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Aluminium – Non-Essential Activator of Pepsin: Kinetics and Thermodynamics

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1. Introduction

The stability of proteins and their interactions with other molecules, especially with toxic ones, is a topic of special interest in biochemistry because many cellular processes depend on that. These interactions have immediate consequences for protein stability, as shown by varying the thermodynamic properties of the system. This fact provides an extra variable to study protein unfolding linked to ligand binding (Lumry et al. 1966, Crothers 1971, Schellman 1975, McGhee 1976).

Porcine pepsin A (EC 3.4.23.1), belongs to the aspartic protease family and plays an integral role in the digestion process of vertebrates. As all aspartic proteases pepsin molecule consist of two homologous lobes composed predominantly of β-sheets separated by a hinged substrate-binding cleft. Two active site aspartic residues, Asp32 and Asp215, occupy the cleft to which admittance is restricted by a hinged flexible flap region (Andreeva et al. 1984, Baldwin et al. 1993, Blundell et al. 1990). One of two Asp residues has to be protonated and the other deprotonated, for the protein to be active (Lumry et al. 1966). Due to the catalytic residues, the active pH ranges from 1.0 to 5.0 (Blundell et al. 1990, Sielecki et al. 1990, Brandts & Lin 1990, Shrake & Ross 1992, Celey et al. 2005).

Pepsin undergoes a conformational transition from the native to the denatured state in a narrow pH range. Refolding of an immobilized form of the denatured pepsin was achieved without the prosequence (Kurimoto et al. 2001), but its refolding mechanism is still unsolved. Thermal denaturation of proteins is being studied intensively today. Investigation of denaturation may help to elucidate mechanisms of the reverse process, i.e. protein folding. Complex structures such as proteins, that undergo transitions originated from conformational changes, are suitable to be studied by Differential Scanning Calorimetry (DSC). DSC is primarily used to characterize stability and folding, and to study binding interactions between proteins and small molecules, drugs, and other proteins (Powers et al. 1977). The consequences of protein interactions with other molecules on interactions on protein stability were shown through varying the thermodynamic properties of the system. This fact provides an extra variable to study protein unfolding linked to ligand binding. Various approaches that describe macromolecular unfolding coupled to ligand binding

were reported by several authors many years ago (Lumry et al. 1966, Crothers 1971, Schellman 1975, McGhee 1976). More recently, with the application of theory, DSC has been proved to be a very useful tool to estimate very tight binding constants (Brandts & Lin 1990, Shrake & Ross 1992,) as well as to characterize the energetic of binding and unfolding (Celey et al. 2005, Celey et al. 2006).

DSC is used to measure the binding constants from temperature melting points (T_m) shifts small molecule binding to a protein. The binding constant of the ligand can be estimated from the T_m in the presence and absence of ligand, as long as the concentration of ligand in the DSC cell is known. Binding of a ligand to a protein occurs only if there is a release of free energy. Accordingly, the protein–ligand complex is more stable than the free partners are. The extent of stabilization depends upon the magnitude of the binding energy. Comparison of stability of the complex with that of the free partners allows estimation of binding energy.

Through calorimetric studies, Privalov showed that thermal denaturation of porcine pepsin is a complex process that proceeds by two distinct stages occurring at different temperatures. 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 (Privalov et al. 1981).

Trivalent aluminium ion, Al^{3+}, is a typical metal ion that exist as a hydrated $A1(H_20)_6{}^{3+}$ in acid pH solutions. Acid digestion in the stomach would solubilise most of the ingested aluminium compounds to the monomolecular species Al^{3+} (e.g. hydrated $Al(H_20)_6{}^{3+}$). After absorption, aluminium distributes unequally to all tissues in humans and accumulates in some. About one-half of the total body burdens of aluminium are in the skeleton and about one fourth is in the lungs (from accumulation of inhaled insoluble aluminium compounds). Aluminium has also been found in most soft tissue organs and its levels have been found to increase with ageing of experimental animals. Aluminium compounds have a wide variety of uses, including production of pharmaceuticals and food additives. A variety of complexes may be formed with the ligands present in biological systems and/or in foods.

The complexes between ligands and aluminium have different physicochemical properties, such as solubility in aqueous medium, stability at different pH, electric charge etc. This can greatly influence the toxicokinetic and toxicodynamic profile of aluminium. Although aluminium is toxic to humans, animals and plants, its biochemistry has been little studied and is poorly understood (Gomez et al.1994, Kerr & Ward 1988.). Because of the lack of quantitative information, it is not easy to assess the biological relevance and possible biological role of such interactions.

The Al^{3+} interacts with a large number of proteins, glycoproteins and carbohydrates, but very little is known about the chemistry, binding strength and binding mode of these complexes. The most likely binding sites of $A1(H_20)_6{}^{3+}$ in bio systems are oxygen donors, and especially negatively charged oxygen donors. Carboxylate, phenolate, catecholate and phosphate are the strongest Al^{3+} binders. Biomolecules containing such functions may be involved in the uptake and transport processes of Al^{3+} (Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Food Contact Materials on a request from European Commission on Safety of aluminium from dietary intake, 2008).

Recently it has been shown that Al^{3+} ions increase pepsin activity (Krejpcio & Wojciak 2002).

Gel electrophoresis is a broad subject encompassing many different techniques and can provide information about the molecular weights and charges of proteins, the subunit structures of proteins and a purity of a particular protein preparation. It is relatively simple to use and it is highly reproducible. The most common use of gel electrophoresis is the qualitative analysis of complex mixtures of proteins. Microanalytical methods are sensitive, linear image analysis system make gel electrophoresis useful for quantitative and preparative purposes as well. The technique provides the highest resolution of all methods available for separating proteins. Polypeptides differing in molecular weights by as little as a few hundreds of Daltons and proteins differing less than 0.1 pH unit in their isoelectric points are routinely resolved in gels.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the most commonly practiced polyacrilamide gel electrophoresis technique used for proteins. The method provides an easy way to estimate the number of polypeptides in a sample and thus assess the complexity of the sample or the purity of a preparation. SDS-PAGE is particularly useful for monitoring the fractions obtained during chromatographic or other purification procedures. One of the more important features of SDS-PAGE is that it is a simple, reliable method with which is easy to estimate the molecular weights of proteins. SDS-PAGE requires that proteins be denatured to their constituted polypeptide chains, so that it is limited in the information it can provide. In those situations where it is desirable to maintain biological activity, non-denaturing systems must be employed. However, the gel patterns from non-denaturing gels are more difficult to interpret than are those from SDS-PAGE. Non-denaturing systems also give information about the charge isomers of proteins.

The subject of electrophoresis deals with the controlled motion of charged particles in electrical fields. Since proteins are charged molecules, they migrate under the influence of electrical fields. From the point of view of electrophoresis, the two most important physical properties of proteins are their electrophoretic mobility and charge and its isoelectric points. The electrophoretic mobility depends on its charge, size, and shape, and it is very different in gels than in free solution. Pepsin has been studied by electrophoresis since its isolation from different sources (Herriot 1940, Porcellii, 1968, Cunningham 1970, Cann 1962, Varilova 2005).

In our previous studies, the in vitro influence of different concentrations of Al^{3+} ions, physiological and toxic ones, on pepsin activity was investigated. Kinetic studies were undertaken to determine the nature of the enzyme modulation (type and mechanism) by investigated metal ion. The mechanism of Al^{3+} ions on pepsin activity evaluated from kinetic studies and was classified as a case of non-essential activation with partial non-competitive character (Pavelkic et al. 2008). With the application of theory, DSC method has been used as a tool to estimate binding constants (Pavelkic et al. 2011) as well as to characterize the energetic of binding and unfolding.

The present paper summarizes the current knowledge of activating and stabilizing effect of Al^{3+} ions on gastrointestinal fluids, especially on main gastrointestinal enzyme - pepsin. Therefore, there is a lack of information about thermal stability of pepsin in a presence of Al^{3+} ions. As the binding mechanism of Al^{3+} ions on pepsin is not still clear the objective of this study is to investigate the *in vitro* conditions the influence of different concentrations of

Al^{3+} ions, physiological and toxic ones, on pepsin conformational stability during the process of thermal unfolding, with a purpose of better understanding of pepsin/aluminium interaction.

2. The effect of activator on the reaction rate and kinetic parameters – Theory

The mode of activation, essential or non-essential, depends on the values of the equilibrium constants, the rate constants of the limiting velocity steps and substrate concentration. Reversible enzyme activation implies the binding of the enzyme to the activator (A) which affects the rate of an enzyme-catalyzed reaction. A simple scheme to describe the interactions between an enzyme (E), a substrate (S) and the activator (A) is presented below.

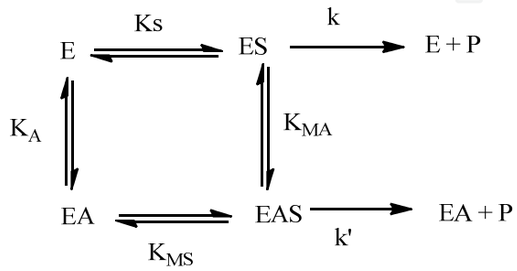

Fig. 1. Reaction scheme representing the mechanism of the enzyme catalyzed reaction and interactions of the enzyme (E) with activator (A) and the substrate (S).

In this model, a molecule of enzyme (E) can bind one molecule of substrate (S) and/or one molecule of activator (A). Equilibrium constants for the dissociation reactions ES ↔ E + S, EAS ↔ EA + S, EA ↔ E + A and EAS ↔ ES + A, are K_S, K_{MS}, K_A and K_{MA} respectively. The rate constants k and k′ are the rate constants for reactions ES → E + P (product) and EAS → EA + P respectively. The reaction scheme is based on the assumption that equilibrium between enzyme, substrate and activator, and their complexes is set up almost immediately and during the time required to measure initial velocity. Also, the higher concentrations of S and A than total enzyme concentration, as well as the velocities of product formation from the enzyme-substrate and enzyme-activator-substrate complexes as a velocity limiting steps in transformation S → P, were assumed. The rate constants k and k′ are related to the parameters V_1 and V_2 through following equations:

$$V_1 = k\left[E_t\right] \tag{1}$$

$$V_1 = k'\left[E_t\right] \tag{2}$$

Where $\left[E_t\right]$ is the total enzyme concentration: $\left[E_t\right] = \left[E\right] + \left[ES\right] + \left[EA\right] + \left[EAS\right]$.

The equilibrium constants K_S, K_{MS}, K_A and K_{MA} for the dissociation reaction mentioned above are respectively:

$$K_S = \frac{[E][S]}{[ES]} \tag{3}$$

$$K_{MS} = \frac{[EA][S]}{[EAS]} \tag{4}$$

$$K_A = \frac{[E][A]}{[EA]} \tag{5}$$

$$K_{MA} = \frac{[ES][A]}{[EAS]} \tag{6}$$

These equilibrium constants are related by the equation:

$$K_S \cdot K_A = K_{MS} \cdot K_{MA} \tag{7}$$

If activator is not present in the reaction solution, the enzyme follows typical Michaelis-Menten kinetics, with apparent values of V_{max} and K_M (V_1 and K_S in the reaction scheme represented in Figure 1, respectively). When activator is present in saturating concentrations, Michaelis kinetics is still obeyed, but with V_{max} and K_M equal to V_2 and K_{MS}. The analysis of such of system (Figure 1), assuming that equilibrium conditions applied to substrate binding, give the following possibilities: partially competitive activation if k = k', partially non-competitive activation if $K_S = K_{MS}$, $K_A = K_{MA}$ and k< k', or partially mixed case if $K_S \neq K_{MS}$, $K_A \neq K_{MA}$ and k< k' (Dixon 1979, Fontes 2000).

Assuming that equilibrium conditions apply, for the above system (Figure 1) the equation that can be derived takes the form:

$$v = \frac{V_{\max}\frac{[S]}{K_S} + V_{\max}\frac{[A][S]}{K_A K_S}}{1 + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{[A][S]}{K_{MA}K_{MS}}} \tag{8}$$

Or

$$v = \frac{V_{\max}[S]}{K_S\frac{\left(1+\frac{[A]}{K_{MA}}\right)}{\left(1+\frac{k'}{k}\frac{[A]}{K_{MA}}\right)} + [S]\frac{\left(1+\frac{[A]}{K_{MA}}\right)}{\left(1+\frac{k'}{k}\frac{[A]}{K_{MA}}\right)}} \tag{9}$$

When activator is not present in the system, i.e. [A]=0 M, the above equation attain the form of well known Michaelis-Menten equation. If the activator is present in the system at any concentration, the reaction rate is defined as apparent so the maximal velocity and dissociation constants too:

$$v_{app} = \frac{V_{\max}\dfrac{\left(1+\dfrac{k'}{k}\dfrac{[A]}{K_{MA}}\right)}{\left(1+\dfrac{[A]}{K_{MA}}\right)}[S]}{K_S\dfrac{\left(1+\dfrac{[A]}{K_{MA}}\right)}{\left(1+\dfrac{k'}{k}\dfrac{[A]}{K_{MA}}\right)}+[S]} \tag{10}$$

And

$$V_{\max app} = V_{\max}\frac{\left(1+\dfrac{k'}{k}\dfrac{[A]}{K_{MA}}\right)}{\left(1+\dfrac{[A]}{k\cdot K_{MA}}\right)} \tag{11}$$

$$K_{Sapp} = K_S\frac{\left(1+\dfrac{[A]}{K_{MA}}\right)}{\left(1+\dfrac{[A]}{k\cdot K_{MA}}\right)} \tag{12}$$

Consequently double reciprocal plots (Lineweaver-Burk) of 1/V = f (1/[S]), will be linear when [A] varied. Secondary plots of the slopes and intercepts of the plots of 1/v =f (1/[S]) against [A] will be hyperbolic.

The linearization of that can be exceeded via plotting double reciprocal plots of the change in slope or intercept (Δ_{slope} or $\Delta_{intercept}$ must be determined by subtracting the values in the presence of activator from that in its absence) will be linear. These give a possibility for easy graphical evaluation of important kinetic constants.

3. The effect of activator on the thermal stability of protein – Theory

Treatment of non-two-state transitions includes both calorimetric and van't Hoff heat changes. Indicating the temperature-dependent parameters heat capacity function can be expressed by the equation:

$$C_p(T) = \left[\frac{K_A(T)\Delta\cdot H_{mA}^{VH}\Delta H_{mA}}{\left(1+K_A(T)\right)^2 RT^2}\right]+\cdots \tag{13}$$

In this case, the equilibrium constant will be:

$$K_A(T) = \exp\left[\frac{-\Delta H_{mA}^{VH}}{RT\left(\frac{1-T}{T_{mA}}\right)}\right] \tag{14}$$

where ΔH_{mA}^{VH} is a van`t Hoff enthalpy for the transition with characteristic T_{mA} .

In order to define the stability of a protein consisting of several structural subunits and assuming that interaction between the units are negligible we estimate the Gibb's energy for a unit from experimental data using the following equation:

$$\Delta G(T) = \Delta H_{mA}^{VH}\left(\frac{1-T}{T_{MA}}\right) \tag{15}$$

To obtain detailed information about thermodynamic properties of pepsin in a strong acid media, DSC profiles were analyzed within the framework of "Non-2-State, with zero ΔC_p model" (that use the Leveberg-Marquardt non-linear least-square method). Non-2-State model involved the next parameters: T_m, H^{cal}, and H^{VH}. The model is applied to transitions with no ΔC_p effects. Before curve fitting, a baseline was subtracted from the experimental data to remove ΔC_p effects and sets C_p to zero at all temperatures (ΔC_p given in Table are evaluated before curve fitting).

The DSC protein stability data contain information on related aspects of protein structure and interactions, and may be used to estimate metal binding affinities in metalloprotein complex.

To estimate the magnitude of Al^{3+} binding affinity to pepsin, we used an expression for equilibrium binding affinity (Brandts et al. 1989, Brandts et al. 1990, Lin et al. 1993) derived from the theory of coupled equilibrium,

$$K_{Al^{3+}}(T_{Al}) = \frac{\exp\left[\frac{-\Delta H_0^{VH}}{R}\left(\frac{1}{T_{Al}} - \frac{1}{T_0}\right) + \frac{\Delta C_p}{R}\left(\ln\frac{T_{Al}}{T_0} + \frac{T_0}{T_{Al}} - 1\right)\right]}{\left[Al^{3+}\right]_{T_{Al}}} \tag{16}$$

Where $K_{Al^{3+}}(T_{Al})$ is the equilibrium binding affinity for formation of the pepsin – Al^{3+} complex at the transition temperature for unfolding Al^{3+}-pepsin, ΔH_0^{VH} is the transition enthalpy for pepsin unfolding, T_0 is the transition temperature for pepsin unfolding, T_{Al} is the transition temperature for pepsin – Al^{3+} complex, and $\left[Al^{3+}\right]_{T_{Al}}$ is the concentration of free metal ion at T_{Al} .

From the shift in T_m, the changes in the apparent stability of the particular units of protein treated with activator (aluminium treated pepsin in investigated model system) relative to the native form $\Delta(\Delta G)$ was evaluated at the transition temperature as a difference between ΔG calculated for native and activator treated protein (Lin et al. 1993, Brewer et al. 2001):

$$\Delta\left(\Delta G\ \right)=\Delta G_{(\text{activator treated protein})}-\Delta G^{0}_{(\text{native protein})} \tag{17}$$

Estimation of the average number of ligands bound to the native protein $\overline{X}_N$ can be accomplished in a term of total Gibbs energy of unfolding that is defined by the difference between the Gibbs energies of the denatured and native state, with the assumption that no ligand - binding occurs in the unfolded state (Brewer & Wampler 2001).

Starting from relation

$$\left(\frac{\partial\Delta G^{0}_{tot}}{\partial\ln[A]}\right)_T = RT\left(\frac{\partial\ln(Q-K_0)}{\partial\ln[A]}\right)_T = RT\overline{X_N} \tag{18}$$

Where,

ΔG^{0}_{tot} is a total Gibbs energy,

Q - partition function of all residual native states which include native ligand free and native ligand bounded protein molecules,

K_0 - the equilibrium constant of unfolding, and

$\overline{X}_N$ - an average number of ligands bound to the native protein,

the average numbers of ligands bound to the native protein could be graphically determined from the slope of G^{0}_{tot} *versus* $\ln[A]$.

Indirect determination of the enthalpy of unfolding assumes the knowledge of the equilibrium as a function of temperature. Starting from spectroscopic data spectroscopic signal for 100% denaturated (random coil) sample and 100% native protein was determined. The temperature range where protein transitions from native to denatured form was covered. Fraction of native protein as a function of temperature and the fraction of unfolded protein as a function of temperature f_N and f_D respectively, were defined in terms of measured absorbance $A(T)$ as:

$$f_D=\frac{A(T)-A_N(T)}{A_D(T)-A_N(T)} \text{ and } f_N=1-f_D \tag{19}$$

N and D refer to the native and unfolded state respectively.

Determined fraction of native and unfolded protein could be used for further determination of enthalpy of unfolding using the relation:

$$\ln\left(K_{eq}\right)=\left(\frac{-\Delta H^{VH}_{unf}}{RT}\right)+\frac{\Delta S}{R} \tag{20}$$

Previous equation give possibility for simple graphical determination equilibrium constant. The plot ln (K_{eq}) *vs. 1/T* describes a straight line with slope equal to $\Delta H^{VH}_{unf} / R$.

The application of gel electrophoresis to the measurement of dissociation constants of protein-ligand complexes has been studied previously (Yen HC et al. 1993). The dissociation constant in the gel (K_d^{gel}) is defined by following equations:

$$K_d = \frac{[P][L]}{[PL]} \tag{21}$$

$$R_S = \frac{r}{R_0} = \frac{[P]}{[P]+[PL]} \tag{22}$$

$$\frac{1}{R_f} = 1 + \frac{[L]}{K_d} \tag{23}$$

$$[L]R_f = K_d - K_d R_f \tag{24}$$

Where $[L]$ is ligand concentration, $[P]$ is concentration of sample protein, R_0 and r protein electrophoretic mobility in the absence and the presence of ligand. Obtained dissociation constant K_d is apparent dissociation constant in gel, and may be different from dissociation constant in solution.

4. Materials and methods

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

4.1 Enzyme assay

The Worthington method based on enzyme-catalyzed measured rate of hydrolysis of denatured hemoglobin (Hb) substrate was used for evaluation of enzyme activity in the absence (control) and presence of Al^{3+} ions (Anson 1938). Pepsin activity was determined in an incubation medium containing 1mL of pepsin solution (20 mg/mL in 0.01M HCl, pH2) and 5 mL of haemoglobin solution (2% solution of hemoglobin 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:

$$U_{units/mg} = \frac{\left(A_{280nm(filtrate)} - A_{280nm(blank)}\right) \cdot 1000}{10\,\text{min} \cdot mg_{\text{enzyme in reaction mixture}}} \tag{25}$$

Kinetic analysis was carried out by following the initial velocity of the enzymatic reaction in the absence and presence of Al^{3+} in concentration range from $1.7 \cdot 10^{-6}$ to $8.7 \cdot 10^{-3}$ M, and increasing concentrations of hemoglobin from $2.5 \cdot 10^{-2}$ to $4 \cdot 10^{-3}$ 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 (Perrela 1988).

4.2 Native PAGE electrophoresis

Native electrophoresis of pepsin and hemoglobin on 10% polyacrylamide gel carried out at 48 °C during 90 min, according to the Laemmli procedure, at pH 8.3 (Laemly 1970). 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^{3+} ion (1, 5 and 10 mM). The samples were diluted with sample buffer in ratio 1:1 (v/v) and applied on gel in volume of 20 µL. Visualization was performed with Commassie 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_S value, where it is defined by:

$$R_S = \frac{\text{Distance of protein migration}}{\text{Distance of tracing dye migration}} \tag{26}$$

4.3 Differential scanning calorimetry

DSC measurements were carried out on a MicroCall "MC-2" Differential Scanning Calorimeter (MicroCall Inc., Northampton, MA) with cell volumes 1.14 mL, at heating rates 1.5 °C/min. DSC scans were obtained in the temperature range from 10 to 100 °C. For all the measurements the protein concentrations were 0.03 mM, pH were set at 2. Degassing during the calorimetric measurements was prevented by additional constant pressure of 1 atm over the liquids in the cells. At first, the solvent was placed in both the sample and reference compartments. A DSC curve corresponding to solvent *vs*. solvent run was used as an instrumental baseline. The calorimetric data were corrected for the calorimetric baseline (by subtracting solvent – solvent scan). The data were converted to molar excess heat capacity by using the protein concentration (0.03 mM pepsin) and cell volume of 1.14 mL and than corrected for the difference in heat capacity between the initial and the final state by using linear baseline. The calorimetric reversibility of thermally induced transition was checked by reheating the protein solution in the calorimetric cell after cooling from the first run.

5. Results and discussion

5.1 The effect of Al^{3+} ions on the reaction rate and kinetic parameters of pepsin

The influence of Al^{3+} ions on porcine pepsin activity were examined in a wide range of Al^{3+} ions concentration included physiological and toxic ones, *in vitro* conditions, and at pH 2. All investigated concentration of Al^{3+} ions cause increase of pepsin activity. The increasing concentrations of metal ions induced increase of enzymatic activity. The observed effects are presented at Figure 2, and it is indicative that increase of pepsin activity follows in a dose dependent manner the concentrations of bounded aluminium. The obtained results

are in agreement with previously reported (Krejpcio 2002) stimulatory effect of Al^{3+} ions on porcine pepsin activity. The authors reported that applying concentration of $1.1 \cdot 10^{-3}$ M Al^{3+} ions induce the activation of 191%, while we obtained 135.8% activation of pepsin activity, in applied concentration of 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).

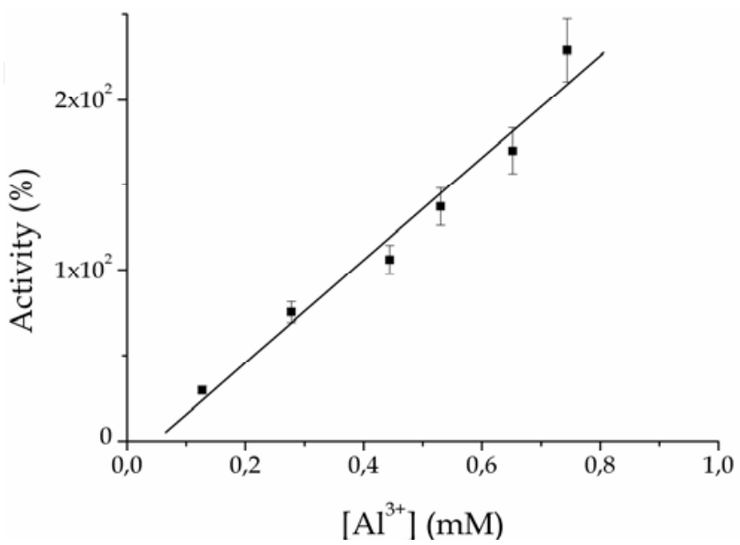

Fig. 2. The *in vitro* effects of Al^{3+} ions on pepsin activity; the effects were investigated in concentration range from $1.7 \cdot 10^{-6}$ to $8.7 \cdot 10^{-3}$ M of Al^{3+} ions. The increasing concentrations of Al^{3+} ions induced increase of enzymatic activity. Values are expressed as the percent of increased activity related to the control, which considered as 100%. Aluminum was found to stimulate the enzyme activity in dose-dependent manner. Investigated concentrations of Al^{3+} ions, induce the increase of pepsin activity from 30.7% to 135.8% ($p < 0.01$), in comparison with corresponding control. The degree of activation is expressed as % of increased activity considering the pepsin activity in the absence of aluminum as 100%. Data are expressed as a mean of at least three independent experiments performed in triplicate. Data from Pavelkic et al. (2008) are modified.

Initial reaction rates were determined by monitoring the change in absorbance at 280 nm due to formed reaction products in a Beckman UV 5260 UV-VIS spectrophotometer, in cells of path length 1cm, thermostatically controlled at 37 ± 0.1 ˚C. A typical kinetic experiment consisted of numerous steady – state rates at different combinations of substrate and activator concentrations was performed and presented at Figure 3.

The results obtained from Lineweaver-Burk plots, are used for calculation of kinetic constants. The secondary plots of the slopes and intersects *vs*. activator concentrations are not linear (data not shown), but the reciprocal of the change in slope and intercept (Δ_{slope} and $\Delta_{intercept}$) that are determined by subtracting the values in the presence of activator from that in its absence, are linear. 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 4). The calculated values for constants are (0.904 ± 0.083) mM and (8.56 ± 0.51) mM, respectively.

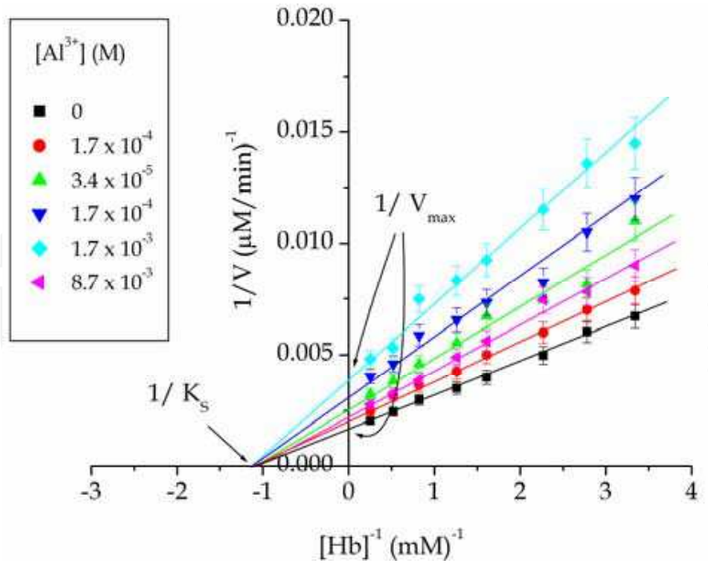

Fig. 3. Double reciprocal Lineweaver – Burk plot of influence of Al^{3+} ions on reaction kinetics of pepsin at pH2; Increase of reaction velocity in a presence of activator (inset: various concentration of Al^{3+} ions) is proportional to increased activator concentration. Increasing of aluminium concentrations increased V_{max} values, without significant change in the value of apparent enzyme affinity for substrate K_S. Graphically determined apparent enzyme affinity for substrate, K_S, in the presence of 1.7 µM activator is (0.907 ± 0.083) mM L^{-1}, while in the presence of maximal activator concentration (8.7 mM) K_S is (0.917 ± 0.073) mM L^{-1}. V_{max} is changed in a concentration depending manner. Without presence of activator maximal reaction rates changes from (254 ± 7) µM min^{-1}, to (599 ± 17) µM min^{-1} at maximal activator concentration. Data from Pavelkic et al. (2008) are modified.

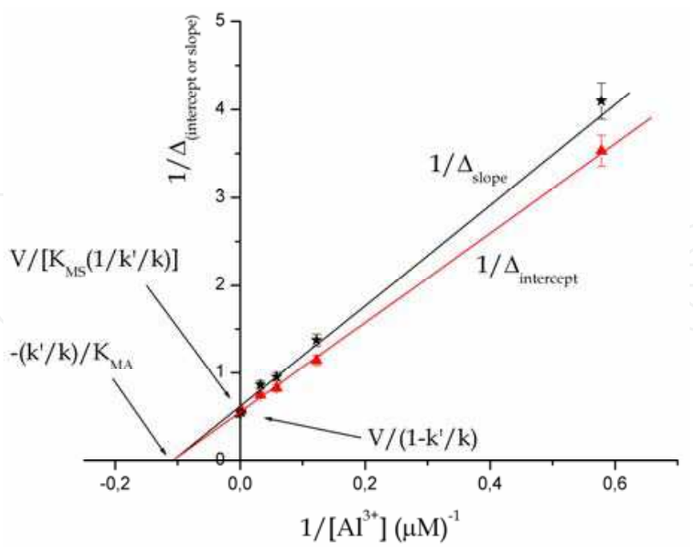

Fig. 4. The reciprocal of the change in slope and intercept (Δ_{slope} and $\Delta_{intercept}$) against reciprocal of activator concentration, where Δ is defined as slope or intercept in the absence of activator, Al^{3+} ions, minus that in its presence. Data from Pavelkic et al. (2008) are modified.

Simple geometrical considerations illustrated in Figure 5 show that kinetic data could be used for graphical determination of the activator concentration that gives a reaction rate equal to the half of saturation concentration of activator (A_{50}), as well as the dissociation constant K_A for enzyme-activator complex. If the abscissa variable is 1/[A], then the intercept is – 1/ [A_{50}], where [A_{50}] is activator concentration that gives a rate equal to the half that at a saturating concentration of activator.

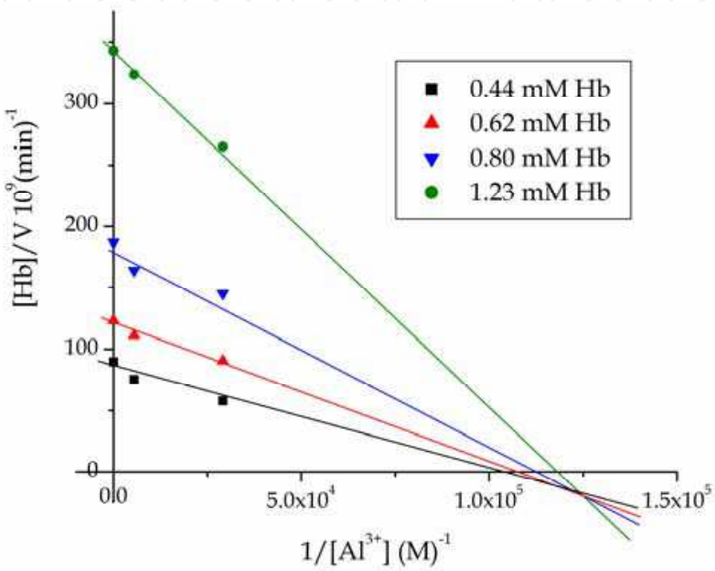

Fig. 5. Activation effects of different aluminium concentrations on pepsin. The intercepts on the abscissa of the plot [Hb]/V *vs.* reciprocal of aluminium concentrations represents the values of A_{50}. The obtained kinetic data were used for determination of dissociation constant K_A for pepsin-aluminium (EA) complex. The calculated values of A_{50} and K_A were (8.05 ± 0.48) μM and (8.82 ± 0.90) μM, respectively. Data from Pavelkic et al. (2008) are modified.

5.2 The influence of Al^{3+} ions on electrophoretic mobility of pepsin

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 samples in absence or in the presence of different concentrations of Al^{3+} ion are presented in Figure 6 and Figure 7, respectively.

The presence of Al^{3+} cause the decrease of pepsin electrophoretic mobility at all investigated concentrations. 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 Rs value for pepsin is 0.47 while in the presence of 1, 5 and 10 mM Al^{3+} ions the obtained Rs values were 0.46, 0.44 and 0.42, respectively.

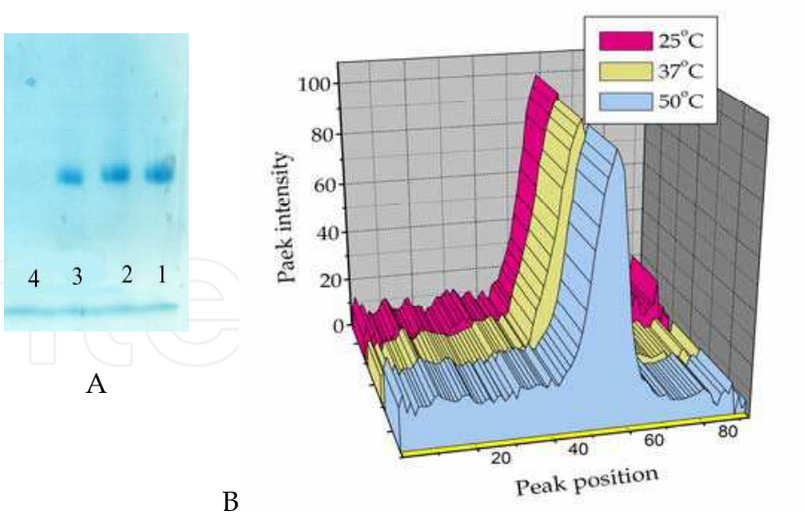

Fig. 6. A - Native PAGE electrophoregram of pepsin without Al^{3+} ions at pH 2. B – Visualization of quantified electrophoretic mobility of pepsin molecule treated at different temperatures.

Native PAGE electrophoresis of pepsin on 10% polyacrylamide gel carried out at 4˚C during 90 min, according to the Laemmli procedure, at pH 8.3. 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 25˚C, 37˚C, 50˚C and 70˚C (band 1 to 4 respectively). Visualization was performed with Commassie Brilliant Blue G-250 dye. B - The gels were scanned and processed using Corel Draw 11.0 software package.

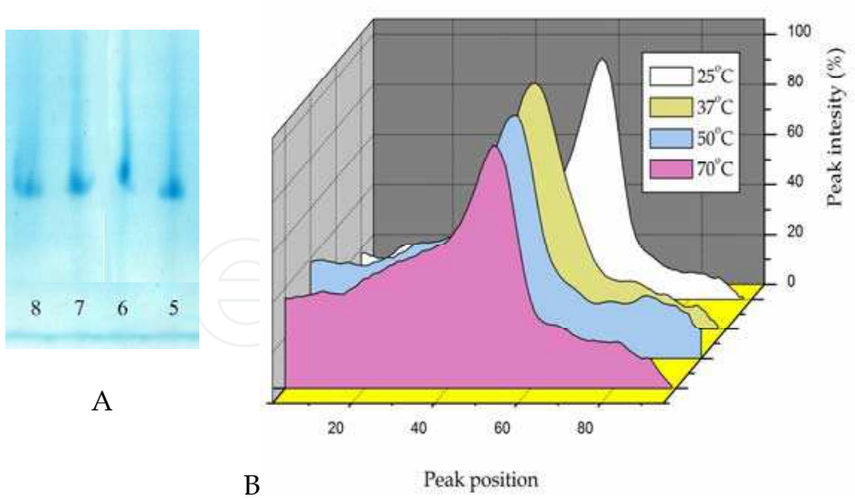

Fig. 7. A - Native PAGE electrophoregram of pepsin in a presence of 5 mM Al^{3+} ions at pH 2. B – Scanned and processed gel of pepsin samples with addition 5 mM Al^{3+}, previously incubated at 25˚C, 37˚C, 50˚C and 70˚C (band 5 to 8 respectively).

Quantification of electrophoretic mobility of the molecule is carried out via R_S value, where it is defined as:

R_S = distance of protein migration / distance of tracing dye migration.

In all cases increasing the temperature causes the decrease in electrophoretic mobility of pepsin. The cause of decrease in electrophoretic mobility can be explained by thermally induced conformational changes in pepsin molecule. The pepsin bend is absent in samples treated at 70°C, in the absence of Al^{+3} ion as well as in the presence of all investigated Al^{+3} concentrations, except 5 mM Al^{+3} . This result is in agreement with previously reported data that temperatures of 70°C and higher induce unfolding of an enzyme (Sepulveda et al. 1975). The degree of pepsin electrophoretic mobility decrease depends on Al^{3+} concentration that the one has been exposed. The difference between Rs values obtained at 25 °C and 50 °C in absence of Al^{3+} ion is 0.02, while in the presence of 10 mM Al^{3+} it is 0.05. If the influence of Al^{3+} ion concentration on pepsin mobility at defined temperature we discuss it could be seen that increase in concentration of Al^{3+} decelerate the migration of pepsin samples on concentration dependent manner. Rs values of pepsin at 37°C in the absence of Al^{3+} is 0.47, while Rs values are 0.46, 0.44 and 0,42 in the presence 1 mM, 5 mM and 10 mM of Al^{3+} respectively (data not shown). The same trend has been obtained for the other tested temperatures, except for 70°C. The slow down in pepsin migration can be explained by conformational changes caused by Al^{3+} binding to enzyme.

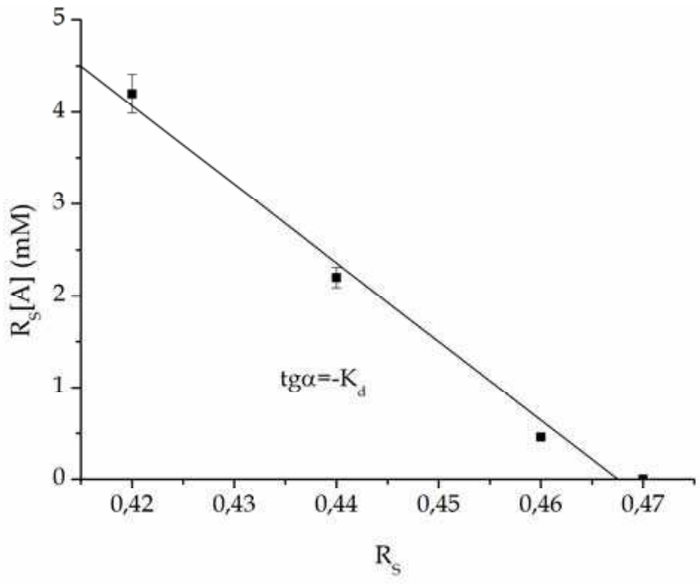

Fig. 8. Graphical determination of dissociation constant from obtained R_s values from electrophoregrams of pepsin in a presence different concentration of Al^{3+} ions. Calculated value of (0.856 ± 0.007) mM is in a good agreement with those determined via kinetic experiments.

5.3 Thermal stability of pepsin in a presence of Al^{3+} ions followed by differential scanning calorimetry

Calorimetric enthalpy for the complete transition was estimated from the total peak area, and also the enthalpy from the temperature dependence of the equilibrium according to the van't Hoff equation, as well as the enthalpy for the each stage of pepsin unfolding. The van't

Hoff enthalpy, ΔH^{VH}, was determined for each scan by subtracting the baseline to remove the heat capacity effect and then curve fitting to a non-two state model (Table 1).

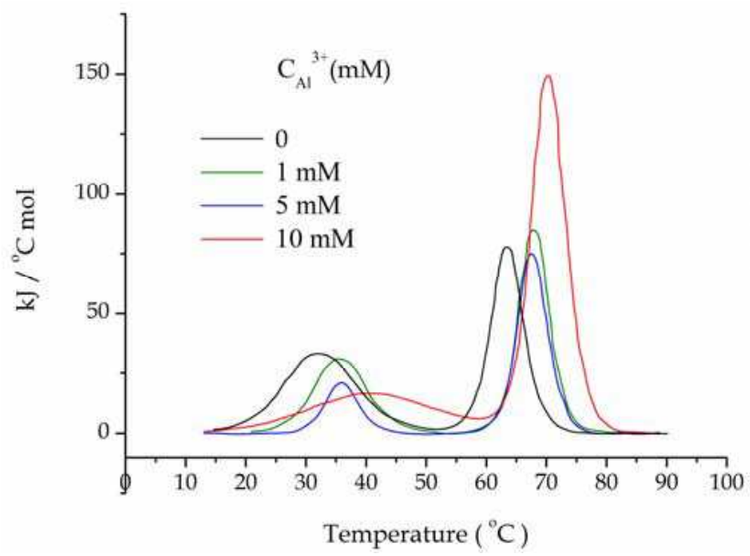

Fig. 9. Thermograms of pepsin with addition different concentrations of Al^{3+} at different concentrations, at pH 2.

A presence of aluminium affects the position of the first peak, and changes its shape. Compared with the DSC profile of pepsin for all Al^{3+} concentrations (1, 5, 10 mM) the first peak becomes more broadened and asymmetric. Van't Hoff enthalpies calculated for the first transition temperature are more than twice larger than calorimetric enthalpies observed for the same transition temperatures. For these transitions calorimetric and van't Hoff enthalpies are calculated and are presented in Table 1.

C_{Al+3} (mM)	peak	T_m (K)	ΔH^{cal} (kJ/mol)	ΔH^{VH} (kJ/mol)	$\Delta(\Delta G^0)$ (kJ/mol)	$K_{L(Tm)}$ (M^{-1})
0	**1**	304.5 ± 0.2	535 ± 21	196 ± 8	0	-
	2	336.9 ± 0.2	556 ± 12	531± 17	0	-
1	**1**	308.6 ± 0.2	138 ± 12	389± 42	7.94±0.76	$1.05 \cdot 10^3$
	2	340.9 ± 0.2	769 ± 21	568 ± 8	-0.52±0.05	$9.26 \cdot 10^8$
5	**1**	309.2 ± 0.2	171 ± 4	422 ± 8	9.32±0.91	$7.30 \cdot 10^2$
	2	342.5 ± 0.2	518 ± 35	627± 42	3.74±0.04	$5.71 \cdot 10^8$
10	**1**	315.6 ± 0.2	159 ± 21	529± 44	8.78±0.88	$6.76 \cdot 10^5$
	2	343.5 ± 0.2	481 ± 17	567± 21	0.23±0.01	$1.43 \cdot 10^6$

Table 1. Thermodynamic parameters of pepsin unfolding in a presence of Al^{3+}. Standard Gibb's energy was calculated at 25°C; K_L is equilibrium-binding affinity; Reported values are the means of minimum three independent replicates.

In contrast to ΔH^{cal} of value 535 kJ/mol calculated for the first transition temperature for intact pepsin, in a presence of Al^{3+} for each applied concentrations values are reduced of approximately 138 to 170 kJ/mol. For the second transition temperature, calorimetric enthalpies are very close to each other. At the same time, calculated values for van't Hoff enthalpies, for both transition temperatures, are close to each other. With the exceptions of the first transition temperature for the aluminium non-treated pepsin, all other examined cases have a ΔH^{VH} that is smaller than ΔH^{cal}. At the first transition temperature, the ratio $n = \Delta H^{cal} / \Delta H^{VH}$ ranges from 0.35 for pepsin treated with 1 mM Al^{3+} to 2.72 for native pepsin. At the second transition temperature, those ratios vary from 0.83 for pepsin treated with 5 mM Al^{3+} to 1.35 for native form of pepsin.

The fact that the ratio is larger than unity for some cases suggests that multiple transitions occur within a single peak and that the transitions are coupled less then 100%. Each lobe of pepsin is composed of two almost identical sub-domains (Andreeva 1989, Brandts 1990, Blundel 1990) forming a part of the binding cleft, and the structural feature might well contribute to the transitions causes deviation from two-state behaviour for most forms. At low temperature, there were essentially no unbound aluminium ions (all excess Al^{3+} was dialysed before DSC experiments). Depending on concentrations of bound Al^{3+} the shifts in T_m values ranges of about 4 to 11 degrees for the first transition, and for the second transition T_m values for all aluminium concentrations are shifted from 4 to about 7 degrees. From the shift in T_m values the changes in apparent stability of the treated pepsin relative to the native form was evaluated from the equation:

$$\Delta\left(\Delta G\right) = \Delta G_{Al^{3+}} - \Delta G^{0}_{(native)} \tag{27}$$

Where $\Delta G^{0}_{(native)}$ is zero at T_m, while $\Delta G_{Al^{3+}}$ was obtained from Gibbs-Helmholtz equation. The obtained values of $\Delta\left(\Delta G\right)$, the Gibbs free energy of aluminium binding, are listed in Table 1. As the process of pepsin unfolding is complex process, and proceeds in two different stages, the free energy of unfolding was calculated at both temperatures of transition. Compared to the temperatures where native pepsin unfolds, and to calculated free energies for the both transition temperatures, aluminium binding causes stabilisation of the pepsin structure for all applied concentrations.

Binding of Al^{3+} at the first part of pepsin molecule causes structural changes and during the melting process, intermolecular interactions are present probably because of partially denaturised initially part of the protein. At the second transition temperature in a presence of various Al^{3+} concentrations at pH 2, the observed values of van't Hoff and calorimetric enthalpies are very close to each other. Unfolding of the second part of the molecule in presence of Al^{3+} take place as a two state process, at which the second part of molecule behave as a single domain.

The DSC curve profiles suggest two kinds of binding sites for Al^{3+} on pepsin. At each transition midpoints, T_{m_1} and T_{m_2}, and from ligand-induced shifts and calorimetric parameters for reversible transitions, an equilibrium binding affinity K_L were calculated (Table 1).

The average number of ligands bound per molecule of native pepsin, as estimated from DSC data, was $\overline{X}_{NI}$ =1.35 for the first transition temperature, and $\overline{X}_{NII}$ =3.58 for the second transition temperature. The obtained values for average number of bound ligands per molecule of pepsin for both transition temperatures are in agreement with calculated binding constants (Table 1).

UV melting experiments, as indirect mode for following thermal unfolding parameters, were conducted in the absence and presence of Al^{3+} ions to assess the impact of bound Al^{3+} ions on the thermal stability of pepsin. The resulting melting profiles of pepsin at 280 nm, pH 2 without and in a presence of 5 mM Al^{3+} ions are presented on Figure 10. Both melting curves showed typical sigmoid behaviour. Addition of 5 mM solution of Al^{3+} produces biphasic denaturation of pepsin at pH 2. Spectroscopic signals for thermal denaturation of pepsin in a presence of Al^{3+} ions at pH 2 are presented at Figure 10.

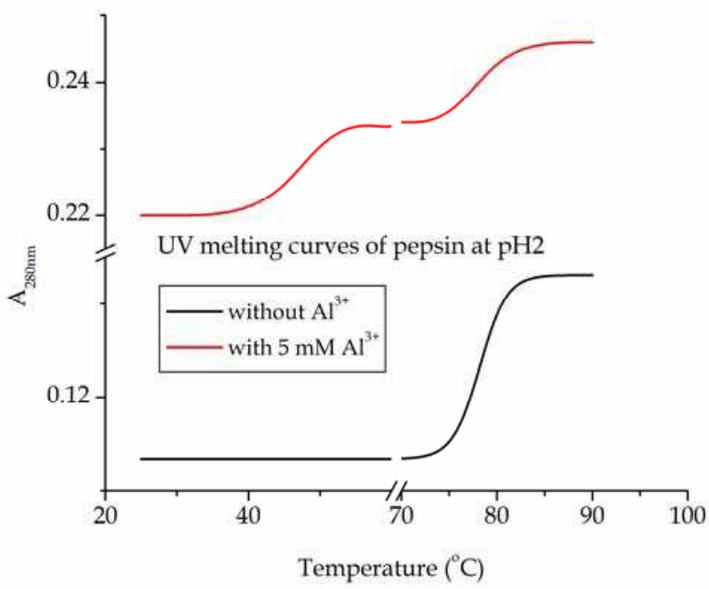

Fig. 10. UV absorbance measurements were carried out on Beckman UV 5260 UV-VIS spectrophotometer with an electro thermal temperature control cell unit. The temperature control was performed with digital voltmeter with thermocouples. A quartz cell with 1 cm path length was used for all the absorbance studies. Absorbance was measured directly as a temperature function. Thermal unfolding of pepsin was monitored by recording absorbance at 280 nm in temperature interval from 20 °C to 90 °C with heating rate of 1 °C/min and samples were allowed to equilibrate for one minute at each temperature setting, while the reference cell, containing just a solvent, was monitored at room temperature. The resulting increase of the absorbance of the sample solution recorded over the temperature range. Pepsin concentration was 0.3 mg / $ml_{solution}$.

The calculated values of van't Hoff enthalpy from spectroscopic data of unfolding of pepsin and corresponding values of temperatue midpoints for thermally induced conformational transitions of pepsin at pH 2 and in a presence of 5 mM Al^{3+} ions were calculated and presented in Table 2.

	λ = 280 nm
T_{m1} (K)	320.5
$\Delta H^{UV}{}_{unf1}$ (kcal/K mol)	97
T_{m2} (K)	352.1
$\Delta H^{UV}{}_{unf2}$ (kcal/K mol)	140

Table 2. Thermodynamic characteristics of pepsin denaturation at pH 2 in a presence of 10 mM Al^{3+} ions, obtained by UV spectroscopy.

6. Conclusion

Results of our study showed that aluminium cause increase pepsin activity. The obtained activation of pepsin is probably a consequence of conformational changes of enzyme molecule induced by bounded aluminium. As it were previously reported in analogy to the other aspartic proteases, bounded aluminium ions could causes significant conformational changes and induce increase in beta structure content (Flaten et al. 1992, Bittar et al. 1992, Clauberg et al. 1993).

The kinetic data implies that activation of pepsin is a non-essential partial non-competitive type. That suggests that bound aluminium do not influence on substrate binding sites on pepsin, but causes conformational changes that increase the rate of substrate converting to the reaction products. The results are consistent with a partial activation system presented in scheme in Figure1. Calculated dissociation constants from kinetic and indirect UV melting assay data are in good agreement to each other.

The present thermodynamic analyses show that DSC method is useful to measure the binding constants from *T*m shifts. The stabilization effect of Al^{3+} binding on pepsin molecule, as well as an average number of ligands bound to the native protein, equilibrium binding affinity K_L were also calculated. The obtained values for binding affinity for site I are lower than on site II, which is in agreement with obtained values for average number of bound ligand. In addition, it can be assumed that the site II has a higher affinity for Al^{3+}than site I.

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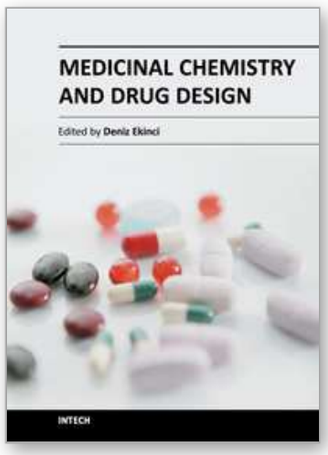

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Over the recent years, medicinal chemistry has become responsible for explaining interactions of chemical molecules processes such that many scientists in the life sciences from agronomy to medicine are engaged in medicinal research. This book contains an overview focusing on the research area of enzyme inhibitors, molecular aspects of drug metabolism, organic synthesis, prodrug synthesis, in silico studies and chemical compounds used in relevant approaches. The book deals with basic issues and some of the recent developments in medicinal chemistry and drug design. Particular emphasis is devoted to both theoretical and experimental aspect of modern drug design. The primary target audience for the book includes students, researchers, biologists, chemists, chemical engineers and professionals who are interested in associated areas. The textbook is written by international scientists with expertise in chemistry, protein biochemistry, enzymology, molecular biology and genetics many of which are active in biochemical and biomedical research. We hope that the textbook will enhance the knowledge of scientists in the complexities of some medicinal approaches; it will stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of medicinal chemistry and drug design.

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