CROSS ANALYSIS OF THE INTERACTION OF α-AMYLASE AND PROTEINASE COMPONENTS OF INSECTS WITH PROTEIN INHIBITORS FROM WHEAT ENDOSPERM

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The components of the digestive proteinases that hydrolyze gelatin, Nα-benzoyl-DL-arginine-p-nitroanilide (BAPNA), acetyl-DL-phenylalanine-2-naphthyl ester (APNE), as well as α-amylases, were studied on eight species of insects that damage cereal grains or grain products, by isofocusing in PAAG. New "cross" methods were used to analyze the interaction of the components of α-amylases and proteinases with protein inhibitors from wheat endosperm. It was shown that the components of insect proteinase complexes are characterized by pronounced species specificity. The components of the spectra differ in their ability to hydrolyze protein and synthetic substrates and with respect to trypsin, chymotrypsin, and thiol proteinase inhibitors. The information obtained by the methods proposed can be used for a preliminary "classification" of the individual enzyme fractions before their detailed investigations, as well as in an assessment of the possible protective role of inhibitors in connection with the peculiarities of complexes of digestive hydrolases in various species of insects.

Key words: insects, α-amylases, proteinases, wheat, endosperm, α-amylase inhibitors, trypsin inhibitors, chymotrypsin inhibitors, and thiol proteinase inhibitors.

Protein inhibitors of α-amylases and proteinases are considered as one of the factors providing for the protection of plants from insects and microorganisms [1-4]. A determination of the role of inhibitors in the protection of wheat and other cereal grasses from pests is hindered by the relative lack of study of digestive enzymes in numerous species of insects that feed on grain or vegetative organs, as well as by the absence of simple methods of analysis.

Inhibitors of the α-amylases of insects and mammals [1, 5, 6], trypsin inhibitors (TI), α-chymotrypsin inhibitors (ChtI), chymotrypsin-subtilisin inhibitors (Cht-StI), endogenous α-amylase-subtilisin inhibitors (A-StI), and thiol proteinase inhibitors have been detected in wheat endosperm [4-9]. In the case of isoelectrofocusing (IEF) of the endosperm proteins in PAAG, followed by detection of the inhibitors according to activity by the corresponding methods [6, 8], the indicated types of inhibitors give characteristic spectra [10]. This, in particular, by comparing the spectra of inhibitors of standard hydrolases and hydrolases of various origin, permits a "classification" of the enzymes to be analyzed with respect to the inhibitors. Certain proteinases of insects and plants have been characterized in this way [8, 9]. However, this approach requires the isolation of individual enzyme fractions, which is extremely laborious and is not always justified. It is known that the multiplicity of components of the enzyme spectrum may be determined genetically or may be associated with posttranslation modifications. In the first case, the most probable differences of the components pertain to important characteristics, including the behavior toward inhibitors or specificity to various substrates; in the latter the components differ only in isopoints (pI).

Another way is to analyze the interaction of hydrolases with inhibitors right at the stage of analytical fractionation. After separation of hydrolases and inhibitor proteins, conditions are created in various gels for a "cross" interaction of the components of the hydrolase and inhibitor spectra. When hydrolases are detected according to activity at the points of intersection of the component of the enzyme with the inhibitor, the band of the enzyme is broken or narrowed. This approach was used in the study of endogenous α-amylases and proteinases of wheat and their inhibitors [11].

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The aim of this work was to study the components of the digestive α-amylases and proteinases of a number of species of insect pests and to use "cross" methods to analyze the interaction of the components of the enzymes with the protein inhibitors from wheat endosperm.

METHODS OF INVESTIGATION

Grain of bread wheat of the varieties "Bezostaya 1," "Diamant," and "Saratovskaya 33" were obtained from the collection of the All-Union Institute of Horticulture. The protein fractions containing α-amylase and proteinase inhibitors were extracted from milled grains, stripped of the germ, with four volumes of water for 1 h at 20°C. β-Amylase was inactivated by heating at 80°C for 5 min. Inhibitors of thiol proteinases were extracted with two volumes of 2 M urea (in this preparation they are ~4 times as active as in an aqueous extract). The insects were grown in cultures obtained from the All-Union Scientific-Research Institute for Grain and Products of Its Processing and the All-Union Institute for Plant Protection (*Tenebrio molitor* L. — the yellow mealworm beetle, *Rhysopertha domina* F. — the lesser grain borer, *Oryzaephilus surinamensis* L. — the saw-toothed grain beetle — *Tribolium confusum* Duv. — the confused flour beetle; *Tribolium castaneum* Herbst. — the rust-red flour beetle; *Sitophilus granarius* L. — the grain weevil; *Sitophilus oryzae* L. — the rice weevil). *Eurygaster integriceps* Put. — the stink bug — was collected in the Krasnodarsk territory. The guts were isolated from the imagos and larvae according to the procedure used in the All-Union Scientific-Research Institute for Plant Protection.

α-Amylases were extracted with 0.1% $CaCl_2$ (from homogenates with a fivefold volume, from guts in an amount of 1-5 organs per 100 μl) at 20°C. Saliva served as the source of human α-amylases.

Proteinases were extracted with 0.01 M dithioerythritol (from homogenates with a fivefold volume, from guts — 2-10 organs per 100 μl). Preparations of trypsin (Sigma, USA) and chymotrypsin (Serva, Federal Republic of Germany) were used.

Isoelectrofocusing of the grain proteins and insect hydrolases was conducted in a plate of 6% PAAG 0.2 mm thick on a Multiphor II instrument (LKB, Sweden) for 1.5-2 h at a final voltage of 2500 V [4-8].

α-Amylases were applied to the gel with paper strips in a volume of 2-10 μl. An amount of the preparation that permitted the production of distinct bands of amylases when developed under the conditions described below was selected. Isoelectrofocusing of α-amylases was conducted in PAAG with 2% servalites, pH 3-7 (Serva), proteinases with servalites, pH 3-10 or 3-7, α-amylase inhibitors with ampholines, pH 5-8 (LKB), and proteinase inhibitors with a mixture of ampholines with pH from 5 to 11. Electrode solutions from Serva were used.

α-Amylases were isolated by applying a plate of 6% PAAG (on a substrate) 0.2 mm thick, containing 0.4% starch, 0.001 M $CaCl_2$, and 0.05 M sodium phosphate buffer, pH 5.5, to the gel after isoelectrofocusing. After 20 min of incubation at 37°C, the replica was immersed in an iodine solution (1 g I and 3 g KI per liter).

α-Amylase inhibitors were detected in the spectra of grain proteins using a 6% PAAG replica containing 0.4% starch, Tris (0.0074 M)-glycine (0.057 M) buffer, pH 8.3, and α-amylase [6].

Proteinases were detected according to the hydrolysis of the gelatin of photofilm, as well as synthetic substrates. A Foto 65 photofilm, moistened with a solution of 0.1 M Na_2HPO_4, pH 9.0 (for trypsin- and chymotrypsinlike proteinases) or 0.1 M NaH_2PO_4 with 0.1 M dithioerythritol (for thiol proteinases), was applied to the gel and incubated for 1 h at 38°C [8, 9]. When synthetic substrates (and sometimes also photofilms) were used, a replica was taken from the gel on a Millipore 0.45 nitrocellulose membrane for 15 min.

For work with the p-nitroanilide of Nα-benzoyl-DL-arginine (BAPNA, Serva), we used our modification of the method of Ohlsson et al. [12]. A nitrocellulose replica was incubated in a solution containing 0.4 ml of a 1% solution of BAPNA in dimethyl sulfoxide and 8 ml of 0.1 M Na_2HPO_4 with 0.001 M EDTA and 0.002 M cysteine (pH 8.0), for 1 h at 38°C. The replica was transferred to a solution of 0.1% $NaNO_2$ in 1 N HCl for 10 min, rinsed in water, and immersed in a 0.05% solution of orcinol (Calbiochem, England) in 0.1 M Na_2HPO_4. The components of the proteinases appeared in the form of yellow-orange bands. A wet replica was photographed with a blue light filter. Nitrocellulose is essential for fixation of readily soluble p-nitroaniline. We used orcinol instead of N(1-naphthyl)ethylenediamine [12] in view of its greater accessibility and also because this facilitates prolonged storage of the dried preparations.

The method that we modified earlier [3] was used to analyze proteinases with acetyl-DL-phenylalanine-2-naphthyl ester (APNE, Sigma, USA). A nitrocellulose replica was incubated for 30 min at 38°C in a solution containing 0.2 ml of 1% APNE in DMSO, 2 ml DMSO, 6 ml 0.1 N sodium phosphate buffer pH 7.0, and 0.1 ml of the 1% dye Fast B (Serva). Proteinase inhibitors were detected by the method of gelatin replicas [7, 8].

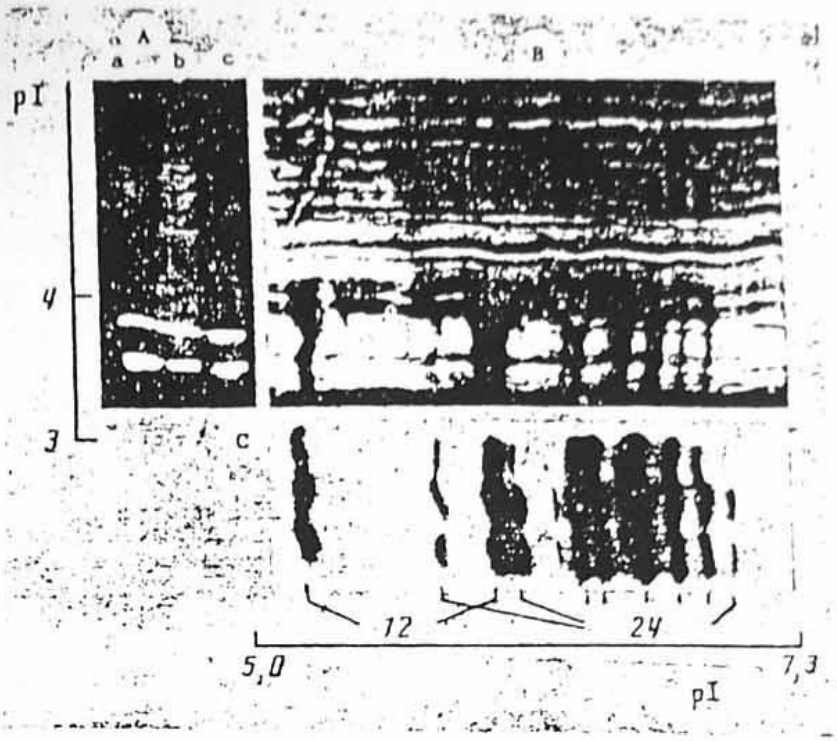

Fig. 1. Cross analysis of the interaction of components of α-amylases of the lesser grain borer *Rhysopertha dominica* with protein inhibitors from endosperm of bread wheat of the Bezostaya 1 variety: A) isoelectrofocusing of α-amylases of the gut (a) and homogenate (b) of the imago and gut of the larvae (c) in the range of pH 3-7; B) spectrum of α-amylases of a homogenate of an imago after cross interaction with components of proteins of a fivefold aqueous extract from endosperm (4 μg protein per mm of band of application); C) spectra of inhibitors detected after isoelectrofocusing of endosperm proteins (pH 5-8) with α-amylase of the larva of *T. molitor* (control). Portions of 5-10 μg endosperm proteins were applied to the track; 12 and 24) molecular weights of components of inhibitors in kD [6, 10].

To analyze the interaction of components of α-amylases and inhibitors, preparations of α-amylases and protein fractions containing inhibitors were applied to various gels in broad bands with paper strips and subjected to isoelectrofocusing. A PAAG replica was placed on the gel with separated inhibitors (just as in the detection of α-amylases), and then it was "cross" applied to a gel with α-amylases, incubated for 20 min at 38°C, pH 5.5, and placed in a solution of iodine.

Proteinases and inhibitors were also applied to the gels in broad bands. A replica on photofilm was taken from the gel in which the grain proteins were separated for 20 min, after which it was applied to gel with proteinases so that the components of the grain proteins and proteinases intersected at right angles, and incubated for 1 h at 38°C (the method was used primarily for "acid" proteinases). In another variant, which gave stabler results in work with trypsin- and chymotrypsinlike proteinases, the gelatin replica-photofilm from the gel with inhibitors was "cross" applied to a nitrocellulose replica from the gel with proteinases, moistened with buffer with the optimum pH for proteinases, and incubated for 1 h at 38°C. In a simplified variant, paper strips 1-2 mm wide, with a length sufficient to overlap all the components of the proteinase spectrum, moistened with solutions of enriched protein inhibitors (1 mg/ml), were applied to the photofilm. After 15 min the photofilm was applied to the gel with proteinases in such a way that the traces from the paper strips intersected the components of the spectra, and incubated for 1 h at 38°C.

Trypsin and chymotrypsin inhibitors from bread wheat endosperm were enriched by affinity chromatography [7, 8]. The activity of the preparations of trypsin inhibitors from endosperm of the Saratovskaya 33 variety was 2.1 mg of trypsin per mg protein. The activity of the soybean trypsin inhibitor (STI) (Serva) under the same conditions was 1.8 mg trypsin per mg protein.

The activity of proteinases and inhibitors was determined according to the hydrolysis of BAPNA in 0.05 M Tris-HCl buffer, pH 7.8, and azocasein in 0.1 M sodium phosphate buffer, pH 5.0 and 8.0, with 0.001 M EDTA and 0.002 M cysteine by micromethods in 96-well culture plates [4]. The activity with respect to BAPNA was expressed in μmoles of p-nitroaniline per ml per min at 38°C (units).

Preparative fractionation of insect proteinases was performed in Ultrodex gel (LKB) [8]. The proteinases of a homogenate of imagos of the lesser grain borer were also isolated by affinity chromatography on STI-agarose (Sigma). A 5-ml portion of the extract from the homogenate (0.5 unit of BAPNA per ml) was applied to a column with gel with a volume of 0.5 ml. Portions of 2.5 ml of the extraction reagent, 0.05 M Tris-HCl buffer, pH 7.8, with 0.2 M NaCl, and again the extraction reagent were passed through the column. Proteinases were eluted with 0.01 N NaOH, pH 11, neutralized by the addition of HCl, and separated by isoelectrofocusing in Ultrodex gel (LKB).

The protein concentration was determined according to the binding of the dye Coomassie G-250 [14].

RESULTS AND DISCUSSION

Figure 1 shows as an example the results of cross analysis of the interaction of the components of α-amylases of the lesser grain borer with components of protein inhibitors. It is evident that the major components of the α-amylases with pI in the region from 3.0 to 4.0, detected both in extracts from the gut and in homogenates, are inhibited by the same components of the grain albumins with molecular weights 12 and 24 kD [5, 6]. Here a replica from the gel with grain proteins, exhibited by a total preparation of α-amylase of a yellow mealworm beetle larva — the most typical and best studied insect α-amylase [5, 15] — was used as the control. Components with pI > 4.5, which also hydrolyze starch, did not react with inhibitors under these conditions. These amylases are present chiefly in extracts from the homogenate and are relatively less intense in extracts from the intestine.

Gut α-amylases of the grain weevil and a number of other insect species are inhibited by the same components of the grain proteins as the α-amylases of the grain borer and yellow mealworm beetle. In turn, all the components of the α-amylase spectrum of human saliva reacted only with the components of albumins with molecular weights 24 and 60 kD (not shown in Fig. 1). Thus, the proposed approach permits an estimation of the components of the complex spectra of the α-amylases of insects and other organisms with respect to inhibitors. Of course, this is a primarily qualitative method, but within definite limits it can be used for a tentative estimation of the specificity of the interaction of various α-amylases with inhibitors. For this purpose preparations of different α-amylases are applied to the gel in amounts providing for approximately the same intensity of the components detected under similar conditions (estimated visually). To obtain the same pictures of "cross" interaction of different α-amylases with inhibitors, different amounts of the inhibitors are needed. Thus, for the sensitive α-amylase of *T. molitor*, the inhibitors contained in a fivefold aqueous extract from the endosperm of bread wheat (2 μg protein per μl) are sufficient when 0.5 μl is applied to a 1 mm band, while for α-amylases of the grain borer, 2-4 times as large an amount of the inhibitor preparation must be used, and for the α-amylases of the gut of the stink bug, more than 10-20 times as much must be used. This corresponds to the sensitivity of the indicated α-amylases to inhibitors, determined by the usual methods [15, 16].

Figure 2 shows the spectra of digestive proteinases of the yellow mealworm beetle *T. tolitor* and the results of the interaction of their individual components with the components of inhibitors contained in an aqueous extract of endosperm proteins (2 μg/μl, 20 μg protein per mm band of application). Here we used a mixture (1:1) of extracts from endosperm of the Beozystaya 1 and Diamant varieties, since the former is characterized by higher activity of chymotrypsin inhibitors, and the latter by higher activity of trypsin inhibitors. (The activity of the inhibitors per ml of mixture was ~80 μg chymotrypsin and 40 μg trypsin.) The components of the proteinases isolated from the intestines of larvae and imagos and the homogenate of larvae are qualitatively similar (Fig. 2A). This permitted the use of the proteinases of the homogenate in cross analysis. Evidently the proteinase component 1a reacts with the components of the spectrum of chymotrypsin inhibitors — ChtI-St and ChtI, while component 2 reacts with trypsin inhibitors. The latter, moreover, hydrolyzes BAPNA (Fig. 2A, D), a substrate widely used for trypsin. Evidently component 1a is a chymotrypsinlike proteinase, while component 2 is a trypsinlike proteinase. It can be noted that the trypsinlike enzymes of *T. molitor* have also been described by other authors [16]. Components with relatively weak BAPNAase activity also appear in the zone of the spectrum with pH ~ 4.0. Perhaps these are also trypsinlike proteinases. Earlier enzymes sensitive to inhibitors of thiol proteinases were detected among the "acid" components of the proteinases of *T. molitor* that hydrolyze gelatin [8]. Distinct spectra of the proteinases that hydrolyze APNE could not be obtained. Evidently the gelatin of photofilm is the most universal substrate for the proteinases of this insect.

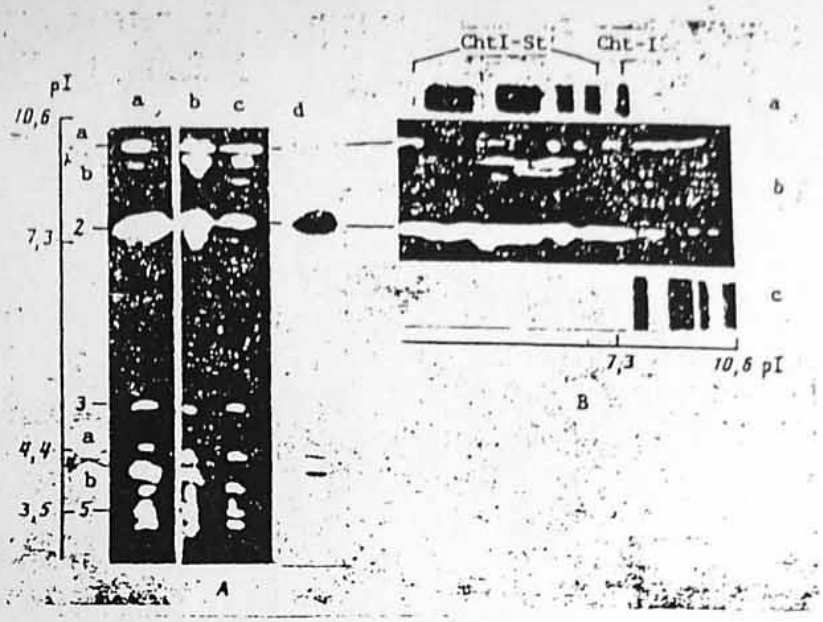

Fig. 2. Cross analysis of the interaction of components of larval proteinases of the yellow mealworm beetle *Tenebrio molitor* with protein inhibitors from endosperm of bread wheat: A) isoelectrofocusing of proteinases from a homogenate of larvae (a, d) and the gut of the imago (b) and larvae (c) in the range of pH 3-10. Portions of 10 μl of extract from the homogenate and gut (one organ per 40 μl) were applied. 1-5) Notations of components of proteinases: a, b, c) proteinases were detected according to hydrolysis of gelatin of the photofilm, d) according to BAPNA. B) Spectrum of proteinases after interaction with components of endosperm proteins (b) [vertical direction: isoelectrofocusing of proteinases; horizontal: isoelectrofocusing of endosperm proteins (20 μg per mm band)]; a) gelatin replica, developed with chymotrypsin; c) with bovine trypsin (control).

Figure 3 shows the results of an analysis of the composition of proteinases of the lesser grain borer *R. dominica*. The proteinase spectra of the gut and homogenate of the imago are similar. All five major components with pI in a wide range from 3.5 to 10 react with trypsin inhibitors. The components of proteinases 1 and 2 were subjected to "cross" analysis with components of isoelectrofocusing of the spectrum of endosperm proteins of wheat of the Diamant variety, and 3 and 5 with fractions of wheat inhibitors enriched by affinity chromatography, and with STI. The reaction proceeded at pH corresponding to the isopoints of the components. All the enumerated components of the lesser grain borer proteinases hydrolyzed BAPNA (components 1 and 2 react significantly more weakly than the rest and are not visible in Fig. 3A). Cross analysis of BAPNAases on nitrocellulose confirmed their sensitivity to trypsin inhibitors.

Of all the insect species studied, the lesser grain borer possesses the most active proteinases with respect to BAPNA (0.5 unit per ml of extract from a homogenate of imagos versus 0.034 for the yellow mealworm beetle and 0.028 for the saw-toothed grain beetle). In turn, with respect to azocasein the activity of the proteinases of the lesser grain borer (at pH 8.0) is at the level of activity of the proteinases of *T. molitor*. Component 3 of the proteinases of the lesser grain borer was isolated by affinity chromatography on STI-agarose, followed by isoelectrofocusing in Ultrodex gel. It was revealed that the activity of enriched TI from wheat endosperm of the Saratovskaya 33 variety toward proteinase 3, expressed in terms of the activity of the enzyme toward BAPNA, was 24 units/mg, while the activity of the STI was 21 units/mg versus 2.7 and 2.3 units/mg with respect to trypsin. Perhaps such a large difference in the values of the activity of the inhibitors is explained by the increased (in comparison with trypsin) specificity of the proteinase of the grain borer for BAPNA. All the components of the proteinases of the lesser grain borer had a pH optimum of 8.0 and higher (with respect to BAPNA, azocasein, and also in a visual estimation according to the gelatin of photofilm).

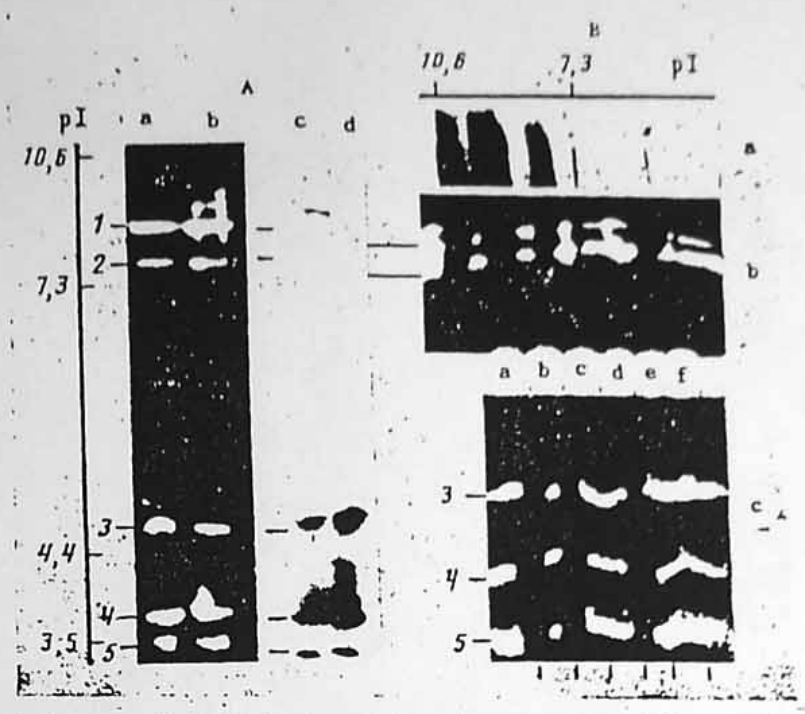

Fig. 3. Cross analysis of the interaction of components of the proteinases of the lesser grain borer *R. dominica* with protein inhibitors from endosperm of bread wheat. A) Isoelectrofocusing of proteinases of homogenate (a, c, d) and gut of imago (b, 1 organ to 40 μl) in the range of pH 3-10. Portions of 10 μl applied. a, b) Proteinases were detected according to the hydrolysis of gelatin of the photofilm, c, d) according to BAPNA. B) Components 1 and 2 of the proteinases after interaction with proteins of the endosperm of bread wheat of the Diamant variety, separated by isoelectrofocusing (b); a) control for isoelectrofocusing of proteins of endosperm, replica developed with trypsin. C) Components of proteinases 3-5, detected with photofilm preliminarily treated with solutions of inhibitors according to bands a-f: a, b) enriched with endosperm TI; c) chymotrypsin inhibitor of endosperm; d) STI (a total of 1 mg per ml); e) 0.002 M p-CMB; f) water.

For the complex of proteinases of the confused and rust-red flour beetles *Tr. confusum* and *Tr. castaneum*, the pH optimum with respect to azocasein (and with respect to gelatin) was in the region of 5.0. The main activity with respect to gelatin (visually) corresponded to the components with pI from 3.0 to 5.0 (Fig. 4A). Earlier it was demonstrated [9] that the "acid" components of the proteinases of *Tr. confusum*, which hydrolyze gelatin and were isolated by preparative isoelectrofocusing, are inhibited by the same components of the endosperm proteins as papain, as well as the thiol proteinase of the germinating wheat grain. (These proteins, denoted as thiol proteinase inhibitors, have a molecular weight of ~11-12 kD [11].) On this basis, the preparation of "acid" proteinases of *Tr. confusum* was used as a control in work by the cross method (Fig. 4B). To characterize the preparation, let us indicate that when azocasein was used as the substrate at pH 5, neither wheat TI nor STI in a concentration in the reaction mixture up to 200 μg/ml had any influence on the proteinase activity. Evidently similar proteinases were isolated from the intestine of *Tr. confusum* by other authors as well [17-19].

Figure 4 shows the picture of the cross interaction of "acid" components of the proteinases of *Tr. confusum* and *Tr. castaneum* with components of the proteins of the endosperm of bread wheat, isolated with 2 M urea and separated by isoelectrofocusing in PAAG. It is evident that the components 3-5 of *Tr. confusum* and components 4-6 of *Tr. castaneum* are inhibited by the same components of wheat proteins. No distinct inhibition of component 3 of the *Tr. castaneum* proteinase is observed, possibly as a result of its high activity (the amount of the inhibitor is insufficient). The possibility also remains that this may be a different kind of proteinase. The "acid" proteinases of these species, which hydrolyze gelatin, did not react with trypsin and

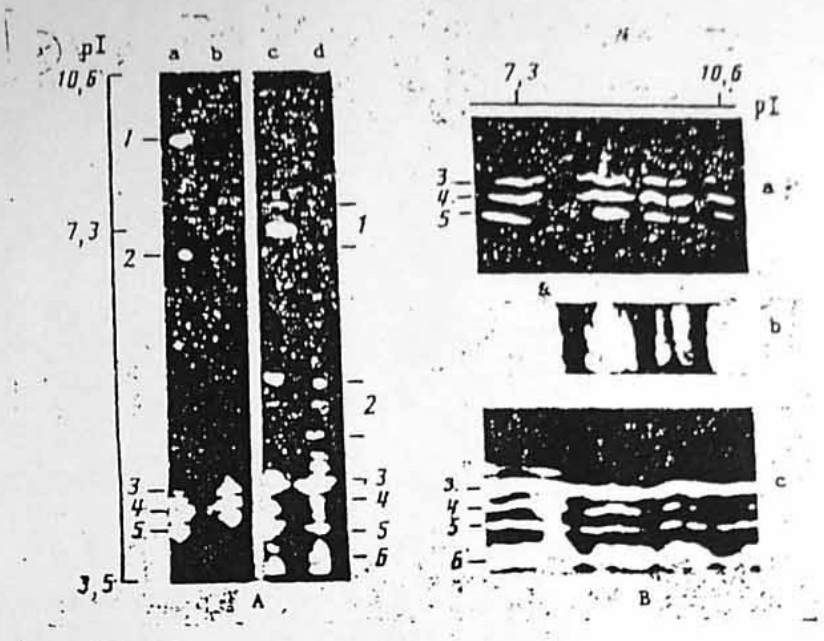

Fig. 4. Analysis of components of proteinases of the confused flour beetle (*Tribolium confusum*) and the rust-red flour beetle (*Tr. castaneum*) with respect to protein inhibitors: A) isoelectrofocusing of proteinases of larvae of the confused flour beetle (a, b) and the rust-red flour beetle (c, d): a, c) guts (10 organs to 100 μl); b, d) homogenates (10 μl applied per track; proteinases detected according to the hydrolysis of gelatin of photofilm); B) cross interaction of proteinase components 3-5 of the confused flour beetle (a) and proteinase components 3-6 of the rust-red flour beetle (c) with components of endosperm proteins of bread wheat of the Diamant variety, separated by isoelectrofocusing; b) gelatin replica from gel after isoelectrofocusing of endosperm proteins, detected with a preparation of "acid" proteinases of the confused flour beetle.

chymotrypsin inhibitors under these same conditions (the reaction occurred at pH corresponding to the isocurrents of the components). In the spectra of proteinases of both species of flour beetles, in the region of pH from 3.5 to 5.0, relatively weak components that hydrolyze BAPNA and APNE were also detected; they reacted with preparations of wheat TI and STI (not shown in Fig. 4).

Weak "alkaline" components of the proteinases of these insect species hydrolyzed only gelatin. The results of cross analysis indicate their possible chymotrypsinlike nature.

It was shown earlier that the "alkaline" components of the proteinases of the grain and rice weevils are chymotrypsinlike [8]. This was also confirmed by cross analysis.

The cross method showed that the major components of the gut proteinases of the saw-toothed grain beetle *O. surinamensis* with pI ~ 5.5-6.0, which hydrolyze gelatin, are inhibited by the endospermal TI and STI and are also capable of hydrolyzing BAPNA. The activity toward APNE corresponds to a different component with lower pI (Fig. 5A).

The gut proteinases of the stink bug *E. integriceps* very weakly hydrolyze gelatin and BAPNA but exhibit appreciable activity toward APNE (Fig. 5C). Cross analysis (strips with inhibitors were applied to a nitrocellulose replica before development) showed that these proteinases are sensitive to wheat TI and STI.

Thus, the components of complexes of the digestive proteinases of insects that feed on grain or grain products is characterized by pronounced species specificity. In turn, the components of the proteinase spectra differ in their ability to hydrolyze protein or synthetic substrates and with respect to protein inhibitors from wheat endosperm.

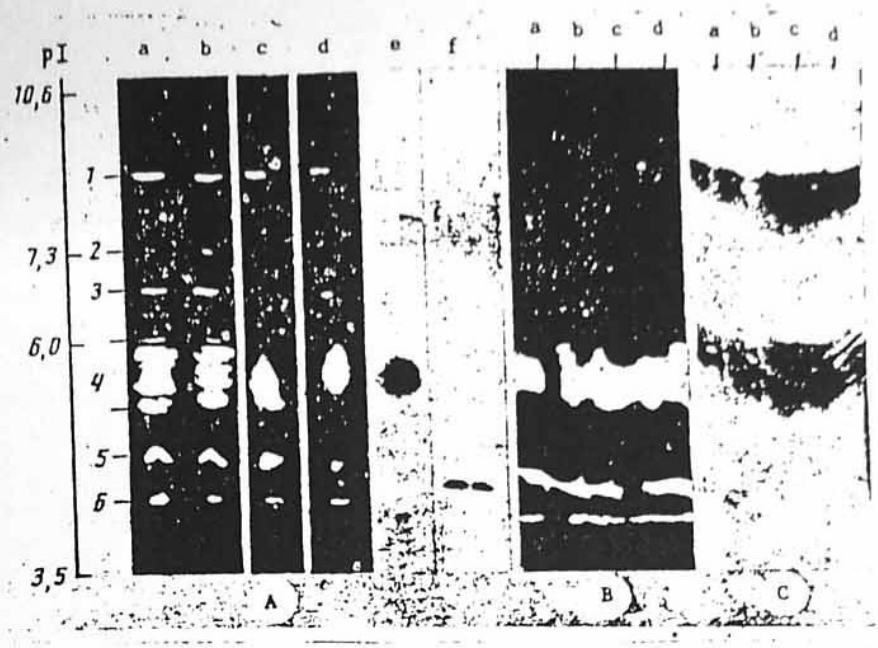

Fig. 5. Analysis of proteinases of the saw-toothed grain beetle *O. surinamenis* (A, B) and the stink bug *E. integriceps* (C) according to substrate specificity and behavior toward inhibitors: A) isoelectrofocusing of a homogenate of larvae (a) and imagos (b, d, f) and guts of imagos (c) and larvae (d) (10 organs to 50 μl) in the range of pH 3-10 (a-d: proteinases were detected according to hydrolysis of gelatin of photofilm; e) BAPNA; f) APNE); B) cross analysis of the interaction of proteinase components 4-6 with fractions of inhibitors: a) enriched TI of endosperm; b) water; c) chymotrypsin inhibitors of endosperm; d) p-CMB; C) isoelectrofocusing of gut proteins of stink bug (proteinases detected according to hydrolysis of APNE; 20 organs to 100 μl, 20 μl of extract applied per mm of band): a-d) strips of paper moistened with solutions of inhibitors and applied to a nitrocellulose replica after development; a) endosperm TI; b) STI; c) endosperm chymotrypsin inhibitor; d) water.

The most universal of the substrates for insect proteinases studied is the gelatin of photofilm, which is hydrolyzed by proteinases of various kinds. Individual components that hydrolyze gelatin also exhibit activity toward BAPNA, APNE, or toward both of them. The proteinase spectrum developed with a synthetic substrate, as a rule, is poorer than when gelatin is used. This is evidently due to the substantial qualitative and quantitative differences of the proteinases with respect to specificity toward definite types of hydrolyzable bonds. The gelatin of photofilm in many cases ensures higher sensitivity of the analysis in isoelectrofocusing (by an order of magnitude and high). Exceptions are the proteinases of the lesser grain borer, specific for BAPNA, and those of the stink bug, specific for APNE.

The proposed modification of the method of analysis with BAPNA is suitable for the detection of trypsinlike proteinases of various insect species. The wide assortment of possible variants of the method of cross analysis permits a qualitative assessment of the interaction of the components of complex mixtures of insect hydrolases (α-amylases, trypsin-, chymotrypsinlike, and thiol proteinases) with protein inhibitors. Such information can serve as a guide for more detailed investigations of individual components of proteinases or α-amylases by classical methods. It is also valuable at the early stages of determination of the peculiarities of the complex of digestive hydrolases of different insect species and, correspondingly, the possible protective role of individual types of inhibitors in plants with respect to concrete pests.

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