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## The influence of Al<sup>3+</sup> ion on porcine pepsin activity *in vitro*

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### Abstract

The *in vitro* effect of Al<sup>3+</sup> ions in the concentration range 1.7·10<sup>-6</sup> M–8.7·10<sup>-3</sup> M on pepsin activity at pH 2, *via* kinetic parameters and its electrophoretic mobility was evaluated. Kinetic study demonstrated the existence of an activation effect of Al<sup>3+</sup> at pH 2 on pepsin molecule. Kinetic analysis with respect to concentrations of haemoglobin showed that Al<sup>3+</sup> ions increase the maximal velocity (V<sub>max</sub>) and k<sub>cat</sub> values rather than apparent affinity for substrate (K<sub>S</sub>) implying the non-competitive nature of activation which indicated that aluminium was a non-essential activator of partial non-competitive type. The values of the equilibrium constants K<sub>S</sub> and K<sub>mA</sub> for dissociation of corresponding complexes were evaluated as 0.904 ± 0.083 mM and 8.56 ± 0.51 μM, respectively. Dissociation constant K<sub>A</sub>, of activator from enzyme-activator complex calculated *via* kinetic and direct measurement of Al<sup>3+</sup> binding data, as well as activation constant A<sub>50</sub>, the activator concentration that gives a rate equal to half at a saturating concentration of activator, were found to be 8.82 ± 0.90 μM, 8.39 ± 0.76 μM, and 8.05 ± 0.48 μM respectively. Native PAGE electrophoresis shows the decrease in electrophoretic mobility of pepsin and confirms modification of the electric charge and conformational changes of pepsin caused by bound Al<sup>3+</sup> on the pepsin molecule. Al<sup>3+</sup> induced conformational changes of pepsin were verified by UV-VIS and IR spectra. Moreover, the absence of conformational changes in the haemoglobin molecule in the presence of Al<sup>3+</sup> ions confirms that the obtained activation is a consequence of conformational changes caused only in the pepsin molecule.

**Keywords:** Pepsin, aluminium, kinetics, activation, electrophoretic mobility

### Introduction

Porcine pepsin A (EC 3.4.23.1), a prominent member of the aspartic protease, is the principal proteolytic enzyme of gastric juice and as the best understood of this family of proteinases it was used as a model to study related enzymes. As all aspartic proteinases pepsin molecule consist of two homologous lobes composed predominantly of β-sheets separated by a hinged substrate-binding cleft. Two active site aspartic residues occupy the cleft to which admittance is restricted by a hinged flexible flap region [1–6]. The pepsin consists of 326 residues with molecular weight of 35000 Da and it derives from its zymogene

pepsinogen, by removal of 44 amino acids, from its amino terminus, to give a single chain enzyme with a low pI and three disulfide bridges. Each lobe of pepsin contains a catalytic aspartic acid residue Asp<sup>32</sup> and Asp<sup>215</sup> located at the centre of the binding cleft between the domains. One of two Asp residues has to be protonated and the other deprotonated, for the protein to be active [1]. Due to the catalytic residues, the active pH ranges from 1.0 to 5.0 [7–11]. Because pepsin has been well structurally characterized, it represents an appropriate model to study the effects of metal ions on structure, function and kinetic behaviour [12,13].

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Aluminium does not belong to essential elements, and as a non-regulatory ion can be toxic to many organisms. Even it is a most abundant metal, comprising almost 8% of the earth's crust; its concentration in living system is very low. Aluminium can occur in a number of different forms in water, and his chemistry in water solutions is very complex where many chemical parameters, including pH, determine which aluminium species are present in aqueous solutions. Aluminium enters the human body from foods, particularly those containing aluminium compounds used as food additives and from drinking water. An additional source of aluminium is Al-containing antacids that have been widely used in therapy for dyspepsia. Average daily dietary aluminium intake is approximately 2–6 mg Al/day in children and 6–14 mg Al/day in adults, according World Health Organization Technical Reports [14,15].

Aluminium toxicity in patients with renal failure is well documented [16,17]. It has been proposed (with some controversy) as a cofactor in the pathogenesis of Alzheimer's disease, as well as other neurodegenerative pathologies. Accumulated aluminium accelerates process of aggregation of amyloid beta peptide and causes activation of present secretases, which belong to aspartic proteases family [18–21]. Some authors perform the studies which included small peptide molecules (13 amino acid residues) which sequence corresponds to those founded in proteins of neuron plaques, shows that bounded aluminium ions cause's significant conformational changes and induce increase in beta structure content (conversion alpha to beta up to 90%) [22].  $Al^{3+}$  ion altered formation and stabilization of beta structure is probably direct consequence of complex formation between  $Al^{3+}$  ion and carboxylate ion in amino acids residues [23]. However, very little knows about whether  $Al^{3+}$  ions, that enter human organism, can affect activity of gastrointestinal enzymes and influence digestion and utilization of nutrients. Aluminium affects the activity of  $\alpha$ -chymotrypsin, in the presence of its specific bovine pancreatic trypsin inhibitor; at the pH 6.5 in the presence of aluminium, the enzyme activity is doubled, and the inhibitor is only 1% as effective as in the absence of the metal ion [24]. Recently it has been shown that  $Al^{3+}$  ions inhibited trypsin activity, but do not pepsin. It was observed highest activation of pepsin activity by 191% in the presence of 25  $\mu$ g  $Al^{3+}$ /mL of reaction mixture [25].

As the mechanism of  $Al^{3+}$  ions on pepsin activity is not still clear, the objective of this study is to investigate the *in vitro* influence of different concentrations of  $Al^{3+}$  ions, physiological and toxic ones, on pepsin activity. Moreover, extensive kinetic studies were undertaken to determine the nature of the enzyme modulation (type and mechanism) by investigated metal ion. Besides, we compared the electrophoretic mobility in the absence and presence of investigated  $Al^{3+}$  ions.

## Materials and methods

### Chemicals

Pepsin, lyophilised powder, was purchased from Sigma–Aldrich, and used without further purification. Haemoglobin from bovine blood was purchased from Sigma–Aldrich and was used as substrate. PAGE-reagents were purchased from Sigma–Aldrich. Other chemicals aluminium chloride ( $AlCl_3 \cdot 6H_2O$ ), hydrochloric (HCl), trichloroacetic acid (TCA) and potassium chloride (KBr) were obtained from MERCK, all of reagent grade were prepared prior to use.

### Enzyme assay

The Worthington method based on enzyme-catalyzed measured rate of hydrolysis of denatured haemoglobin (Hb) substrate was used for evaluation of enzyme activity in the absence (control) and presence of  $Al^{3+}$  ions [26]. Pepsin activity was determined in an incubation medium containing 1 mL of pepsin solution (20  $\mu$ g/mL in 0.01M HCl, pH 2), 5 mL haemoglobin solution (2% solution of haemoglobin in 0.01M HCl). The working solutions were incubated for 10 min at 37°C. The reaction was stopped by addition of 10 mL 5% TCA. The absorbance of clear filtrates recorded at 280 nm, and activities were calculated by the equation:

$$U_{\text{units/mg}} = \frac{[A_{280}(\text{Filtrate}) - A_{280}(\text{Blank})] \times 1000}{10 \text{ min} \times \text{mg enzyme in reaction mixture}} \quad (1)$$

### Estimation of kinetic parameters

Kinetic analysis was carried out according to a slightly modified method of Anson [26], by following the initial velocity of the enzymatic reaction in the absence and presence of  $Al^{3+}$  ( $1.7 \cdot 10^{-6}$ – $8.7 \cdot 10^{-3}$  M) and increasing concentrations of haemoglobin ( $0.025$ – $4 \cdot 10^{-3}$  M). All the assays were performed at pH 2. The data analyzed by the software package Origin 6.1 and the results were recalculated using EZ FIT program [27].

### Elemental analysis

For direct measurement of  $Al^{3+}$  binding, sample of 5 mL of pepsin solution ( $3 \cdot 10^{-5}$  M of in 0.01 M HCl, pH 2), was extensively dialyzed. Solution was placed in dialysis bags (9 mm flat width) with a molecular cut of 12 to 14 kDa (Sigma- Aldrich) and dialysed against 50 mL  $AlCl_3 \cdot 6H_2O$  solution (0.01 M pH 2), for 36 h at 4°C with gently stirring. All solutions were prepared in distilled-deionised water. Dialysis bags were prepared by boiling and rinsing in distilled-deionised

water. The concentration of aluminium ions inside and outside the dialysis bag was carried out by inductively coupled plasma atomic emission spectroscopy (ICP-AES), (Spectroflame ICP, 2.5 kW, 27 MHz). ICP-AES analysis was performed by measuring the intensity of radiation of the specific wavelength emitted by aluminium at 396.152 nm and with a sample flow rate of 1 mL min<sup>-1</sup>. Integration times were 1 per increment (*i.e.*, analysis time of 1 minute per sample).

#### UV absorbance measurements

UV absorbance measurements of pepsin and haemoglobin samples, both in the absence and in the presence of Al<sup>3+</sup> (water solutions acidified with 0.01M HCl, pH2), carried out on Beckman UV 5260 UV-VIS spectrophotometer with an electro-thermal temperature control cell unit. The temperature control performed with DANA – Digital voltmeter model 4800 with chromel-alumel thermocouples. A quartz cell with a 1 cm path length was used for all the absorbance studies. All measurements were carried out at 37°C.

#### IR studies

Pepsin-Al<sup>3+</sup> and haemoglobin-Al<sup>3+</sup> samples were lyophilized and used for the further experiments. The removal of any traces of water from the samples was insured by drying samples over silica gel over night and heating during 2 hours at 100°C. For infrared spectroscopy, the samples were prepared in the form of standard potassium bromide (KBr) pallet (the ratio between pepsin and KBr and pepsin – Al<sup>3+</sup> and KBr were 1:100); the pallets of haemoglobin samples in KBr were prepared in the same manner. Infrared spectra between 4000 and 400 cm<sup>-1</sup> obtained on a Specord 75 IR, Carl Zeiss in double-beam operation *vs.* KBr as a reference. IR spectra of both proteins in KBr matrices recorded at room temperature (25°C).

#### Polyacrylamide gel electrophoresis (PAGE)

Native electrophoresis of pepsin and haemoglobin on 10% polyacrylamide gel carried out at 4°C during 90 min, according to the Laemmli procedure, at pH 8.3 [28]. Water solutions of all samples of enzyme (pepsin dissolved in water to final concentration of 2 mg/mL) were titrated with HCl to pH 2 and incubated at 37°C, with addition of different concentrations of Al<sup>3+</sup> ion (1, 5 and 10 mM). The samples of pepsin and haemoglobin were diluted with sample buffer in ratio 1:1 (v/v) and applied on gel in volume of 20 µL. Visualization was performed with Comassie Brilliant Blue G-250 dye. The gels scanned and processed using Corel Draw 11.0 software package. Quantification of electrophoretic mobility of the molecule is carried out *via* R<sub>S</sub> value,

where it is defined by:

$$RS = \frac{[\text{distance of protein migration}]}{[\text{distance of tracing dye migration}]} \quad (2)$$

#### Statistical analysis

Graphs were plotted by using Microcal Origin program (version 6.1). Values including K<sub>S</sub>, V<sub>max</sub>, K<sub>mS</sub>, K<sub>mA</sub>, K<sub>A</sub>, and A<sub>50</sub> and their standard errors are presented as means ± SEM (obtained by the linear regression analysis). The statistical comparisons were performed by Students t-test for paired observations. The means of at least five observations was quoted in the text and p < 0.01 was considered statistically significant.

## Results

#### *In vitro* effects of Al<sup>3+</sup> ions on porcine pepsin activity

The influence of Al<sup>3+</sup> ions on porcine pepsin activity was investigated in the concentration range 1.7·10<sup>-6</sup> M–8.7·10<sup>-3</sup> M at pH 2. We tested wide range of Al<sup>3+</sup> concentration that included physiological as well as toxic doses of metal ion because of lack of similar information in available literature. The effects of chosen concentrations of Al<sup>3+</sup> ions on *in vitro* pepsin activity are presented in Figure 1.

It is obviously from Figure 1 that all investigated concentration of Al<sup>3+</sup> ions cause increase of pepsin activity. The increasing concentrations of metal ions induced increase of enzymatic activity. Values are expressed as the percent of increased activity related to the control, which considered as 100%. Aluminium was found to stimulate the enzyme activity

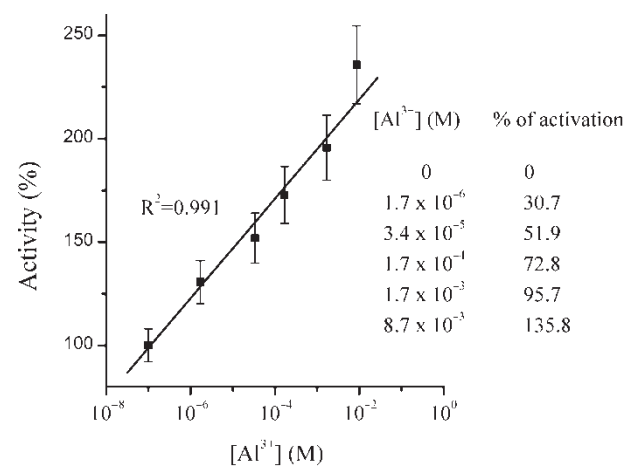


Figure 1. Relative activity of pepsin in the presence of Al<sup>3+</sup> ions. Proteolytic activity of pepsin expressed as percentage depends on Al<sup>3+</sup> ions present. The degree of activation is expressed as % of increased activity considering the activity of pepsin in the absence of Al<sup>3+</sup> ions as 100%. All results are expressed as a mean percentage of enzyme activity relative to the corresponding control value, from at least three independent experiments performed in triplicate.

in dose-dependent manner and linear curve (the correlation coefficient was 0.991) was obtained and presented on Figure 1. The presence of  $1.7 \cdot 10^{-6}$  M  $Al^{3+}$  in incubation milieu causes increase in pepsin activity for 30.7%. Increasing the amount of  $Al^{3+}$  led to a more significant increase of proteolytic activity. So,  $3.4 \cdot 10^{-5}$  M of  $Al^{3+}$  increases pepsin activity for 51.9% ( $p < 0.01$ ),  $1.7 \cdot 10^{-4}$  M for 72.8%, while in the presence of  $1.7 \cdot 10^{-3}$  M  $Al^{3+}$  in incubation milieu the activity of pepsin was doubled (95.7%,  $p < 0.01$ ). Maximal investigated concentration of  $Al^{3+}$  ions, induce the increase of pepsin activity for 135.8% ( $p < 0.01$ ), in comparison with corresponding control.

### Kinetic analysis

In order to evaluate the nature of porcine pepsin activation induced by  $Al^{3+}$ , kinetic parameters  $K_S$  and  $V_{max}$  were determined by varying the concentration of substrate—denatured haemoglobin. The kinetic properties of enzyme were determined in the presence of desired concentration of metal ions (from  $1.7 \cdot 10^{-6}$  to  $8.7 \cdot 10^{-3}$  M, pH 2). The dependence of the initial reaction rate *vs.* substrate concentration in the presence and the absence of aluminium exhibited typical Michaelis–Menten kinetics that is presented in Figure 2 inset. A double reciprocal plot of the velocity *vs.* substrate concentrations at increasing concentrations of  $Al^{3+}$  ions resulted in a family of linear plots intersecting at  $1/[Hb]$  axis (Figure 2).

The Lineweaver-Burk plots show that increase of reaction velocity in the presence of  $Al^{3+}$  ions is proportional to increased  $Al^{3+}$  ions concentration.

Kinetic constants obtained from Lineweaver–Burk plot at variety of different activator concentrations, are presented in Table I.

As can be seen, increasing of aluminium concentration increased  $V_{max}$  values, without producing a significant change in the value of apparent enzyme affinity for substrate ( $K_S$ ). The results obtained from Lineweaver–Burk plots, are used for calculation of kinetic constants. The secondary plots of the slopes and intercepts *vs.* activator concentrations are not linear (data not shown), but the reciprocal of the change in slope and intercept ( $\Delta$ slope and  $\Delta$ intercept) that are determined by subtracting the values in the presence of activator from that in its absence, are linear [29,30].

The intercepts of a plot  $1/\Delta$ slope and  $1/\Delta$ intercept *vs.*  $1/[Al^{3+}]$  on  $1/\Delta$  axis, and intercepts of both plots on  $1/[Al^{3+}]$  axis are used for calculating equilibrium constants  $K_{mS}$  and  $K_{mA}$  for dissociation of formed binary enzyme-activator ( $Al^{3+}$ ) and ternary enzyme-activator-substrate complexes (Figure 3, Scheme 1). The calculated values for constants are  $0.904 \pm 0.083$  mM and  $8.56 \pm 0.51$   $\mu$ M, respectively.

For further comparison of pepsin catalytic behaviour in absence and presence of aluminium the catalytic constant  $k_{cat}$  and catalytic effectiveness  $k_{cat} \cdot K_S^{-1}$  were calculated. It could be seen (Table I) that increasing concentrations of activator causes the increase of turnover numbers. In the presence of  $1.7 \mu$ M of  $Al^{3+}$ , the  $k_{cat}$  is  $3378 \pm 81$   $min^{-1}$ , while in the presence of maximal concentration of activator (8.7 mM)  $k_{cat}$  is  $6112 \pm 173$   $min^{-1}$ . In addition, the higher values of catalytic effectiveness were obtained in the presence of  $Al^{3+}$ .

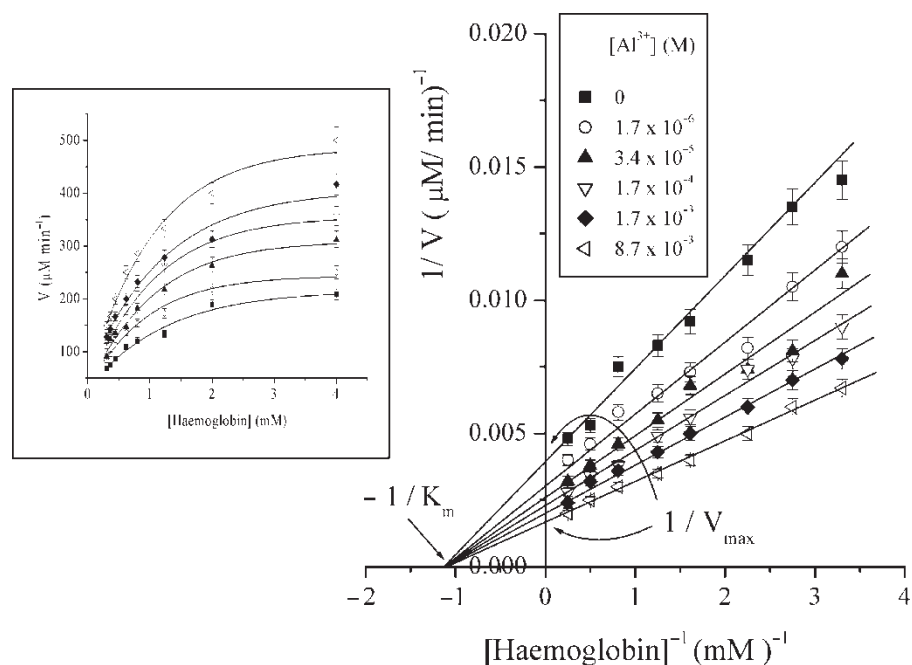


Figure 2. Lineweaver–Burk plot of a series of kinetics measurements in a presence of different  $Al^{3+}$  ions concentrations at pH2; (inset) Hyperbolic plots (sigmoid fit) representing initial pepsin velocity versus haemoglobin concentration in the absence and presence of different  $Al^{3+}$  ions concentrations at pH2.



Table I. Influence of  $\text{Al}^{3+}$  on  $K_m$ ,  $V_{\max}$ ,  $k_{\text{cat}}$  and catalytic effectiveness of enzyme ( $k_{\text{cat}} K_m^{-1}$ ).

$[\text{Al}^{3+}]$ (M)	$K_S$ ( $\text{mM L}^{-1}$ ) <sup>a</sup>	$V_{\max}$ ( $\mu\text{M min}^{-1}$ ) <sup>a</sup>	$k_{\text{cat}}$ ( $\text{min}^{-1}$ ) <sup>b</sup>	$k_{\text{cat}} K_S^{-1}$ ( $\text{min}^{-1} \text{mM}^{-1}$ )
0	$0.904 \pm 0.083$	$254 \pm 7$	$2591 \pm 70$	$3264 \pm 121$
$1.7 \cdot 10^{-6}$	$0.907 \pm 0.072$	$332 \pm 8$	$3378 \pm 81$	$3724 \pm 126$
$3.4 \cdot 10^{-5}$	$0.912 \pm 0.072$	$386 \pm 10$	$3938 \pm 102$	$4318 \pm 154$
$1.7 \cdot 10^{-4}$	$0.918 \pm 0.073$	$439 \pm 13$	$4479 \pm 132$	$4879 \pm 191$
$1.7 \cdot 10^{-3}$	$0.921 \pm 0.074$	$497 \pm 14$	$5071 \pm 143$	$5506 \pm 209$
$8.7 \cdot 10^{-3}$	$0.917 \pm 0.073$	$599 \pm 17$	$6112 \pm 173$	$6665 \pm 254$

<sup>a</sup> The  $K_m$  and  $V_{\max}$  were determined by their respective regression equations; <sup>b</sup>  $k_{\text{cat}}$  (turnover number) was calculated using the equation  $k_{\text{cat}} = V_{\max} / [E]_{\text{t}}$ , assuming that the molecular mass of the enzyme is 35 kDa.

Using UV spectral and aluminium binding assays data (experimental data not shown) the fraction of bound aluminium to pepsin  $\nu$  and dissociation constant for pepsin-aluminium complex  $K_A$ , were found to be  $0.96 \pm 0.09$  and  $8.39 \pm 0.76 \mu\text{M}$  respectively [31].

Kinetic data was used also for calculating the activator concentration that gives a rate equal to the half at a saturating concentration of activator ( $A_{50}$ ), and also dissociation constant  $K_A$  for enzyme-activator complex (Figure 4) [32]. The calculated values of  $K_A$  and  $A_{50}$  for pepsin-aluminium complex were  $8.82 \pm 0.90 \mu\text{M}$  and  $8.05 \pm 0.48 \mu\text{M}$ , respectively, that is consistent with a non-essential partial non-competitive activation system (Scheme 1).

#### Influence of $\text{Al}^{3+}$ ions on pepsin and haemoglobin conformation

*UV studies.* The pepsin- $\text{Al}^{3+}$  and haemoglobin- $\text{Al}^{3+}$  interactions were followed by UV spectra. The absorption spectra of pepsin and haemoglobin in the absence and presence of 10 mM  $\text{Al}^{3+}$  are presented in Figures 5 A and B. It is obvious that the characteristic absorption maximum for pepsin at 280 nm is shifted in a presence of  $\text{Al}^{3+}$  to 255 nm by formation pepsin -  $\text{Al}^{3+}$  complex for all applied concentrations of aluminium

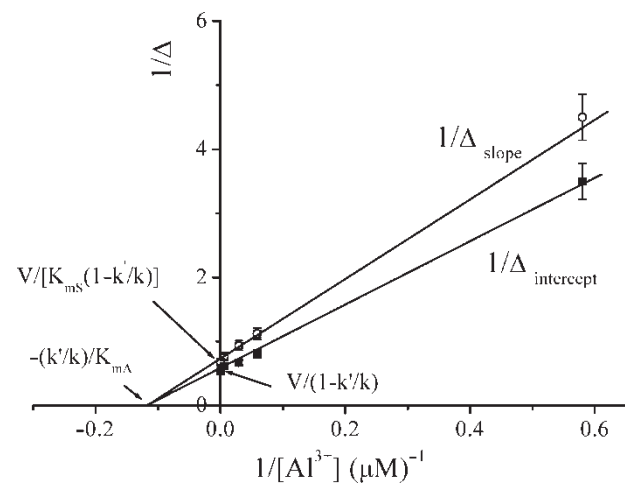
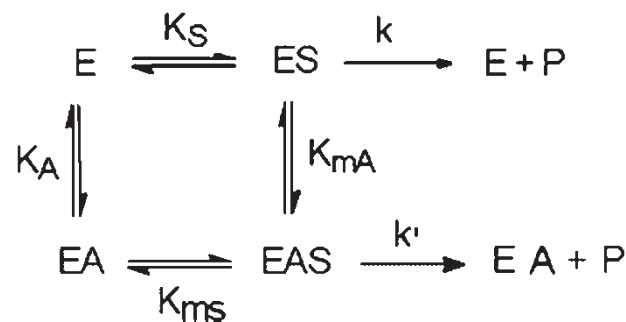


Figure 3. The plots of  $1 / (\text{change in slope or intercept})$ ,  $(1/\Delta)$  against  $1 / [\text{Al}^{3+}]$ , where  $\Delta$  is defined as slope or intercept in the absence of activator ( $\text{Al}^{3+}$  ions) minus that in its presence.

(1, 5, 10 mM) and intensity of characteristic peaks have been decreased in a dose dependent manner (Figure 5 A). However, the presence of  $\text{Al}^{3+}$  ions did not induce any shifts of characteristic peaks positions (273 nm and 369 nm) (Figure 5 B). In UV spectra of haemoglobin, in the presence of all applied concentrations of aluminium (1, 5, 10 mM), intensity of characteristic peaks decreased in a dose dependent manner (Figure 5 B). Figures 5 A and B shows only UV spectra in a presence of 10 mM  $\text{Al}^{3+}$ .

*IR studies.* IR spectra of pepsin and haemoglobin in KBr matrices in absence (solid curve 1) and in a presence 10 mM  $\text{Al}^{3+}$  ions (dotted curve 2) are presented in Figures 6 A and B.

As can be seen, presence of 10 mM  $\text{Al}^{3+}$  ion causes noticeable changes in infrared spectra of pepsin. Typical features were: the disappearance of the vibration bands in the  $3500\text{--}2750 \text{ cm}^{-1}$  region, especially bands at  $3256$  and  $2920 \text{ cm}^{-1}$ ; decrease of intensity of the vibration band at  $1600 \text{ cm}^{-1}$ ; the disappearance of the vibration bands in the  $1500\text{--}1200 \text{ cm}^{-1}$  especially band at  $1491 \text{ cm}^{-1}$  in respect to spectrum of pepsin (Figure 6 A). However, IR spectra of haemoglobin demonstrate that the treatment with 10 mM  $\text{Al}^{3+}$  ions does not cause noticeable changes in its conformation as shown in Figure 6 B. The both spectra of haemoglobin (Figure 6 B, curve 1) and haemoglobin treated with 10 mM  $\text{Al}^{3+}$  ions (Figure 6



Scheme 1. Reaction scheme for non-essential activation; Abbreviations are E—enzyme, S—substrate, A—activator, P—product.

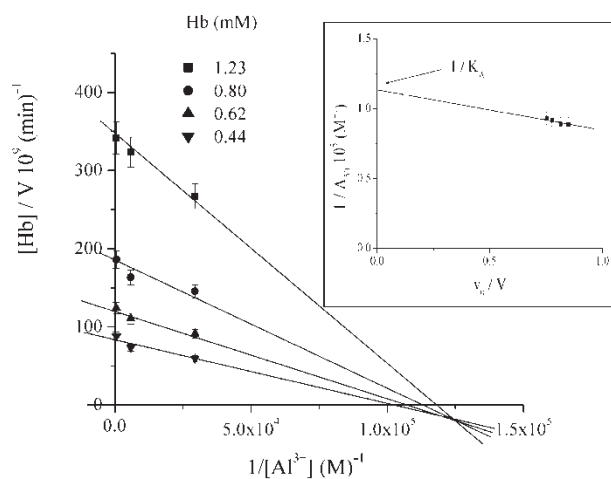


Figure 4. Activation of pepsin by  $Al^{3+}$  ions; The intercepts on the abscissa of the primary plot of  $[Hb]/V$  against reciprocal of activator concentrations provide the values of  $A_{50}$  used to obtain the plots of  $1/A_{50}$  against  $v_0/V$  shown in the inset.

B, curve 2) contain characteristic bands of haemoglobin without changes in its positions and intensity.

*Electrophoretic analysis of pepsin and haemoglobin.* Native PAGE profiles of untreated and aluminium treated pepsin solutions at pH 2 were studied to verify the conformational changes of pepsin induced by  $Al^{3+}$  ions that resulting in activation effect. Electrophoretic mobility in the presence of  $Al^{3+}$  ions (from 1 to 10 mM) inducing the highest activation (producing around the 100% activation or more upon the enzyme assay data) and in the absence of activator were compared. The electrophoregrams of pepsin and

haemoglobin samples in absence or in the presence of different concentrations of  $Al^{3+}$  ion are presented in Figure 7.

The presence of  $Al^{3+}$  cause the decrease of pepsin electrophoretic mobility at all investigated concentrations. In addition, the degree of decrease is proportional to  $Al^{3+}$  concentrations, which the one has been exposed. In the absence of  $Al^{3+}$  ion, the electrophoretic mobility of pepsin under the physiological conditions the obtained  $R_s$  value for pepsin is 0.47 while in the presence of 1, 5 and 10 mM  $Al^{3+}$  ions the obtained  $R_s$  values were 0.46, 0.44 and 0.42, respectively. It is obviously that the highest concentration of  $Al^{3+}$  results in the lowest electrophoretic mobility. Electrophoretic mobility of haemoglobin in the absence of  $Al^{3+}$  ion was characterized with  $R_s$  value of 0.20, while in the presence of 1, 5 and 10 mM  $Al^{3+}$  ions the obtained  $R_s$  values were 0.20, 0.20 and 0.19 respectively. The presence of all investigated concentration of aluminium does not cause changes in electrophoretic mobility of haemoglobin.

## Discussion

In the available literature, information about the influence of toxic metal ions on the activity of extracellular enzymes is scant and controversial. Variability of data results from the chemical properties of the element and the formation of various chemical species, depending on the pH, ionic strength, presence of competing elements and complexing agents within the gastrointestinal tract. To date, however, there have been no papers about investigations with gastrointestinal enzymes and  $Al^{3+}$  ions and the mechanism of this action.

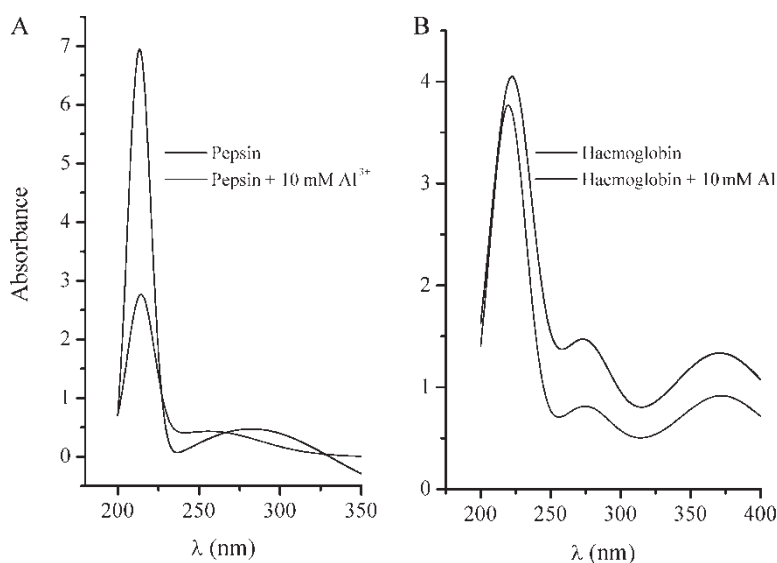


Figure 5. **A**—UV absorption spectra of pepsin (solid curve) and pepsin treated with 10 mM  $Al^{3+}$  (dotted curve). **B** - UV absorption spectra of haemoglobin (solid curve) and haemoglobin treated with 10 mM  $Al^{3+}$  (dotted curve). Other spectral curves obtained in a presence of 1 mM and 5 mM  $Al^{3+}$  are not presented because of clarity of the graphs.

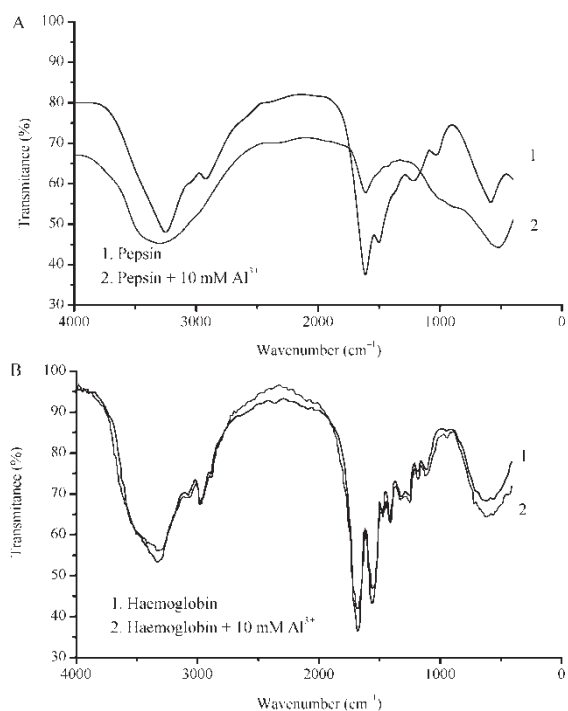


Figure 6. **A**—IR spectra of pepsin (solid curve,1) and pepsin in a presence of 10 mM  $\text{Al}^{3+}$  ions (dotted curve,2) and **B** - IR spectra of haemoglobin (solid curve,1) and haemoglobin in a presence of 10 mM  $\text{Al}^{3+}$  ions (dotted curve,2). IR spectra were recorded in KBr matrices.

Aluminium is a toxic ion that is partly absorbed from the mammalian gastrointestinal tract. It was noted that the gastrointestinal fluids have their own intrinsic metal binding properties, especially for copper ( $2^+$ ) and iron ( $3^+$ ). It is known that  $\text{Al}^{3+}$  ions interact with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -dependent enzymes. Binding of  $\text{Al}^{3+}$  ion with ATP is stronger than with  $\text{Mg}^{2+}$ , which is a natural activator of ATP-ase; therefore the  $\text{Al}^{3+}$  ion is a strong inhibitor of all ATP-ases. It has been documented that  $\text{Al}^{3+}$  inhibits activity of hexokinase, adenylat-cyclase, calmodulin, NAD-kinase and other Mg-dependent enzymes [33]. In addition, there is lack of data referring about mechanism and kinetics of aluminium binding to gastrointestinal fluids, especially to main enzyme of gastric juice—pepsin. In particular, we simulate the effects of  $\text{Al}^{3+}$  ions on pepsin activity *in vitro* conditions.

Results of our study showed that aluminium stimulate pepsin activity. The obtained results are in agreement with previously reported [25] stimulatory effect of  $\text{Al}^{3+}$  ions on porcine pepsin activity. The authors reported that applying concentration of  $1.1 \cdot 10^{-3}$  M  $\text{Al}^{3+}$  ions induce the activation of 191%, while we obtained 135.8% activation of pepsin activity, in applied concentration of  $\text{Al}^{3+}$  ions of  $8.7 \cdot 10^{-3}$  M. The observed disagreements could be explained by differences in experimental conditions (different pH, enzyme/substrate ratio). The obtained

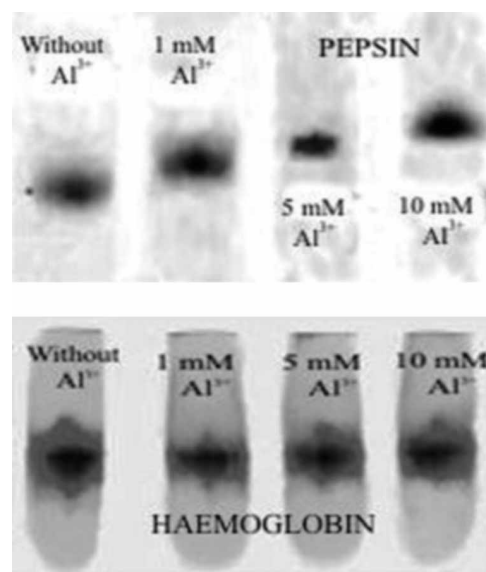


Figure 7. Native PAGE electrophoregram of pepsin (upper) and haemoglobin (lower) without and in a presence of 1, 5 and 10 mM  $\text{Al}^{3+}$  at pH 2.

activation probably is consequence of changes in conformation of enzyme molecule induced by bonded  $\text{Al}^{3+}$  ions. Upon analogy to the other aspartic proteases, it is possible that bounded aluminium ions causes significant conformational changes and induce increase in beta structure content [22,23,34].

As  $\text{Al}^{3+}$  ions occur mostly in an ionic form in the acidic milieu (as  $[\text{Al}(\text{H}_2\text{O})_6]^{3+}$ ) it probably affect electric charge of the molecules of enzyme and cause the differences in its electrophoretic mobility. The decrease in the electrophoretic mobility of pepsin molecule may be assigned to the change in net electric charge of the pepsin caused by binding of  $\text{Al}^{3+}$  ions to corresponding binding sites of pepsin molecule and probably to conformational changes evaluated by UV and IR spectroscopy. The obtained effect is proportional to the increase of  $\text{Al}^{3+}$  ions concentration. Overall, it can be concluded that  $\text{Al}^{3+}$  ions binding to pepsin molecule induced conformational changes resulting in increased enzyme activity and decrease of electrophoretic mobility.

Analysis of obtained kinetic data [29,35] implies the activation as a non-essential partial non-competitive type. It suggests that aluminium do not influence on substrate binding at specific binding sites on pepsin, but causes conformational changes (Figures 5 and 6) that increase the rate of substrate converting to the reaction products. The results are consistent with a partial activation system (Scheme 1), in which the kinetic constants  $K_{mS}$  and  $K_{mA}$  for dissociation of corresponding binary and ternary complexes can be determined [29].

At equilibrium conditions partially non-competitive activation exists if the following conditions are



satisfied, i.e.  $K_S = K_{mS}$ ,  $K_A = K_{mA}$  and  $k < k'$  (Scheme 1) [29,35]. Constant  $K_A$  was calculated from kinetic and equilibrium binding assay data; the obtained values are in good agreement to each other, and are  $8.82 \pm 0.90 \mu\text{M}$  and  $8.39 \pm 0.76 \mu\text{M}$  respectively. Other constants,  $K_{mA} = 8.56 \pm 0.51 \mu\text{M}$  for binding of aluminium to pepsin-haemoglobin complex and  $K_{mS} = 0.904 \pm 0.083 \text{ mM}$  for binding of haemoglobin to pepsin-aluminium complex are in agreement with proposed conditions for partially non-competitive activation mechanism.

At the same time bound  $Al^{3+}$  could induce conformational changes of enzyme responsible for enzyme ability to convert substrate to product (increase in  $V_{max}$ ) rather than apparent affinity for substrate ( $K_S$ ) obtained by kinetic analyses.

In summary, the present kinetic analyses, which classify this phenomenon as a case of non-essential activation, indicated that the activation by  $Al^{3+}$  ions was of partial non-competitive type.

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