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Full-text article

HETEROLOGOUS EXPRESSION OF TWO ACETYLCHOLINESTERASES OF COLORADO POTATO BEETLE *LEPTINOTARSA DECEMLINEATA* IN BACTERIA *ESCHERICHIA COLI* AND PRODUCTION OF FORM-SPECIFIC ANTIBODIES

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The Colorado potato beetle *Leptinotarsa decemlineata* is a widespread pest of plants of the Solanaceae family. Huge economic loss caused by *L. decemlineata* around the world is multiplied by its ability to develop resistance to all major insecticide classes. Previously, such resistance was found to be associated with mutations in the target enzyme LdAChE2, orthologous to *Drosophila melanogaster* acetylcholinesterase. However, discovery of the second form of *L. decemlineata* acetylcholinesterase LdAChE1 has changed this view. In order to compare the role of two acetylcholinesterase forms in the Colorado potato beetle physiology and in pest resistance to insecticides, gene copies were cloned and their heterologous expression in bacteria *E. coli* was followed by production of polyclonal antibodies against the recombinant proteins. Immunoblotting with produced antibodies demonstrated the absence of cross-reactivity, a lower content of LdAChE1 in the tissues of *L. decemlineata* adults compared with the second form, and the association of LdAChE2 with membranes. Further immunoaffinity purification of natural enzymes from the beetle tissues as well as their heterologous expression in insect cell cultures should help to evaluate the role of each form in physiology of the pest and in its resistance to insecticides.

Keywords: Colorado potato beetle, *Leptinotarsa decemlineata,* acetylcholinesterase, heterologous expression, polyclonal antibodies, immunoblotting

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Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is a widespread pest of crops of the family Solanaceae, that may cause complete defoliation of potato plants. Huge economic loss caused by CPB in North America, Europe and Asia is multiplied by its ability to develop resistance to all major insecticide classes (Alyokhin et al., 2008). In many cases such resistance may be associated to mutations in their target enzyme acetylcholinesterase (AChE, EC 3.1.1.7) (Yao et al., 1997; Kim et al., 2006; Malekmohammadia et al., 2012; Pang, 2014). The enzyme AChE plays an important role in the cholinergic synapses, participating in the hydrolysis of the neurotransmitter acetylcholine. Finding of the single gene in *Drosophila melanogaster* (Hall, Spierer, 1986; Harel et al., 2000) has suggested that insects have only one AChE form (Toutant, 1989). However, the genes encoding the second enzyme has been found later in *Anopheles gambiae* and other insect genomes (Weill et al., 2002; Villatte, Bachmann, 2002) except for the true flies. To date, the orthologs of *D. melanogaster* genes are named as *ace2* and the orthologs of *A. gambiae ace1* gene are named as *ace1* (Huchard et al., 2006).

Although the first studies of *L. decemlineata* AChE (Zhu, Clark, 1995) mutations in insecticide-resistant populations of the pest were performed on the gene orthologous to *D. melanogaster ace2* (Kim et al., 2006; Malekmohammadi et al., 2012), in 2011, the second enzyme form was identified in CPB (Revuelta et al., 2011). In comparison with LdAChE2, this enzyme demonstrated higher expression level, its RNAi resulted in a reduction of about 50 % of total enzyme activity and in increasing insect mortality by 43 %. RNAi of the LdAChE2 gene reduced AChE activity to 85 % and increased mortality by 29 %. Based on these data, the authors proposed to revise the association between CPB resistance to insecticides and mutations in the gene encoding LdAChE2 (Revuelta et al., 2011).

Later, it was shown that in different systematic insect groups, the first or second AChE form may be responsible for the acetylcholine hydrolysis (Lu et al., 2012; Kim et al., 2013, Pang, 2014). Insect acetylcholinesterase is a target for effective and environmentally safe insecticides. Two enzyme forms extracted from the heads of 100 species belonging to 18 insect orders have been separated by native PAGE. Immunoblotting with form-specific antibodies (Abs) against conserved peptide fragments and AChE activity staining in gels has demonstrated that 67 insect species, including *L. decemlineata* and other beetles, predominantly expressed AChE1 and 33 species predominantly expressed the AChE2 enzyme. One of the results of this study is a discrepancy between results of Western blotting and enzyme activity staining. In most samples, Abs has recognized major bands corresponding to both enzyme forms. However, only one of them often demonstrates AChE activity (Kim et al., 2013).

In order to continue the comparative analysis of AChE forms of CPB we have overexpressed LdAChE1 and LdAChE2 in bacteria *Escherichia coli,* produced form-specific polyclonal Abs and have compared the content of two proteins in the tissues of adult insects.

Materials and methods

DNA constructs

Fat bodies and ventral nerve cords were isolated from *L. decemlineata* adults and total RNA was extracted using Trizol reagent (Thermo Fisher Scientific, MA). Synthesis of cDNA was carried out for 1 h at 37 °C in PCR tubes with 20 µl of the reaction mixture containing 2.5 µg RNA, 10 mM Tris-Cl (pH 8.8), 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 1 µg oligo (dT) as a primer, 200 U of RevertAid M-MuLV-reverse transcriptase (Thermo Fisher Scientific) and 5 U of RNAase inhibitor (Thermo Fisher Scientific). At the following step, the mixture was heated at 95 °C for 5 min and 1 µl was used for PCR with Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific). The gene of LdAChE1 (GenBank ID: JF343436.1 and XM_023156211.1) was amplified with forward gtacCTCGAGATGACRACAACGCTACGAGTATT CTG (*Xho*I site is underlined) and reverse agggGAATTCTT ACTGGTAGCGTTTCCATCCAATTC (*EcoR*I site and stop codon are underlined) primers. The forward primer gagaCT CGAGATGGGCCAGCTTTCGATCCTGTGCT (*Xho*I site is underlined) and reverse primer agggGAATTCCTACAA AGCGTTAAGTAGTGTCATG (*EcoR*I site and stop codon are underlined) were used for PCR-amplification of DNA fragment encoding LdAChE2 (GenBank ID: L41180.1). The PCR products were gel purified, digested with *Xho*I/*EcoR*I restriction enzymes, and inserted into the pRSETa vector (Thermo Fisher Scientific) linearized by the same enzymes. About 750 bp of both constructed plasmids were sequenced using T7 forward and reverse primers to verify the correct amplification and insertion of protein-encoding fragments.

Heterologous expression and analysis of LdAChEs in E. coli

To express LdAChEs, *E. coli* BL21(DE3)-derived C41 cells (Miroux, Walker, 1996) were electroporated by obtained constructs at 1700 V using Electroporator 2510 (Eppendorf, Germany). Bacterial colonies from agar plates with LB medium containing 0.15 mg/ml ampicillin were inoculated into flasks with 25 ml of the same liquid medium. The cultures were grown to OD_{600} 0.6 and expression was induced by the addition of 0.2 mM IPTG (final concentration) with the following incubation for 15 h at 25 °C.

After cultivation, bacterial cells were pelleted by centrifugation at 3 000 g for 10 min and sonicated in 1 ml of 50 mM Tris-Cl buffer solution (TB, pH 7.5). To analyze whether LdAChEs accumulates in bacteria in soluble form or as insoluble inclusion bodies, homogenates were centrifuged at 14 000 g for 15 min and 0.1 ml of supernatants and pellets resuspended in TB to homogenate volume were used to prepare the samples for SDS-PAGE. The rest of the pellets were used to isolate the recombinant AChEs forming insoluble inclusion bodies (IBs). The upper layer of the pellets (bacterial membranes) was removed by careful pipetting and the lower white layer of IBs significantly enriched in the recombinant proteins was washed in TB.

To assay solubility of recombinant enzymes in the presence of 8 M urea, IBs isolated from 0.2 ml of bacterial homogenate were resuspended in 0.25 ml of TB containing 8 M urea, incubated for 10 min, centrifuged at 14 000 g for 5 min and supernatants and pellets resuspended in TB to supernatant volume were also analyzed by SDS-PAGE.

Production of polyclonal Abs

To get recombinant antigens for rabbit immunization, heterologous expression of LdAChEs was repeated in 250 ml of LB medium. In the case of LdAChE1 expression, we increased IPTG final concentration in the culture medium to 0.5 mM (instead of 0.2 mM) and cultivation temperature to 37 °C (instead of 25 °C). Isolated IBs were carefully washed in TB with 8M urea, dissolved in sample buffer for SDS-PAGE at 95 °C for 15 min, carefully dialyzed against TBS (50 mM Tris-Cl pH 7.4, 150 mM NaCl) and used for production and purification of polyclonal Abs using previously described methods (Dolgikh et al., 2009).

Preparation of CPB protein samples

L. decemlineata guts, fat bodies, flight muscles and ventral nerve cords of eight adults were isolated, homogenized in 0.3 ml of TB with 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and heated in sample loading buffer for SDS-PAGE. To pellet cells, hemolymph of these beetles was centrifuged at 3 000 g for 15 min and supernatant (plasma) was used for sample preparation. In other experiments, fat bodies of ten CPB adults were homogenized on ice in 2.5 ml of TB with 0.5 mM PMSF and centrifuged at 1 000 g for 15 min to pellet rude debris. The supernatant was carefully removed and centrifugated at 30 000g for 15 min at 4 °C. The pellet was dissolved in the equal volume of TB with 0.5 mM PMSF, 0.5 % Triton X-100 for 2 min and centrifuged again at the same rate. The both supernatants, rude debris and precipitate after Triton X-100 extraction resuspended in TB with 0.5 mM PMSF to homogenate volume were analyzed by immunoblotting.

SDS-PAGE and immunoblotting

The samples by SDS-PAGE were heated at 95 °C for 10 min with one fifth of the volume of 6 × sample buffer containing 375 mM Tris-Cl (pH 6.8), 12 % SDS, 6 % 2-mercaptoethanol and 60 % glycerol. 5 µl of protein samples were separated by SDS-PAGE in 12 % or in 4–20 % gradient gels, transferred on nitrocellulose membrane and stained with Ponceau S. Immunoblotting with rabbit polyclonal Abs and monoclonal antibodies against polyHis sequence was performed as previously described (Dolgikh et al., 2009; Dolgikh et al., 2019).

Results

The isolation of RNA from *L. decemlineata* fat bodies and ventral nerve cords followed by cDNA synthesis with oligo-dT primer allowed us to amplify two DNA fragments about 1900 bp in size and to clone them in pRSETa vector (Fig. 1). Sequencing of their 3'- and 5'- regions (about 750 bp) demonstrated identity with the LdAChEs genes as well as confirmed correct insertion into the expression vector.

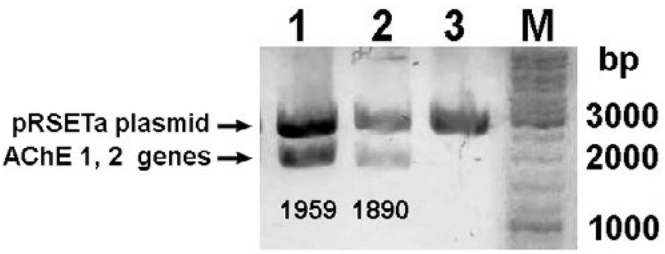

Fig. 1. Restriction analysis of constructs after inserting of genes encoding LdAChE1 (lane 1) and LdAChE2 (lane 2) into pRSETa vector followed by agarose gel analysis. Plasmids were digested by XhoI / EcoRI restriction enzymes. Lane 3 – linearized vector without any insert. The size of the insertion corresponding to the AChE2 gene (1890 bp) is slightly smaller than the size of the insertion corresponding to the AChE1 gene (1959 bp)

Transformation of C41 *E. coli* cells by these constructs, heterologous expression, sonication of bacteria and centrifugation of homogenates followed by SDS-PAGE analysis and immunoblotting with anti-polyHis Abs demonstrated accumulation of both recombinant LdAChEs in the form of insoluble IBs. More effective production of recombinant product was observed, in case of LdAChE2. LdAChE1 protein band showed a slightly higher molecular weight (66–65 kDa) compared to LdAChE2 (about 64 kDa) (Fig. 2A).

Centrifugation of sonicated bacteria, removal of the upper pellet layer of bacterial membranes by careful pipetting and repeating this procedure enabled us to isolate IBs enriched in the expressed proteins. Resuspending of isolated IBs in TB with 8M urea showed that the most part of both recombinant enzymes was not solubilized by this chaotropic agent (Fig. 2B). At the same time, very poorly soluble with 8M urea recombinant LdAChEs were completely dissolved during heating in sample loading buffer for SDS-PAGE containing SDS and 2-mercaptoethanol. Proteins solubilized in the sample buffer were carefully dialyzed and used for rabbit immunization, immune sera raising and purification of specific Abs.

Preparation of samples from five organs and tissues of CPB adults followed by their immunoblotting with Abs against two forms of LdAChEs demonstrated specific recognition of single protein bands of about 70–75 kDa (Fig. 3).

An interesting result of this experiment was a lower intensity of LdAChE1 staining by specific Abs compared to LdAChE2 in the same samples. The most distinctions were observed in fat body and nerve cord samples. This result was not due to different sensitivities of the produced Abs. Firstly, approximately the same level of immunolabeling by two types of Abs was found in the insect gut samples. Secondly, anti-LdAChE1 Abs effectively stained the recombinant form of this enzyme (Fig. 4).

Centrifugation of fat body homogenate followed by extraction of membrane fraction with 0.5 % Triton X-100 showed that a significant part of LdAChE2 was localized in the membrane fraction and could be effectively extracted by this nonionic detergent (Fig. 5).

In case of LdAChE1, the enzyme band was found in rude debris, as well as in soluble fraction, but not in the membrane fraction. This experiment also confirmed the accumulation of significantly larger amounts of LdAChE2 in beetle fat bodies in comparison with LdAChE1. Separation of proteins in a 4–20 % gradient gel showed that the proteins differ in size and produced Abs did not show a cross reactivity.

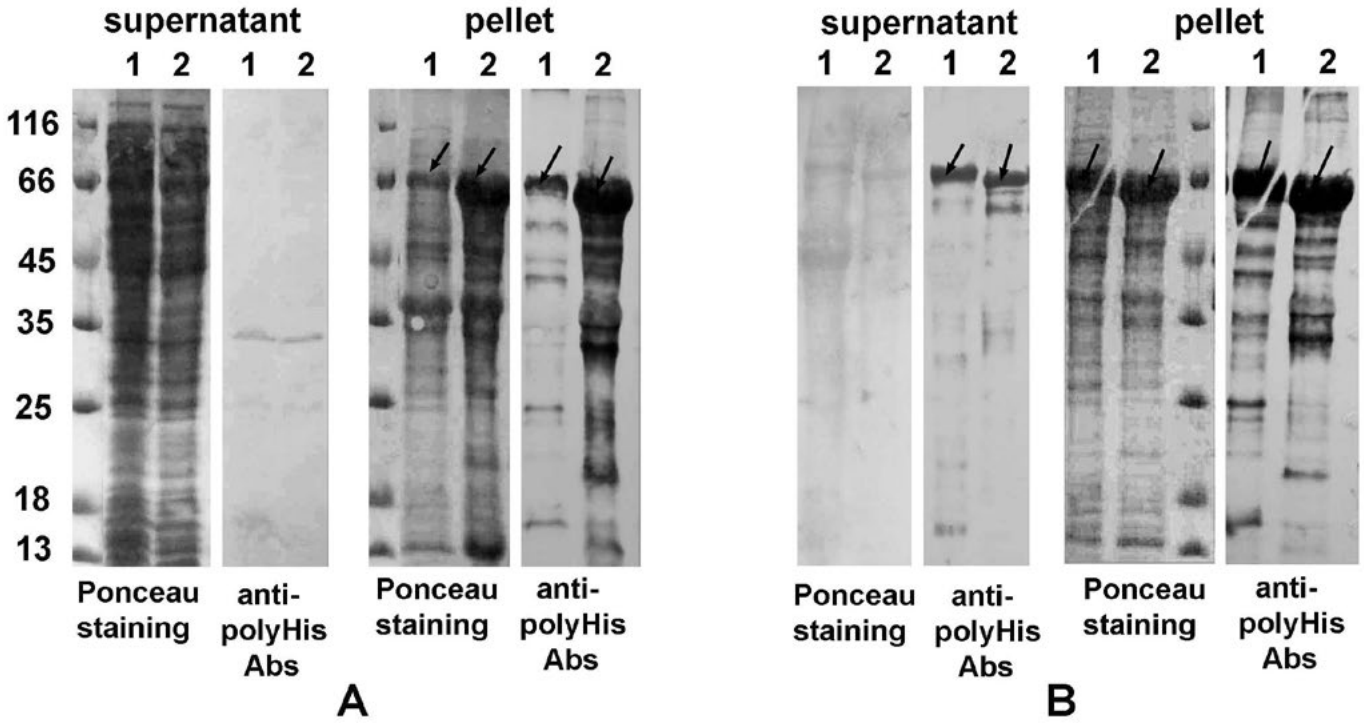

Fig. 2. Immunoblotting of recombinant LdAChE1 (lanes 1) and LdAChE2 (lanes 2) expressed in *E. coli* with anti-polyHis Abs. A. Bacteria after expression were sonicated in TB and centrifugation of homogenate was followed by immunoblotting of supernatants and pellets resuspended in TB to homogenate volume. B. Treatment of isolated IBs with 8 M urea was followed by centrifugation and immunoblotting of supernatants and pellets resuspended in TB to supernatant volume. Proteins separated by SDS-PAGE were transferred on nitrocellulose membrane, stained with Ponceau S and analyzed by immunoblotting with anti-polyHis Abs conjugated with horseradish peroxidase. Recombinant enzymes are indicated by arrows

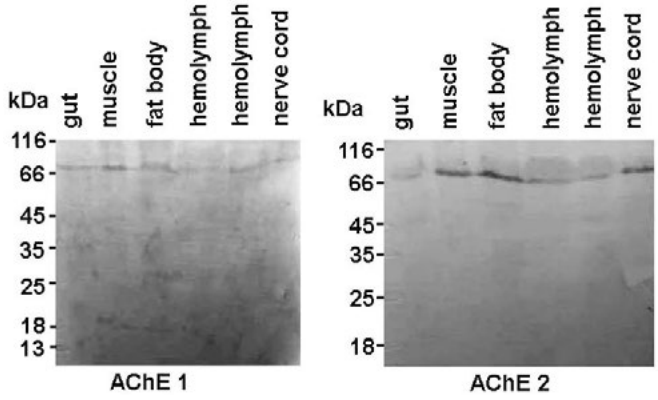

Fig. 3. Immunoblotting of protein samples of five *L. decemlineata* adult organs with anti-AChE1 and anti-AChE2 Abs

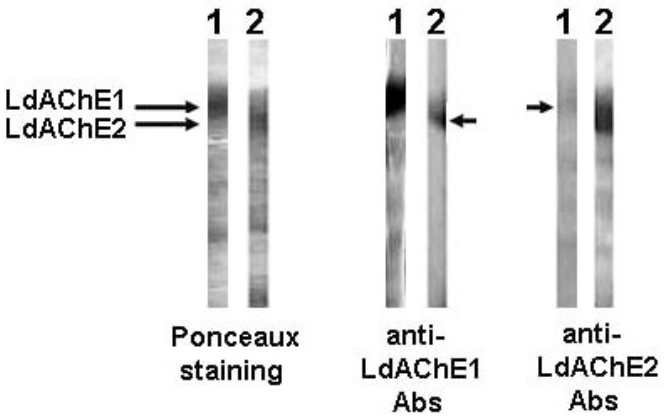

Fig. 4. Immonoblotting of recombinant LdAChE1 (lanes 1) and LdAChE2 (lanes 2) with produced polyclonal Abs. Recombinant proteins are noted by arrows

Discussion

In this study we cloned DNA fragments encoding full-size LdAChEs and expressed them in bacteria *E. coli*. As it was expected, SDS-PAGE analysis of recombinant proteins showed slightly higher molecular weight of LdAChE1 (65–66 kDa) compared to LdAChE2 size (about 64 kDa). However, both of these values were lower than the predicted size of the enzymes (73 kDa and 71 kDa respectively). At the same time, immunoblotting of CPB proteins with anti-LdAChE1 and anti-LdAChE2 Abs demonstrated a larger size of both natural proteins (70–75 kDa) compared with those expressed in bacteria. Since the proteins produced in *E. coli* had an additional 4 kDa tag, the net size difference between recombinant and native forms of LdAChEs seemed to be even greater. Moreover, N-terminal signal peptide responsible for the secretion of AChEs should be removed in the mature forms of both enzymes in insect cells. The higher molecular weight of natural forms of LdAChEs may be explained by their glycosylation, which is typical for the human enzyme (Velan et al., 1993) and requires further study. Since electrophoretic mobility of LdAChE2 with predicted size 71 kDa was lower than the mobility of 73 kDa LdAChE1, the first enzyme may contain more carbohydrate residues.

The absence of cross-reactivity between anti-LdAChE1 and anti-LdAChE2 Abs was expected because two forms demonstrate only 37% of amino acid sequence identity. Linkage of *D. melanogaster* AChE to membranes via

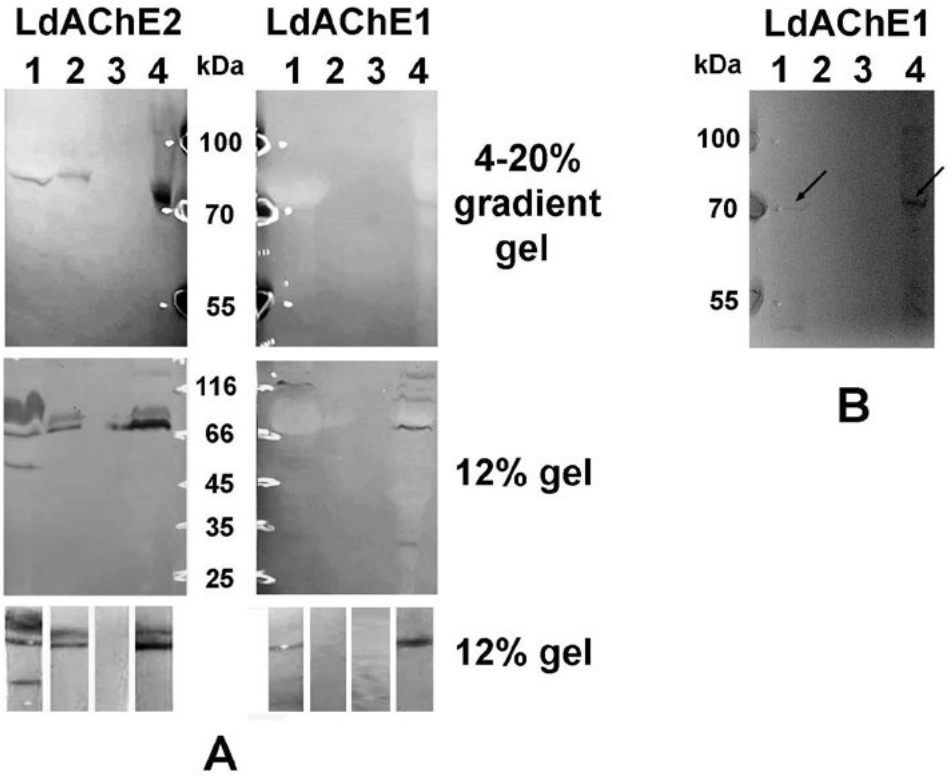

Fig. 5. Immunobolltig of *L. decemlineata* fat body proteins with Abs against LdAChEs. Lane 1 – supernatant of fat body homogenate centrifuged at 30 000 g for 15 min. Lane 2 – supernatant after extraction of pelleted membranes with 0.5% Triton X-100 and re-centrifugation. Lane 3 – pellet after extraction of membranes and re-centrifugation. Lane 4 – rude debris pelleted by clarification of the original homogenate at 1000 g for 15 min. Both pellets (lanes 3,4) were resuspended in TB to homogenate volume before sample preparation. A. Fat body proteins were separated by SDS-PAGE in 4–20% gradient or 12% gels and analyzed by immunoblotting. B. To visualize minor bands, the image of proteins separated in gradient gel and labeled with anti-LdAChE1 Abs was contrasted

a glycolipid anchor (Fournier et al., 1992; Incardona, Rosenberry, 1996) is consistent with membrane localization of its ortholog LdAChE2.

Despite the fact that LdAChE2 was considered as the target for many insecticides for a long time, discovery of the second enzyme form (Revuelta et al., 2011) changed this view. The higher level of LdAChE1 gene expression in *L. decemlineata* embryos, larvae and adults, stronger effect of its RNAi on total AChE activity and insect mortality confirmed an essential role of LdAChE1 in CPB physiology (Revuelta et al., 2011). Later, Kim and co-authors demonstrated that AChE1 was the main catalytic enzyme in heads of *L. decemlineata*, other beetles, and most other insect groups (Kim et al., 2013). A comparison of these data with the results of our study, demonstrating a lower content of LdAChE1 in *L. decemlineata* tissues, suggests the need for new studies using form-specific Abs. Heterologous expression of both AChE forms in insect cell cultures, immunoaffinity purification of natural enzymes from CPB tissues should help to evaluate the role of both enzymes in physiology of the pest and in its resistance to insecticides.

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Полнотекстовая статья

ГЕТЕРОЛОГИЧНАЯ ЭКСПРЕССИЯ АЦЕТИЛХОЛИНЭСТЕРАЗ КОЛОРАДСКОГО ЖУКА *LEPTINOTARSA DECEMLINEATA* В БАКТЕРИЯХ *ESCHERICHIA COLI* И ПОЛУЧЕНИЕ АНТИТЕЛ К ОТДЕЛЬНЫМ ФОРМАМ ФЕРМЕНТА

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Колорадский жук *Leptinotarsa decemlineata* является широко распространенным вредителем пасленовых (сем. Solanaceae). Огромный экономический ущерб, вызываемый этим видом по всему миру, усиливается способностью *L. decemlineata* приобретать устойчивость к основным классам инсектицидов. Ранее такую устойчивость связывалась с мутациями в гене, кодирующим LdAChE2 – ортолог ацетилхолинэстеразы *Drosophila melanogaster*. Однако обнаружение у колорадского жука и многих других видов насекомых второй формы фермента LdAChE1 заставило пересмотреть эти представления. С целью сравнения роли двух форм ацетилхолинэстеразы в физиологии колорадского жука и его устойчивости к инсектицидам, кДНК копии обоих генов были клонированы и экспрессированы в *E. coli*. Иммуноблоттинг с поликлональными антителами против выделенных рекомбинантных белков показал отсутствие перекрестной реакции, более низкое содержание LdAChE1 в тканях имаго колорадского жука по сравнению со второй формой и ассоциацию LdAChE2 с мембранами. В дальнейшем планируется использовать полученные антитела для иммуноаффинной очистки природных форм фермента из тканей жука, а также после экспрессии активных форм в культуре клеток насекомых. Это поможет в оценке роли LdAChE1 и LdAChE2 в физиологии вредителя и в приобретении им устойчивости к инсектицидам.

Ключевые слова: колорадский жук, *Leptinotarsa decemlineata,* ацетилхолинэстераза, гетерологичная экспрессия, поликлональные антитела, иммуноблоттинг

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