THE INFLUENCE OF SALT OF HEAVY METALS ON THE ACTIVITY OF ALKALINE PEPTIDEHYDROLASE FROM *DROSOPHILA MELANOGASTER*

Iryna Leonidivna Ryzhko[1], Oleksandr Mykhaylovych Andriyeskyi[2], Sergii Anatoliyovych Petrov[2]*

[1]Department of Hydrobiology and General Ecology, Odessa I.I. Mechnykov National University, Odessa, Ukraine

[2]Department of Biochemistry, Odessa I.I. Mechnykov National University, Odessa, Ukraine

*Correspondence author e-mail: i.l.ryzhko@onu.edu.ua

S u m m a r y. The influence of salts of heavy metals on the partially purified methods of salting-out, gel chromatography and alkaline electrophoresis of trypsin-like peptidehydrolase of Drosophila larvae was studied. It has been established that the largest inhibitory effect is characterized by cadmium chloride, and the smallest is zinc chloride. The alkaline peptidehydrolase of the *Normal* line and the *Muller-5* line are similar in molecular weight, substrate specificity and response to the negative effects of $CoCl_2$, $CuCl_2$, $ZnCl_2$ i $CdCl_2$, and differ in pH optimum, temperature optimum and affinity to the substrate of BAPNA.

K e y w o r d s: heavy metals, peptidehydrolase, Drosophila.

INTRODUCTION

Proteolytic enzymes play an important role in the exchange of proteins, because their action is the replenishment of the amino acid pool of cells, the degradation of abnormal molecules, as well as the regulation of metabolic processes in the ontogenesis of animals [Vernet et al., 1991; Chougule et al., 2008], which ensures the physiological functioning of living organisms. At present, the role of peptidehydrolase in individual development and adaptive reactions of organisms is one of the few studied biochemistry problems of ontogenesis and general enzymology [Santos et al., 2008]. In this connection, the study of peptidehydrolase in the ontogenesis of animals is important because of the influence on them of certain factors of the environment, in particular – salts of heavy metals.

It is known that metal ions, depending on their nature and concentration, can either activate or inhibit proteolysis [Elpidina et al., 2001; Morty et al., 2005], which can have a decisive influence on the metabolism in general and, consequently, on the processes of growth and development. In addition, heavy metals are one of the most harmful factors of environmental pollution, so studying the laws of their action on animal organisms is extremely relevant.

One of the most convenient model objects is *Drosophila melanogaster* Meigen [Deweikis et al., 2001; Totsky et al., 2001], which has a short generational cycle and gives a large number of descendants. Since, when the environment is polluted by heavy metals, their toxic activity is primarily directed to the gastrointestinal tract, then the functional state of the enzymes of the gastrointestinal tract, the main of which is alkaline peptidehydrolase, can serve as one of the earliest markers for assessing the degree of metabolic disorders in poisoning with salts of heavy metals.

THE AIM OF THE STUDY

The purpose of the presented work is to investigate the biochemical properties and functional activity of the alkaline peptidehydrolase in the intestine of Drosophila in the ontogenesis of the flies, with keeping them under standard conditions and in the presence of heavy metal salts in the environment.

MATERIALS AND METHODS

The studies were conducted on the fly of the species *Drosophila melanogaster* Meigen. In experiments, the wild-type flies – *Normal* and the *Muller-5* mutant – were used. The choice of lines is due to the fact that *Normal* is a strong line with homozygous-dominant manifestations of individual features, while *Muller-5* is weak with homozygo-recessive manifestations of eye and body color.

The flies were kept under standard conditions at + 25 °C [Medvedev, 1968] and on an experimental environment containing salt components (Co2+, Cu2+, Zn2+, Cd2+). The larvae, pupae or imago were homogenized at room temperature for 1-3 minutes in 0.1 M glycine buffer (pH 9.0) in a ratio of body weight to a 1:10 volume of extractant. The resulting homogenates were centrifuged at 10 000 g and +4 °C for 15 minutes. The anilidase activity of alkaline peptidehydrolase was determined by the hydrolysis of 1.0 mM chromogenic substrate N, α-benzoyl-L-arginine-p-nitroanilide (BAPNA, Serva, Germany), in 0.1 M glycine-NaOH buffer pH 9.0 at 382.5 nm [Erlanger et al., 1961; Andriyevskyi, 2002]. The esterase activity of the alkaline peptidehydrolase was determined by the hydrolysis of 1.0 mM N, α-benzoyl-L-arginine-ethyl ether (BAEE, Reanal, Hungary) in 0.1 M glycine-NaOH buffer pH 9.0 at 253 nm [Trautschold , Werle, 1961; Andriyevsky, 2002]. For 1 milli Unit (mU) of activity, the amount of alkaline peptidehydrolase was taken, resulting in the formation of 1 μmol of aromatic product for 1 minute incubation at +37 °C. Specific activity of alkaline peptidehydrolase was determined in mU related to 1 mg of total protein of the investigated tissue extract or enzyme preparation.

Fractionation of proteins was carried out by staged precipitation with ammonium sulfate from 0.5 M to 5.0 M at an interval of 0.5 M. Separation of the fractions of proteins deposited at 3.0 M saturation (NH4)2SO4 was performed by gel chromatography on a column (4.5 x 16.0 cm) filled with Sephadex G-100 (Pharmacia, Sweden) at +4 °C in the low temperature cabinet (Combicoldrac 11). 0.01 M trisacetate buffer pH 7.05 was used for elution. Ion exchange chromatography was carried out on a column (2.8 x 10.0 cm) filled with spherical DEAE-cellulose (Reanal, Hungary), balanced with 0.01 M trisacetate buffer (pH 7.5). The proteins were eluted in a step gradient of KCl solution (from 0.01 M to 1.0 M). In the fractions, the protein content was determined [Lowry et al., 1951] and the activity of the alkaline peptidehydrolase.

For separation and purification of drugs, lamellar vertical electrophoresis in polyacrylamide gel (PAAG) under alkaline and acidic conditions of protein distribution was also used.

The influence of inhibitors and activators on the activity of alkaline peptidehydrolase of larvae was investigated using (in final concentrations) the following reagents: 2-mercaptoethanol (0.04 and 0.06% solution); 0.1 mM solutions of parachlormercurybenzoate (PCMB), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetate (EDTA), methylene blue, 0.1 μg/ml soybean trypsin inhibitor, leupeptin, pepstatin; 0.1 mM solution of L-cysteine and dietotreitol. Pre-incubated 0.1 ml of reagent and 0.1 ml of alkaline peptidehydrolase solution for 30 minutes at + 37 °C. The level of inhibition or activation of the enzyme was measured as a percentage relative to its activity in the samples without the addition of inhibitors or activators.

The influence of metal cations on the activity of alkaline peptidehydrolase was investigated using solutions of chlorides of Co2+, Cu2+, Zn2+ и Cd2+ at the final concentration: 0.2, 0.4, 1.0 and 2.0 mM. The action of metal cations was estimated by the hydrolysis efficiency of 1.0 mM BAPNA solution in glycine-NaOH buffer pH 9.0 for 45 minutes of incubation at +37 °C.

Substrate specificity of purified alkaline peptidehydrolase preparations was determined by hydrolysis: 1.0% native and denatured hemoglobin, 2.0% casein, 1.0% gelatin solution, 2.0 mM phenylalanylphenylalanine solution, prolilalanine, glutamylcyrosine, glutamylphenylalanine; 1.0 mM BAPNA solution and 1.0 mM BAEE solution.

The dependence of the reaction rate on the substrate concentration was analyzed according to the Linouiver-Burke coordinates.

The results were statistically processed. The obtained results were processed using computer programs Microsoft Excel 2007.

RESULTS AND DISCUSSIONS

Isolation and purification of alkaline peptidehydrolase from tissues of Drosophila larvae

For the separation of alkaline peptidehydrolase, the larvae of 72-75 hours of development of the *Normal* and the *Muller-5* Drosophila line was used. The best extraction solution for the production of alkaline peptidehydrolase from the acetone

powder of larvae tissues of both lines was 0.1 M glycine buffer, pH 9.0 when used in the ratio of body weight to extract of 1:6 volume. The suppressant fluid was used for salt fractionation of alkaline peptidehydrolase for using a step gradient (NH4)2SO4. The highest percentage of the yield of enzyme was 64.0-67.0% and the purification factor was 6.8-7.2 (for the *Normal* line and the *Muller-5* line respectively) were obtained at 3.0 M saturation (NH4)2SO4. The protein fraction obtained with 3.0 M saturation (NH4)2SO4 was used for chromatographic studies. The alkaline peptidehydrolase of the larvae of both lines was eluted with the second protein peak (Fig. 1).

Enzymes of the larvae tissues of both lines were eluted under DEAE-cellulose chromatography with 0.01 M trisacetate buffer pH 7.05 after two protein peaks that did not show enzymatic activity (Fig. 2).

The use of ion exchange chromatography allowed to clear the alkaline peptidehydrolase of the wild type Drosophila larvae tissues by 7.5 times, and the alkaline peptidehydrolase of the tissues of the Drosophila larvae of the mutant line was 6.0 times, but the loss of enzyme was 92-94%. Electrophoretic studies confirmed that the enzyme obtained at 3.0 M deposition (NH4)2SO4 and purified by gel chromatography on sephadex G-100 is a protein consisting of a single polypeptide chain (with a distribution coefficient Rf = 0.27).

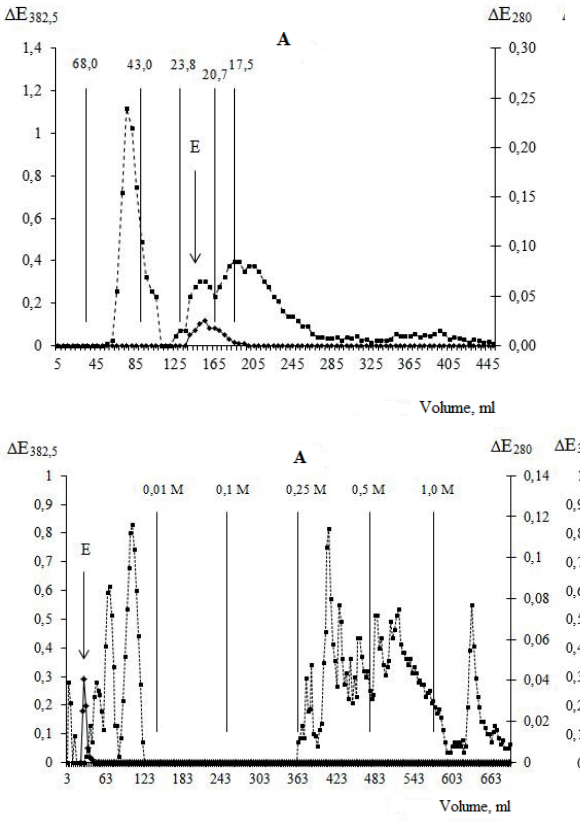


Fig. 1. Chromatographic profile of the distribution of alkaline peptidehydrolase in tissues of larvae on Sephadex G-100 in the analysis of fractions of total proteins obtained at 3.0 M deposition $(NH_4)_2SO_4$. A – line *Normal*, B – line *Muller-5;* Sephadex G-100 (4.5 x 16.0 cm). 8.22 mg of protein was applied; The volume of the fraction was 5.0 ml, the rate – 28.0 ml/h, eluting with 0.01 M trisacetate buffer pH 7.05. Molecular masses of marker proteins are indicated in kDa. E is the yield of alkaline peptidehydrolase; 1 – activity of alkaline peptidehydrolase ($\Delta E_{382,5}$); 2 – extinction of protein solution (E_{280}).

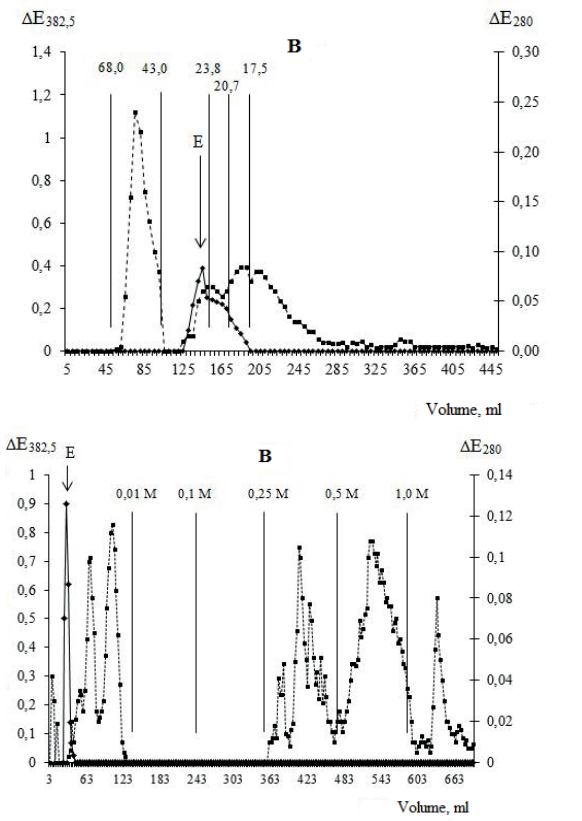


Fig. 2. Profile of ion-exchange distribution of tissue proteins and alkaline peptidehydrolase in tissues of larvae on Sephadex G-100 in the analysis of fractions of total proteins obtained at 3.0 M deposition $(NH_4)_2SO_4$. A – line *Normal*, B – line *Muller-5;* Sephadex G-100 (4.5 x 16.0 cm). 0,227 mg of protein was applied; The volume of the fraction was 3.0 ml, the rate – 28.0 ml/h, eluting with 0.01 M trisacetate buffer pH 7.05. Molecular masses of marker proteins are indicated in kDa. E is the yield of alkaline peptidehydrolase; 1 – activity of alkaline peptidehydrolase ($\Delta E_{382,5}$); 2 – extinction of protein solution (E_{280}).

Physical-chemical and biochemical properties of alkaline peptidehydrolyse of drosophila larvae tissues

In determining the molecular mass of alkaline peptidehydrolase tissues of the Drosophila larvae of the *Normal* and *Muller-5* lines, it was found that its value for alkaline peptidehydrolase for Drosophila for the *Normal* and *Muller-5* is 22 919 and 25 438 Da, respectively.

It turned out that the molecular weight of the alkaline peptidehydrolase of the Drosophila larvae of the *Normal* and *Muller-5* lines are close to each other and practically do not differ from the molecular weight of trypsin from the pancreas of cattle – 23 800 Da [Parshina, 2008].

Determination of the temperature optimum of the alkaline peptidehydrolase tissues of the Drosophila larvae of the *Normal* and *Muller-5* lines revealed that the synthetic substrate of BAPNA most actively cleaves alkaline peptidehydrolase of both lines at +40 °C. The enzymes of the larvae of the flies of the *Muller-5* line have a higher sensitivity to temperature and are active in a more narrow range of temperatures. The maximum activity of the alkaline peptidehydrolase of the larvae tissues of both lines with respect to the substrate of BAPNA is set at pH 9.0.

In the presence of pepstatin, an aspartic protease inhibitor, the activity of the alkaline peptidehydrolase of the *Normal* larvae line decreased, while the activity of the alkaline peptidehydrolase of the larvae of the *Muller-5* line increased by 20%. The activity of the alkaline peptidehydrolase of the larvae of the *Normal* line was greater than the enzyme activity of the larvae of the *Muller-5* line, suppressed in the presence of PMSF. Protein trypsin inhibitor from soy significantly (on 76 and 82%, respectively) reduced the activity of alkaline peptidehydrolyse of both lines. Pre-incubation with PCMB led to a decrease in the activity of the enzyme of the *Normal* line by 32.5 and of the *Muller-5* line by 53.3%. Pre-incubation with 0.04% and 0.06% β-mercaptoethanol resulted in 100% inhibition of the enzyme activity of both lines. The photooxidation of histidine with methylene blue resulted in inhibition of enzyme activity up to 42% in the *Normal* line and up to 23% (*Muller-5* line).

Alkaline peptidehydrolase activity of the Drosophila of both lines was not changed in the presence of L-cysteine and increased slightly (by 11 and 14%) in the presence of dietotreitol.

The obtained results indicate that in the hydrolysis of BAPNA the alkaline peptidehydrolase of both lines is involved: the hydroxyl group of serine, the carboxyl group of aspartic acid, the SH-group of cysteine and the NH-group of histidine. The results obtained are in agreement with the results of studies of enzyme isolated from *Tenebrio molitor* [Elpidina et al., 2005], *Sitophilus zeamais* [Silva et al., 2010] and *Catharsius molossus* [Ahn et al., 2005].

Influence of heavy metal salts on the activity of the purified form of alkaline peptidehydrolase of the drosophila larvae of *normal* and *muller-5* lines

Pre-incubation with 0.2-0.4 mM CoCl2 and CuCl2 increased the activity of the alkaline peptidehydrolase of the original extract of tissues of the Drosophila larvae of the *Normal* line, respectively, by 21.6 and 11.2% (Fig. 3, A) and led to a decrease in purified enzyme activity by 10.8 and 27.5% (Fig. 3, B). In the presence of 1.0 and 2.0 mM CoCl2, the activity of both purified and non-purified enzyme was suppressed by more than 50%. Reduced purity of enzyme activity by 88-95% and complete inhibition of the activity of the crude enzyme were established by pre-incubation with 2.0 mM CuCl2 and ZnCl2 solutions (Fig. 3).

The activity of the alkaline peptidehydrolase of the extract of the *Muller-5* line increased by 15.0-25.7% in the presence of 0.2 mM CoCl2 and CuCl2 (Fig. 4). In the presence of 1.0-2.0 mM CoCl2, the purified and untreated enzyme activity was suppressed by 20% and 67%, respectively.

Complete inhibition of the activity of crude and purified enzyme was determined by pre-incubation with 2.0 mM of ZnCl2 solution and 2.0 mM of CuCl2 solution (Fig. 4). The obtained data indicate inhibition of activity of alkaline peptidehydrolase of Drosophila larvae of both lines in vitro with high concentrations of chlorides Co, Cu, Zn and Cd.

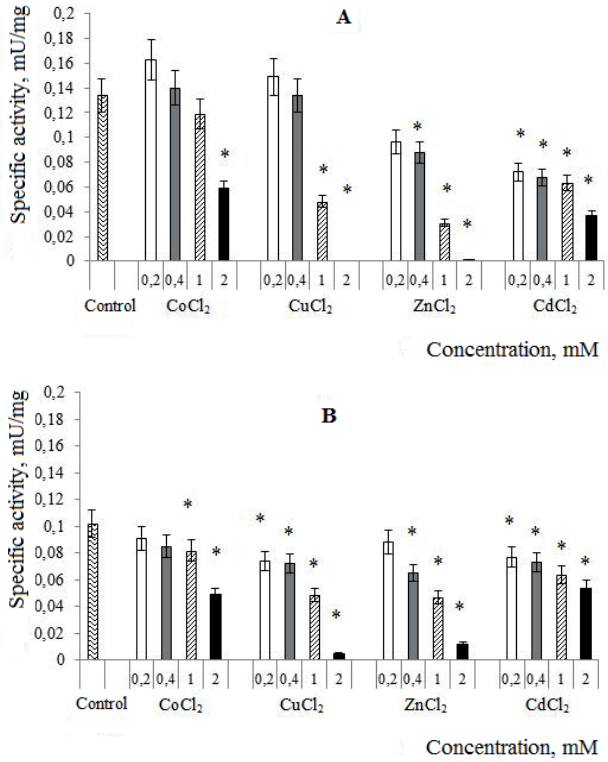
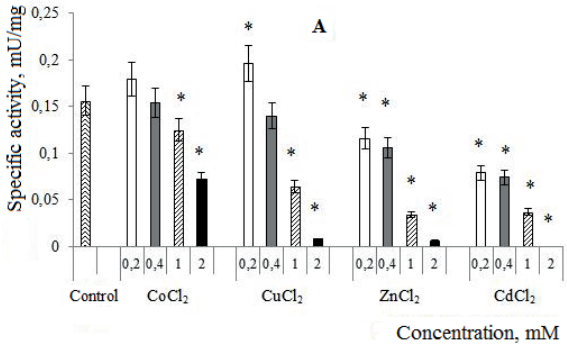


Fig. **3.** Influence of heavy metal salts on the peptidehydrolytic activity of the exit extract (A) and purified (B) alkaline peptidehydrolase of the Drosophila larvae of the *Normal* line * – a significant decrease in the activity of alkaline peptidehydrolase in relation to control. P <0.05; M ± m, n = 6.



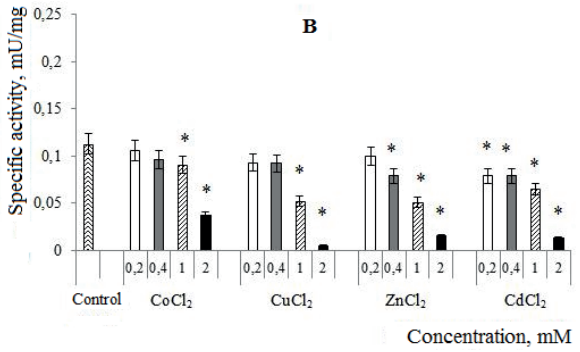


Fig. **4.** Influence of heavy metal salts on the peptidehydrolytic activity of the exit extract (A) and purified (B) alkaline peptidehydrolase of the Drosophila larvae of the *Meller-5* line. * – a significant decrease in the activity of alkaline peptidehydrolase in relation to control. P <0.05; M ± m, n = 6

The influence of metal cations on the activity of alkaline peptide glycosylates of larvae of both lines of Drosophila can be explained by non-specific irreversible inhibition associated with the adsorption of metal ions on the surface of the protein molecule and their interaction with functional active groups, which leads to conformational changes in the enzyme molecule or their effect on the active center. Thus, for example, serine proteases showed an inhibitory effect of metal cations that interact with histidine of the catalytic triad [Varfolomeev, Pozhytkov, 2000].

Substrate specificity of alkaline peptidehydrolase in drosophila larvae of *normal* and *muller-5* lines

The enzyme of both lines had a higher activity in relation to BAEE than in relation to BAPNA. The esterase activity of the alkaline peptidehydrolase of the *Normal* line was 8.1 times higher than the activity of the alkaline peptidehydrolase of the *Muller-5* line, while the amidase activity on the contrary was 2.5 times higher in the alkaline peptidehydrolase of the *Muller-5* line. This indicates that the enzyme possesses both estradiol and amidase activity. The alkaline peptidehydrolase of both lines is also capable of exhibiting endopeptidase activity, confirmation is the hydrolysis of the native protein substrates of casein and hemoglobin. Synthetic dipeptides practically did not hydrolyze alkaline peptidehydrolase in both lines, indicating no peptidase activity in the enzyme. The enzyme of the *Muller-5* line exhibited higher activity in

protein and synthetic substrates than the *Normal* enzyme.

Kinetic characteristics of the alkaline peptide of the hydrolases of tissues of drosophila larvae of the *normal* and *muller-5* lines

It was found that for the alkaline peptidehydrolase of the *Normal* V_{max} line, it was 27.8 mM BAPNA per minute with saturation of the enzyme by substrate at a concentration of 2.0 mM. For the alkaline peptidehydrolase, the *Muller-5* V_{max} line was 35.0 mM BAPNA per minute, and saturation of the enzyme by substrate was observed at a BAPNA concentration of more than 2.0 mM. The definition of Km showed that for alkaline peptidehydrolase of the *Normal* line larvae is 0.88 mM, while *Muller-5* is 0.39 mM for the BAPNA hydrolysis. This indicates that the alkaline peptidehydrolase of the *Muller-5* line has a 2.26 fold greater affinity for the BAPNA substrate than the *Normal* alkaline peptidehydrolase (Fig. 5).

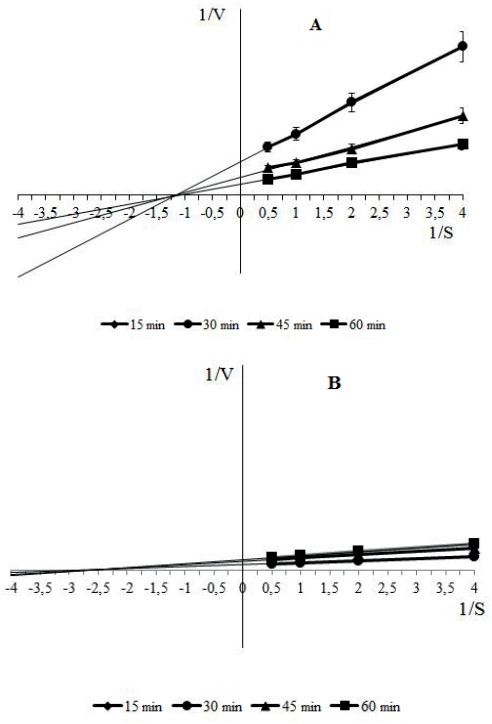


Fig. 5. The reaction rate, which is catalyzed by the alkaline peptidehydrolase of the larvae tissues of the *Normal* (A) line and the *Muller-5* (B) line, depending on the concentration of the substrate in the Linouiver-Burke coordinates.

One can assume that one of the most probable explanations for the differences in molecular weights and physical-chemical properties of the alkaline peptidehydrolase of the wild type Drosophila and the *Muller-5* mutants is the various ways of alternative splicing and biochemical modifications of the precursors of these enzymes by expression of the corresponding genes. Scientific literature has a lot of data that suggests that alternative splicing can lead to the synthesis of substantially different structure and function of products of the same gene depending on specific conditions. This is precisely what happens in the synthesis of some mitochondrial enzymes [Shpakov, Derkach, 2014], proteins of nerve tissues [Zhernosekov, 1999], for the synthesis of proteins regulating sex, etc.

The results of the performed studies indicate an important role of alkaline peptidehydrolase in homeostasis Drosophila ontogenetic development, which may be a promising marker for the detection of environmental pollution, especially heavy metal salts.

CONCLUSIONS

1. It has been established that flies of wild-type *Normal* and mutant flies of the *Muller-5* line have intrinsic properties, but not identical forms of alkaline peptidehydrolase.

2. It has been established that the alkaline peptidehydrolase of Drosophila tissues has peptidehydrolase, BAPNA-azine (amidase) and BAEE-ester activity, while its activity depends on the OH group of serine, the COOH group of aspartic acid, the SH-group of the cysteine, and the NH-group of histidine.

3. It was found that the flies of the *Normal* line had a high esterase activity of the enzyme at the development stage of the larvae and significant amidase activity at the stage of the imago, whereas in the alkaline peptidehydrolase of the flies of the *Muller-5* line, there was a high BAPNA activity at the larva stage at its absence in the imago.

4. It has been established that enzymes isolated from wild and mutant flies differ significantly in molecular weights, electrophoresis mobility, pH and temperature optimizations, Vmax, Km, Ki sensitivity to heavy metal ions, substrate specificity, and the like.

REFERENCES

1. Ahn, M.Y., Hahn B.S., Ryu K.S., Hwang J.S., Kim Y.S. 2005. Purification and characterization of a serine protease (CPM-2) with fibrinolytic activity from the dung beetles. Archives of pharmacal research, 28(7): 816-822.
2. Andriyevskiy O.M., Ryzhko I.L., Radionov O.O. 2002. Effect of metal ions on the level of proteolytic activity in the digestive system of Drosophila ontogeny. Bulletin ONU. Biology. **7**(1): 15-21. (In Ukrainian)
3. Chougule N.P., Doyle E., Fitches E., Gatehouse J.A. 2008. Biochemical characterization of midgut digestive proteases from *Mamestra brassicae* (cabbage moth; Lepidoptera: Noctuidae) and effect of soybean Kunitz inhibitor (SKTI) in feeding assays. J. Insect Physiol. 54(3): 563-72.
4. Deweikis D.N., Nikol'chenko Z.T., Shakhbazov V.G. 2001. Use of the Drosophila test system to detect mutagenic activity of azodyes. Works on fundamental and applied genetics. Kharkiv: 275-285. (In Russian)
5. Elpidina E.N., Tsybina T.A., Dunaevsky Y.E. et al. 2005. Chymotrypsin-like proteinase from the midgut of *Tenebrio molitor* larvae. Biochimie. 87(8): 771-779.
6. Erlanger B., Kokovsky N., Cohen W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. and Biophys. 95(2): 271-278.
7. Lowry O., Rosebrough N., Farr A., Randall R. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 1: 265-275.
8. Medvedev N.N. 1968. The practical Genetics. M.: Nauka: 294. (In Russian)
9. Morty R.E., Vadász I., Bulau P. et al. 2005. Tropolysin, a new oligopeptidase from *African trypanosomes*. Biochemistry. 44 (44): 14658-69.
10. Parshina V.V. 2008. Activity of amylolytic and proteolytic enzymes of chyme in cows under the action of feed additives with adsorption properties. Agricultural Biology. 2: 72-77. (In Russian)
11. Santos A.L., Soares R.M., Alviano C.S., Kneipp L.F. 2008. Heterogeneous production of met allotype peptidases in parasites belonging to the family Trypanosomatidae. Eur. J. Protistol. 44(2): 103-13.
12. Shpakov A.O., Derkach K.V. 2014. Soluble forms of adenylate cyclase of spermatozoa. Cytology. 56(1): 5-13. (In Russian)
13. Silva L.B., Reis A.P., Pereira E.J. et al. 2010. Partial purification and characterization of trypsin-like proteinases from insecticide-resistant and -susceptible strains of the maize weevil, *Sitophilus zeamais*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 155(1): 12-9.
14. Totsky V.N., Khaustova N.D., Gandiruk N.G. 2001. Gene balance and adaptation of natural and artificially created genotypes of *Drosophila melanogaster*. Works on fundamental and applied genetics. Kharkiv:140-155. (In Russian)
15. Trautschold I., Werle E. 1961. Spectrophotometrische Bestimmung des Kallikreins und seiner Inaktivatoren. Z. Phys. Chem. 325: 48-59.
16. Varfolomeev S.D., Pozhitkov A.E. 2000. Active centers of hydrolases: the main types of structures and the mechanism of catalysis. Herald of Moscow Un. 41(3): 147-156. (In Russian)
17. Vernet T., Khouri H. E., Laflamme P., Tessier D.C., Musil R., Gour-Salin B.J., Storer A.C., Thomas D.Y. 1991. Processing of the papain precursor. Purification of the zymogen and characterization of its mechanism of processing. J. Biol Chem. 266(32): 21451-7.
18. Zhernosekov D.D. 1999. Structural and functional features of neurospecific adhesion proteins of the immunoglobulin family. Biopolymers and cell. 15(2): 143-148. (In Russian)