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THE EFFECT OF SEVERAL BIVALENT CATIONS ON THE SENSITIVITY OF *RANA RIDIBUNDA* FROG LIVER ARGINASE TOWARDS PROTEOLYSIS

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In the current study the products of *Rana ridibunda* adult frog liver arginase hydrolysis by trypsin were investigated in order to obtain information about the polypeptide chain scroll level, as well as conformation of the enzyme under consideration. The results revealed that under the influence of trypsin (18 hour, pH=9.5, 25^0C) 4 relatively large fragments were appeared. The augmentation of divalent ions` Mn, Co, Ni, Mg in liver extracts increases both the enzyme activity and the sensitivity towards proteolytic inactivation. According to the results, Mg ions do not have an effect on the sensitivity of the enzyme towards proteolysis and are partly considered a protector against the effect of trypsin, which probably is the result of the changes in enzyme's conformation, which leads to trypsin's sensitive bonds blockade.

Arginase – bivalent cations – limited trypsinolysis

Ժել-ֆիլտրացիայի եղանակով (Sephadex G-200) հետազոտվել են *Rana ridibunda* հասուն գորտի լյարդի տրիպսինով հիդրոլիզի արգասիքները, նպատակ ունենալով ստանալ տվյալներ պոլիպեպտիդային շղթայի պարուրման աստիճանի և հետազոտվող ֆերմենտի կոնֆորմացիայի մասին: Ցույց է տրված, որ տրիպսինի ազդեցությամբ (18 ժ, pH=9.5, 25^0C) կատարվում է պոլիպեպտիդային շղթայի 4 համեմատաբար խոշոր հատվածների առաջացում: Երկվալենտ իոնների՝ Mn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+} ավելացումը լյարդի էքստրակտներին բերում է ֆերմենտի ակտիվացմանը և միաժամանակ պրոտեոլիտիկ ինակտիվացման նկատմամբ զգայնության բարձրացմանը: Երկվալենտ Mg^{2+}-իոնները չեն ազդում լյարդի արգինազի պրոտեոլիտիկ ինակտիվացման զգայնության վրա և որոշ չափով ունեն պաշտպանիչ դեր տրիպսինի ազդեցության նկատմամբ, ինչը հավանաբար կապված է ֆերմենտի մոլեկուլի կոնֆորմացիոն փոփոխությունների հետ, որը բերում է տրիպսինի նկատմամբ զգայուն պեպտիդային կապերի շրջափակմանը:

Արգինազ – երկվալենտ կատիոններ – սահմանափակ տրիպսինոլիզ

С целью получения информации о степени свернутости полипептидной цепи и о конформации макромолекулы аргиназы печени взрослых лягушек *Rana ridibunda* методом гель-фильтрации на колонках с сефадексом G-200 исследовали продукты триптического гидролиза изучаемого фермента. Показано, что под действием трипсина в течение 18 ч при pH 9.5 и 25^0C происходило образование 4 фрагментов. В результате инкубирования экстрактов печени в присутствии Mn^{2+}, Co^{2+}, Ni^{2+} происходила активация аргиназы, при этом возрастала чувствительность ее к протеолитической инактивации. Ионы Mg^{2+} не влияли на чувствительносить фермента к трипсину и в определенной мере выступали в роли протектора, что, очевидно, связано с конформационными изменениями в молекуле фермента, которые приводят к блокированию некоторых чувствительных к трипсину пептидных связей.

Аргиназа – двухвалентные катионы – частичный трипсинолиз

Studies on the subject of reversible inactivation of frog liver arginase conducted at the Chair of Biochemistry of YSU have revealed the structural characteristics of native and reactivated enzyme's subunits. 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 [1, 2, 3]. The native protein limited proteolysis is considered one of the prevailing methods for investigation of proteins and isoproteins structural and functional characteristics. The mentioned method allows determining 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 may provide valuable information about the polypeptide chain scroll level, as well as conformation of macromolecules. Different proteins display diverse sensitivity towards the influence of proteases, for instance, serum albumin splits into relatively 4 large fragments by trypsine [4], meanwhile one of the molecular forms of glycogensynthase gets completely degradated into low molecular fragments after 30 min. of trypsinolysis [5]. This method was successfully used to investigate structural and functional features of certain enzymes such as, creatine kinase [6], adenosine triphosphatase [7], myosin [8] and bovine liver arginase [9].

Herein the effects of several bivalent cations (Mn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}) on the sensitivity of *Rana ridibunda* frog liver arginase towards proteolysis is described.

Materials and methods. *Rana ridibunda* Pallas adult frogs are used as a research object. The study was implemented in accordance with the Helsinki convention on animal studies. The *Rana ridibunda* frog liver arginase preparation final concentration is 5 %, which were prepared by distilled water. Enzyme activity was measured by Ratner and Pappas method [10], subsequently the urea, which emerges by substrate dissection, was measured according to the method of Archibald, which was modified by Moore and Kauffman [10]. 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) in 25^0C, 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 (fig. 1). 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. Bivalent cations were added 25 μmol per 1 ml to the studied sample.

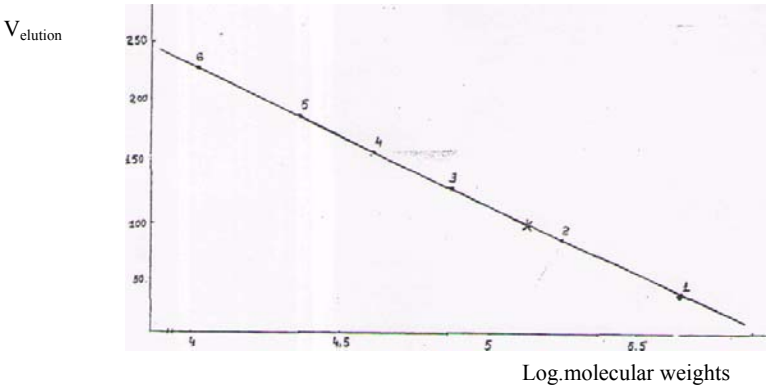

Fig.1. Molecular weights of Rana ridibunda frog liver arginase determined by gel-filtration method (Sephadex G-200).
1.Urease, 2.alcohol dehydrogenase, 3.human serum albumin, 4.pepsin, 5.trypsin, 6.ribonuclease, *Arginase

Results and Discussion. In the present report, we investigated the effect of several bivalent cations` Mn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+} on the sensitivity of liver arginase inactivity. Mn^{2+} ions are necessary for the stability of enzyme, activity expression and maintenance of molecular charge and olygomer structure.

At the begining, 2 hours pre-incubation of arginase in the presence of studied cations has been done. As shown in tab. 1 and fig.2 after 2 hours of pre-incubation with Mn^{2+} ions arginase activity increases by 97%.

Table 1. The effect of several bivalent ions Mn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+} on the sensitivity of arginase inactivity n=5

Control (without ion)	Activation per 1 ml Activation and inhibition %			
	Mn^{2+}	Co^{2+}	Ni^{2+}	Mg^{2+}
3.4	6.7	4.7	3.94	2.54
Increase of activity (%)	+97	+38	+16	-25.3

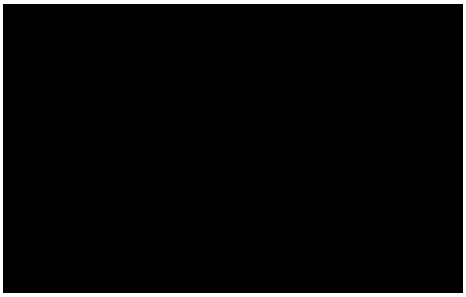

Fig.2. The effect of several bivalent ions Mn2+, Co2+, Ni2+, Mg2+ on the proteolytic inactivation of arginase.

As one can notice from the results, 2 hours pre-incubation of arginase with Co^{2+} and Ni^{2+} also activates the enzyme 38% and 16%, respectively, in the case when Mg ion inhibit the activation by 25.3%. The indispensable time for providing complete restoration of activity depends on the ion concentration, temperature and pH. According to the author, the enzyme connection segment is inaccessible and for ion connection it requires the reunion of the atoms [9].

Subsequently after enzyme pre-incubation with bivalent cations, hydrolysis is performed by trypsine, of which brief characteristics are presented in tab. 2.

Table 2. The effect of Mn^{2+} ions on the sensitivity of *Rana ridibunda* adult frog liver arginase towards proteolytic inactivation (Initial activity 29000 μmol/g)

	Limited proteolysis by trypsin without pre-incubation by Mn^{2+}		Limited proteolysis by trypsin after pre-incubation by Mn^{2+}	
Activation	0		8700	
Sample	Extent of trypsinolysis	Molecular weights of fragments	Extent of trypsinolysis	Molecular weights of fragments
Partly purified	2	85000, 35000	2	68000, 36000, 25000
	4	85000, 24000	4	68000, 36000, 13700
	8	82000, 40000, 13700	8	35000, 25000, 13700
	18	62000, 40000, 33000, 13700	18	36000, 30000, 13700, 10000, 8000

Unlike the non-incubated samples by Mn^{2+}, in pre-incubated samples the activation is preserved for 30%. In pre-incubated samples by Mn^{2+}, after 2 hours hydrolysis, appeared 3 fragments, which molecular weights are less than molecular weights of non incubated preparations (tab. 2, fig. 3-6, Blue dextran and trypsin corresponding peaks ere not represented in the curves). After 4 hours of trypsinolysis the fragments with 68000, 36000 and 13700 Da molecular weights were appeared, which means the further dissect-

tion of the 3rd fragment. As well as, after 8 hours 3 fragments with lower molecular weights (35000, 25000 and 13700 Da) were appeared. Furthermore, after 18 hours trypsinolysis 5 fragments with 36000, 33000, 13700, 10000 and 8000 Da molecular weights were appeared.

It has been previously shown in tab. 1 that, Mn ions 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 in the sensitivity of enzyme towards proteolysis. The results indicate that although the initial activation of enzyme (Co^{2+} 38%, Ni^{2+} 16%), these ions also increase the sensitivity of arginase towards the influence of trypsin more than Mn^{2+} (fig. 7, 8, 9).

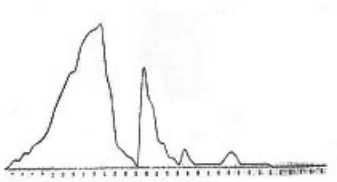

Fig.3. Trypsinolysis after 2 hours

Fig.4. Trypsinolysis after 4 hours

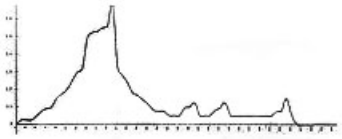

Fig.5. Trypsinolysis after 8 hours

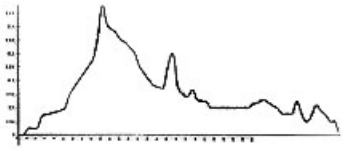

Fig.6. Trypsinolysis after 18 hours

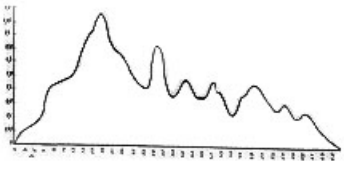

Fig.7. Effect of Co ions (After 18 h Trypsinolysis)

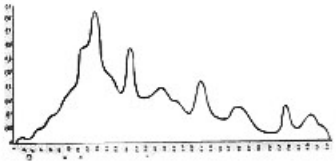

Fig.8. Effect of Ni ions (After 18 h trypsinolysis)

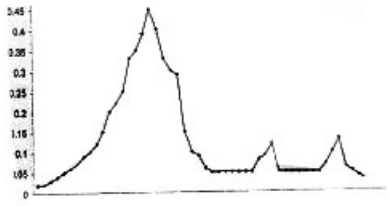

Fig.9. Effect of Mg ions (After 18 h Trypsinolysis)

Fig.3. The effect of Mn ions on the sensitivity of *Rana ridibunda* liver arginase towards proteolysis

Compared to the Mn^{2+}, trypsin shows a stronger degradation effect on Co^{2+} and Ni^{2+}. In addition, pre-incubation by Mg^{2+} prohibits the arginase activation by 25.3%; nevertheless,it is considered a protector during the limited trypsinolysis by protecting arginase from trypsin's proteolytic effect therefore, quantity of fragments decreases to 3, which supposedly is considered as the result of changes in enzyme's conformation (tab.3).

Table 3 .The effect of of bivalent cations on the sensitivity of *Rana ridibunda* frog liver arginase towards proteolytic inactivation (Extent of trypsinolysis` 18 hours) n=4

Ions	Molecular weights of fragments						№ of fragments
Trypsinolysis without pre-incubation by ions	62680	40960	33740	12320			4
Mn^{2+}	36000	30000	13700	10000	8000		5
Co^{2+}	44700	37500	19800	13700	10300	8200	6
Ni^{2+}	40600	34500	26000	15800	11800	8000	6
Mg^{2+}	72860	28180	16790				3

It is notable, that our results do not coincide with the reports of Bond relating to bull liver arginase, where it is noted that Mg^{2+} does not activate enzyme and does not effect on inactivation sensitivity. However, it corresponds with the Bond results about rat liver arginase, according to pre-incubation with Mn^{2+} and Co^{2+} increases enzyme sensitivity against proteolytic inactivation [11]. There is evidence that Mn^{2+} is considered as a protector for rat liver arginase against protease effects [12]. In human liver arginase loss of Mn^{2+} makes the enzyme more sensitive towards proteolytic inactivation [13]. Thus, it can be stated that Mn^{2+} and Co^{2+} ions are stimulating the activity of *Rana ridibunda* liver arginase, but do not protect enzyme against the effect of trypsin during the limited inactivation, which coincides with the evidence reported by Carvajal relating to human liver arginase [13], and do not coincide with the Diez studies about rat liver arginase [12].

In conclusion, we consider that it is necessary to carefully transfer particular data of arginases by different origins from one class of animal to another. Further studies will allow discovering the molecular variation in similar enzyme, structural and dimensional disposal differences and simultaneously investigate the species specificity.

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