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INVESTIGATION OF THE *RANA RIDIBUNDA* TADPOLE'S LIVER ARGINASE TRYPTIC HYDROLYSIS PRODUCTS BY GEL-FILTRATION METHOD

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In the current study we investigated the *Rana ridibunda* tadpole's liver arginase limited trypsinolysis in order to obtain information about the sensitive bonds towards proteases, as well as about the structural and functional interrelations during metamorphose. After 18 hours of limited trypsinolysis in the early stages of tadpole development (21–27) appeared relatively massive fragments, the degradation of fragments deepen concomitant to the real metamorphose approach (28–29 stages). Enzyme pre-incubation with Mn^{2+} leads to stabilization of activity and protects the enzyme from proteolytic inactivation.

Keywords: limited trypsinolysis, Mn^{2+} ions metamorphose, proteolytic inactivation, enzyme pre-incubation.

Introduction. Studies of Biochemistry Chair of YSU on the subject of frog liver arginase revealed the structural characteristics of native and reactivated enzyme's subunits before and after metamorphose. It has been shown that studied enzyme has structural and consequently functional characteristics, which distinguishes it from various arginases in different levels of evolution development. We investigate the *Rana ridibunda* frog liver arginase functional and structural characteristics during ontogenetic development [1–3]. Formerly we investigated the limited hydrolysis of adult *R. ridibu*nda frog liver arginase by trypsin. After 2 to 22 hours of limited hydrolysis of arginase appeared relatively large fragments, which further degradation occurred with time elongation. Depending on the extent of trypsinolysis and enzyme purity, from 2 to 5 fragments can be detected. It has been shown that thermal refinement partly affects on trypsinolysis process, and further degradation of enzyme occurs at the expense of low molecular versions [1].

The native protein's limited proteolysis is considered as one of the prevailing methods of investigations of proteins and isoproteins structural and functional characteristics. The mentioned method allows determination of the position of the sensitive bonds towards proteases, which exists on the surface of the molecule. Research about the type and quantity of derived fragments can give valuable

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information about the stage of the polypeptide chain scroll, as well as conformation of macromolecules [4]. Different proteins display diverse sensitivity towards the influence of proteases. This method was successfully used to investigate structural and functional features of certain enzymes such as serum albumin [5], one of the molecular forms of glycogen synthesis [6], creatine kinase [7], adenosine triphosphatase [8], myosin [9] and bovine liver arginase [4].

In this study, we attempted to investigate the *R. ridibunda* tadpole's liver arginase limited hydrolysis by trypsin.

Materials and Methods. *R. ridibunda* Pallas tadpoles are served as a research object. The study was approved by the ethics committee of the Biochemistry Chair of YSU and implemented in accordance with the Helsinki convention related to animal studies. Tadpoles were collected from the lake Ayghrlich and Metsamore River and maintained in glass aquariums with faucet water at room temperature. The development classification was done by Terentev, which was based on the external morphology of frogs [10]. The first 19 stages are embryonic and the 30th stage corresponds to the beginning of the real metamorphose. For tadpoles from 21 to 27 stages of development, genesis of teeth and gills, differential, but not functional hind limbs development are typical, and from 28 to 29 stages of development, limbs are movable and well developed, fore limbs are formed however the signs of tail disappearance are not visible yet. The *R. ridibunda* tadpole's liver arginase preparations final concentration is 5%, which were prepared by distilled water. Enzyme activity was measured by Ratner and Pappas method [3], subsequently the urea, which emerges by substrate dissection, was measured according to the method of Archibald, modified by Moore and Kauffman [3].

The absorbance of protein was measured in a spectrophotometer (Genesys 10S UV-VIS) at 280 *nm*. The molecular weights of fragments were determined by Gel-filtration method (Sephadex G-200, Uppsala, Sweden). The equilibration and elution was done with buffer solution containing 0.05 *mol* glycine-NaOH (pH 9.5) at $25^0 C$, elution velocity is 20 *ml* per hour.

Marker proteins (urease, alcohol dehydrogenase, human serum albumin, pepsin, trypsin and ribonuclease) are used for determining the molecular masses. During the experiments we used purified, chymotrypsin free trypsin preparations, which was added by 1 *mg*/*ml* concentration to 5% enzyme preparation. The reaction stopped by soybean antitrypsin with 0.2% concentration in the solution.

Results and Discussion. In the current study the limited trypsinolysis of *R. ridibunda* tadpole's liver arginase was investigated; it is notable that the tadpoles were in certain phase of development (21–27 and 28–29 stages). The results are presented in theTable.

Furthermore, in the present report we investigate the effect of bivalent Mn^{2+} ions on the sensitivity of *R. ridibunda* tadpole's liver arginase proteolytic inactivity. According to the results obtained from our previous study of adult frog liver arginase, Mn^{2+} increases the sensitivity of studied enzyme towards trypsin activity, and inactivates the pre-incubated samples. The increase in the number of fragments and decrease of fragments weights proves that point. Probably, after pre-incubation by Mn^{2+} conformation changes occur in arginase, which reflects on the sensitivity of enzyme towards proteolysis [11].

The effect of Mn^{2+} ions on the sensitivity of Rana ridibunda tadpole's liver arginase towards proteolytic inactivation

Sample	Development Phase	Conditions	Molecular Weights of Fragments, *Dalton*	№ of Fragments
Partially purified enzyme	21–27	18 *h*, $-Mn^{2+}$	36000, 18000, 8200	3
		18 *h*, $+Mn^{2+}$	36000, 18000, 8200	3
	28–29	18 *h*, $-Mn^{2+}$	37050, 22910, 15850, 6918	4
		18 *h*, $+Mn^{2+}$	37050, 15140, 8511	3

Comparing the results of the presented investigation with the results of limited proteolysis of adult frog's liver arginase can be observed, that after 18 *h* of trypsinolysis in the early stages of tadpole development (21–27) appeared 3 fragments, while concomitant with the pre-metamorphic stages (28–29) in the preparation without Mn^{2+} appeared 4 fragments, which corresponds to the number of derived fragments of adult frog liver arginase trypsinolysis in the same conditions [11]. The number of derived fragments are the same in the presence of Mn^{2+} at the early stage of tadpole development (21–27), as well as premetamorphic stages (28–29).The results will lead us to the conclusion that enzyme pre-incubation with Mn^{2+} protected the premetamorphic tadpole's liver arginase from proteolysis.

According to the obtained results we can assume, that further characteristic investigation of *R. ridibunda* frog liver arginase limited trypsinolysis and structural research of derived fragments will allow us to obtain information about the polypeptide chain, amino acids structure and successions, likewise about the position of proteolysis sensitive bonds during ontogenesis. However, our data allow us to discuss the structural differences and dimensional array of two molecular models of *R. ridibunda* frog's liver arginase, which distinguishes it from various arginases in different levels of evolution development.

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