution and left to stand for half an hour for polymerization. The resulting gel was homogenized with knife homogenizer, selected fine fraction and left to stand in 1.6 M carbonate buffer (pH 9) with 0.5% glutaric aldehyde at 37 °C for 10 hours for the formation of covalent crosslinks between polyacrylamide and ligang. The sorbent was then transferred to the Tris-HCl buffer solution, was introduced into the column of size 10x2 cm and balanced with buffer.

The expression of enzymes and their inhibitors was performed on low pressure liquid chromatograph BioLogic LP (Biorad, USA) at 4 °C. The elution speed was 2 ml/min.

Separation of hydrolases and their inhibitors by the method of two-dimensional and one-dimensional electrophoresis

To determine the values of isoelectric points and molecular masses of proteins we used method of denaturing two-dimensional electrophoresis in polyacrylamide gel. Proteins (enzymes or inhibitors) was precipitated with twice volume of acetone for 10 hours at -20 °C. The precipitate was washed twice with acetone, were resuspended in buffer (0.7 M sucrose, 0.5 HEPES-KOH (pH 7.5), 0.1 M KCl, 2% mercaptoethanol, 1 mm EGTA, 1 mm PMSF, 0.1 mm sodium orthovanadate) and incubated for 30 minutes at 4 °C. Thereafter, proteins were extracted with phenol according to the method described in [27]. To 1 ml of protein solution was added 2 ml of phenol saturated with Tris-HCl, the resulting mixture was incubated at -20 °C for 30 minutes, then centrifuged for 30 minutes at 20000 g. Proteins from the phenol phase was precipitated in four volumes of 0.1 M ammonium acetate in ethanol at -20 °C for 10 hours.

The resulting precipitate was washed three times with ammonium acetate and dissolved in lysis buffer solution (8M urea, 2M thiourea, 1% CHAPS, 30 mm DTT, 20 mm Tris, 0.3% solution of ampholytes (pH 3-10).

Isoelectrofocusing proteins was performed on the Protean IEF device (Biorad, USA). For protein separation in the first direction (isoelectric point) used ready 7 cm strips (Biorad, USA), pH range 3-10. Before focusing was performed passive rehydration for 12 hours at 20 °C. Focusing used a voltage of 4000 V for 22 hours, then maintained a voltage of 500 V to the end of the process. After isoelectrofocusing strips stand for 15 minutes consecutively in solutions of 2% dithiothreitol and 2.5% iodoacetamide in buffer solution with 25% glycerol, then washed in 0.025 M Tris-glycine buffer, pH 8.3.

To separate proteins by molecular weight was performed SDS-electrophoresis in 10% page by Laemmli (concentration in the concentrating gel – 4 %). Strip and the marker proteins on filter paper was placed on a polyacrylamide gel and shed with 1% agarose in Tris-glycine buffer solution. Electrophoresis was carried out at the 90-120 V using the power supply "Elf" (DNA-technology, Russia) in the camera for vertical electrophoresis (Biorad, USA), gel then stabilized in 50% ethanol for 10 minutes, then stained with 0.1% solution of Coumassie G-250 for 10 hours at room temperature. Gel were washed from the dye in a solution containing 8% acetic acid and 25% ethanol.

ISSN: 0975-8585

Evaluation of the activity of proteinases and their inhibitors

Activity of proteolytic enzymes of insects and their inhibitors in plant leaves was determined by the rate of hydrolysis of gelatin immobilized in the agarose gel plate [28].

The activity of the enzyme and the inhibitor was calculated by the size of the area of the gel with hydrolyzed substrate. As 1 activity unit (U) was adopted such activity, which causes the hydrolysis of the substrate in the 1 sq. mm of the gel. To measure the activity of inhibitors in the hole was placed a mixture of the enzyme and inhibition. Inhibitor activity was determined as the difference of the squares of the hydrolyzed area on gel with a free enzyme and the enzyme with the inhibitor.

Statistical processing of the results

Statistical processing of experimental results, including the calculation of mean values, standard deviation and confidence interval, regression analysis and determination of reliability of differences between sample means was performed in a computer program MS Excel. Experiments with quantitative definitions was repeated not less than four times for experimental and control samples. In the tables and charts are shown the average values of the investigated variables and the values of the confidence interval of the sample mean.

RESULTS AND DISCUSSION

In fig. 1 shown the results of separation by two-dimensional electrophoresis of *L. decemlineata* larvae proteinases and inhibitors of these enzymes from the potato leaves. The image shows that the studied enzymes of insects represented by two heterogeneous groups of proteins that differ in molecular weight. The group of low molecular weight proteinases (M of about 25 kDa) consists of three components, distinguished by the values of isoelectric points. The group of relatively high molecular weight proteinases represented by 5 components with molecular weights of about 70 kDa. The values of the isoelectric points of low molecular weight and high molecular weight proteases are in a weakly acidic region of pH, in the range of from 5 to 6. Judging by the values of molecular masses and isoelectric points, the group of high molecular weight proteinases probably represent the various forms of cathepsin-like proteinases. Representatives of this type of enzymes are widely distributed in the digestive system of insects and is responsible for the hydrolysis of the main leaf tissue proteins, such as RUBISCO [29, 30].

Low molecular weight proteinases of larvae may represent isoforms of trypsin-like and chymotrypsin-like proteinases found in the gut and hemolymph of insect larvae [31 - 33]. They can carry out the hydrolysis of proteins both in the intestine and in the hemolymph of insects. It is shown that some plant proteins are not completely hydrolyzed in the midgut of insects: the peptides can be transported through the intestinal wall into the hemolymph and be subjected to hydrolysis [34].

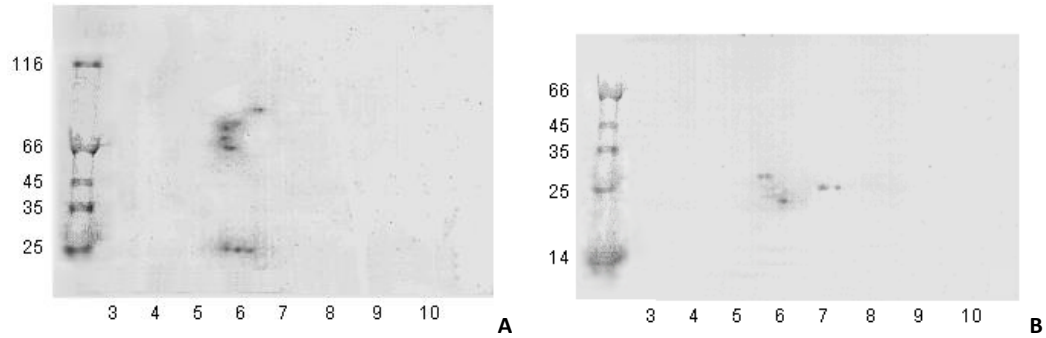

Figure 1: Division of proteinases of the Colorado potato beetle larvae and their inhibitors from potato leaves by two-dimensional electrophoresis in 10% SDS-PAGE. (A) Proteins of larvae (4 stage of development), purified on a column with immobilized gelatin. (B) Proteins of potato leaves (variety Nevsky), purified on a column with immobilized proteinases of larvae. Horizontally indicated the pH, vertically – molecular weight, kDa.

Inhibitors of *L. decemlineata* proteinases in potato leaves submitted 5 low-molecular-weight forms with the values of molecular masses of about 25-30 kDa (Fig. 1, B). The values of the isoelectric points of these

ISSN: 0975-8585

proteins are weakly acidic and neutral regions (pH 5.5 – 7.5). These molecules may represent inhibitors of Kunitz-like serine proteases and inhibitors of cathepsin-like enzymes. These types of inhibitors are widely represented in solanaceous plants, including potato [35 - 38].

One of the mechanisms of adaptation of insects to plant substrates with different chemical composition is the high plasticity of the digestive system. According to our data, feeding by larvae of the Colorado potato beetle leaves of plants of different species and varieties leads to changes in the level of proteolytic activity (Fig. 2). Therefore, feeding by insect larvae leaves of potato or eggplant increases the activity of gelatinolytic enzymes, comparing to starving insects, by 10-40 %. Eating leaves tomatoes, on the contrary, decreases the activity of these proteinases in 2 times.

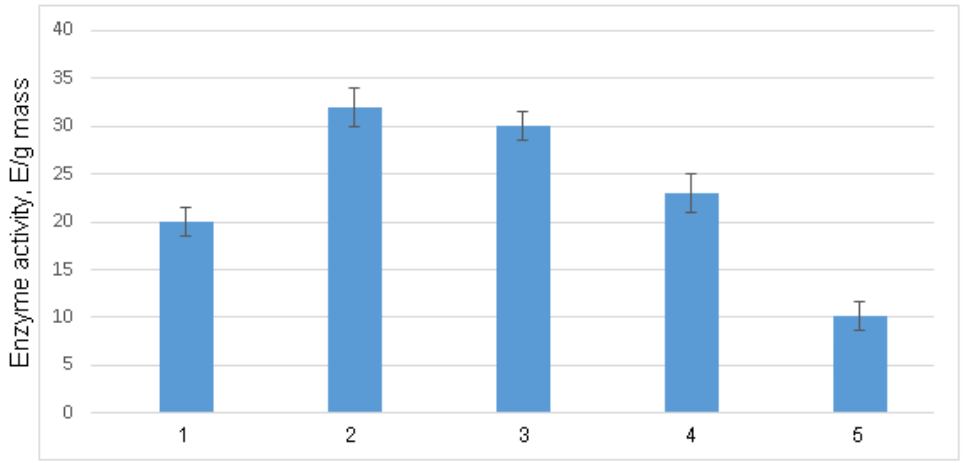

Figure 2: Proteolytic activity in homogenates of the Colorado potato beetle larvae (3-4 stage of development) when feeding on leaves of various plants within 48 hours before check-in. 1. without food; 2. potato (varietiy Nevskiy); 3. potato (variety Bashkir); 4. eggplant; 5. tomato.

The results of SDS electrophoresis of L. decemlineata proteins obtained on gelatin sorbent from homogenates of larvae reveals 5 molecular forms of proteinases (Fig. 3). Thus, the electrophoretic pattern of separation of samples obtained from the hungry larvae, and insects feeding on leaves of different plant species, did not differ. Observed changes in the level of activity of these proteinases may be due to a change in the relative activity of single molecular components of the larvae proteolytic complex. On the pest of grain crops *Callosobruchus* maculates shown that insects are capable of altering the level of proteolytic activity through the modulation of gene expression of gene activity, and by posttranslational modifications of the major digestive proteinases [39, 40].

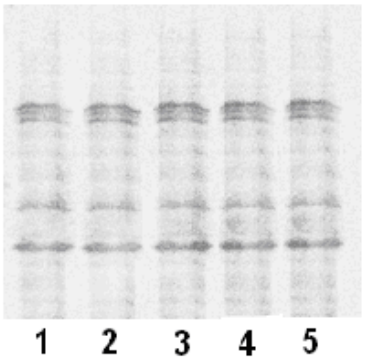

Figure 3: Separation of affine-purified proteinases of the Colorado potato beetle larvae by electrophoresis in 10% SDS-PAGE at feeding insects on leaves of various plants: 1. without food; 2. potato (varietiy Nevskiy); 3. potato (variety Bashkir); 4. eggplant; 5. tomato.

Thus, the larvae of the Colorado potato beetle have a set of digestive proteinases, the activity of which changes when feeding on leaves of various species of Solanaceae. The potato leaves are characterized by a complex of proteins and inhibitors demonstrating specificity to proteolytic enzymes of larvae of the

Colorado potato beetle. The obtained results allow detailing the mechanisms of the relationship of the Colorado potato beetle and potato, to identify the varietal characteristics of the demonstration of plant resistance to insect.

ACKNOWLEDGEMENTS

This work was performed with financial support from the Ministry of education and science of the Russian Federation (task No. 01201456414).

REFERENCES

[1] Vilkova NI, Ivashchenko SL. Modern systems and new directions in enhancing the resistance to the Colorado potato beetle: proceedings of the conference. M., 2000;pp. 25-35.
[2] Forgash AJ. Mass Agr Exp Stn Res Bull 1985: 704:35-52.
[3] Udalov MB, Benkovskaya GV. Vavilov J Genet Breed 2011; 15(1): 156-172.
[4] RS Ushatinskaya, EP Gulyaev, SS Izhevsky M. Science 198;375 .
[5] Walling LL. J Plant Growth Reg 200;19:195–216.
[6] Zagrobelny M, Bak S, Rasmussen AV, Jørgensen B, Naumann CM, Lindberg Møller B. Phytochem 2004;65;293–306.
[7] Lankau RA. New Phytologist 2007;175:176–184.
[8] Hopkins RJ, Van Dam NM, Van Loon JJA. Ann Rev Entomol 2009;54:57–83.
[9] Chen H, Gonzales-Vigil E, Wilkerson CG, Howe GA, Chen H, Gonzales-Vigil E, Wilkerson CG, Howe GA. Plant Physiol 2007;143:1954–1967.
[10] Shpirnaya IA, Umarov IA, Ibragimov RI. Bull Bashkir Univer 2006;3:49-52.
[11] Jinwon Kim, Hélène Quaghebeur, Gary W Felton. Phytochem 2011; 729 (13): 1624–1634.
[12] Joe Louis, Michelle Peiffer, Swayamjit Ray, Dawn S. Luthe and Gary W. Felton. New Phytologist 2013 199(1):66–73.
[13] Kessler A, Baldwin IT. Annu Rev Plant Biol 2002;53:299–328.
[14] Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H. Plant Physio. 2004;134: 420–431.
[15] Kempema LA, Cui X, Holzer FM, Walling LL. Plant Physiol 2007;143:849–865.
[16] Green TR, Ryan CA. Science 1972;175:776–777.
[17] Walker-Simmons M., Hadwiger L., Ryan C. Am Soc Plant Biol 1983;110(1):194–199.
[18] Hildmann T., Ebneth M., Peiia-Cortth H., Sánchez-Serrano J., Willmitzer L., Prat S. The Plant Cell 1992. V. 4, P. 1157-1170.
[19] Lawrence S, Novak N, Ju C, Cooke J. J Chem Ecol 2008;34:1013–102.
[20] Hines M, Nielsen S, Shade R, Pomeroy M. Entomologia Experimentalis et Applicata 1990;57(3):201–207.
[21] Oppert B, Morgan T, Culbertson C, Kramer K. Comp Biochem Physiol 1993;105(3):379–385.
[22] Bolter C, Jongsma M. J Insecr Physiol 1995;41(12):1071-1078.
[23] Mazumdar-Leighton S, Broadway RM. Insect Biochem Mol Biol 2001;31:645–657.
[24] Brunelle F, Cloutier C, Michaud D. Arch Insect Biochem Physiol 2004;55:103–113.
[25] Guidelines on mass valuation of breeding material of potato resistance to Colorado potato beetle. Moscow: FNIISH. 1994. 42 C. (in Russian)
[26] Tsvetkov VO, Ryabtseva ND, Shpirnaya IA, Bashirova RM, Valiakhmetova KI, Ibragimov RI. Proceedings of Samara scientific center of the Russian Academy of Sciences 2011;13(3):221-224.
[27] Vincent D, Wheatley M, Cramer G. Electrophoresis 2006;27:1853–1865.
[28] Shpirnaya IA, Umarov IA, Shevchenko ND, Ibragimov RI. Applied Biochem Microbiol 2009; 45(4): 497-501.
[29] Chen H, Wilkerson CG, Kuchar JA, Phinney BS, Howe GA. Proc Natl Acad Sci USA 2005;102:19237–19242.
[30] Chen H, Gonzales-Vigil E, Wilkerson CG, Howe GA. Plant Physiol 2007;143:1954–1967.
[31] Lopez-Ordonez T, Rodriguez M, Hernandez-Hernandez D. Insect Mol Biol 2001; 10(5): 505-511.
[32] Nikola Lončar, Zoran Vujčić, Nataša Božić, Jelisaveta Ivanović and Vera Nenadović. Arch Insect Biochem Physiol 2010;74(4) :232–246.
[33] Zulfiqar A. Malik, Sumaira Amir and István Venekei. Arch Insect Biochem Physiol 2012;80(1) :26-41.
[34] Jeffers LA, Thompson DM, Ben-Yakir D, Roe RM. Entomol Exp Appl 2005;117:135–146.
[35] Ryan CA. Annu Rev Phytopathol 1990;28:425–449.

[36] Valueva TA, Mosolov VV. Adv Biological Chem 2002;42:193-216.
[37] Heibges A, Glaczinski H, Ballvora A, Salamini F, Gebhardt C. Mol Genet Genom 2003;269(4):526-534.
[38] Rivard D, Cloutier C, Michaud D. Arch Insect Biochem Physiol 2004;55(3):114-23.
[39] Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H , 2004; Zhu-Salzman K, Salzman RA, Ahn JE, Koiwa H. Plant Physiol 2004;134:420–431.
[40] Ahn JE, Salzman RA, Braunagel SC, Koiwa H, Zhu-Salzman K. Insect Mol Biol 2004;13:649-650.