

The Influence of Al³⁺ Ions on Pepsin and Trypsin Activity in Vitro

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Abstract

The objective of this study was to investigate the influence of the concentration of Al³⁺ ions and the substrate/enzyme ratio on pepsin and trypsin activity in vitro. The experimental design was a combination of three Al³⁺ ion concentrations (0.25; 2.5 and 25.0 µg Al³⁺/ml of reaction solution) and two substrate/enzyme ratios (S/E = 10 and 100 for pepsin, and 100 and 1000 for trypsin). Enzymatic activity was determined by the Folin method based on the reaction of tyrosine with the Folin reagent.

It was found that the concentration of Al³⁺ ions influenced activity of pepsin which increased with the increasing metal ion concentration in the reaction milieu. Al³⁺ ions did not affect activity of trypsin. Proteolytic activity of pepsin and trypsin depended on substrate/enzyme ratio. Higher concentration of substrate decreased efficacy of enzymatic protein breakdown in vitro.

Keywords: aluminium, pepsin, trypsin, enzymatic activity

Introduction

Aluminium is the most abundant metal, comprising almost 8% of the earth's crust; however, its concentration in living systems is very low [1]. Aluminium does not belong to essential elements, and as a non-regulatory ion can be toxic to many organisms.

Aluminium enters the food chain through soil and water. The main sources of this metal to humans are food and water. According to WHO Technical Reports [2] the average daily dietary aluminium intake is approx. 2-6 mg Al/day in children and 6-14 mg Al/day in adults. The Provisional Tolerable Weekly Intake established for Al is 7 mgAl/kg body weight [3]. An additional source of aluminium are food additives (Al stearinate, palmitate, silicate) that are components of emulgators, baking powders, pH-regulators and anticaking agents [2, 3]. Besides, aluminium can migrate in acidic conditions to water and food from Al-alloys used for cutlery, containers and foil

[4, 5]. Aluminium can also enter the human body with Al-containing antacids that have been widely used in therapy for dyspepsia peptic ulcer, and phosphate-binding gels for non-dialyzed and dialyzed uremic patients as a method to control serum phosphorus concentration [6-9]. Aluminium toxicity in uremic patients is well documented [10]. Elevated levels of Al in brain and bone have been associated with several neurological diseases and in hemodialysed patients suffering from renal failure [11, 12]. Aluminium interferes with metabolism of some nutrients, especially minerals such as calcium, phosphorus, magnesium and iron and may cause osteodystrophias and anaemia [3]. However, very little is known about whether Al³⁺ ions that enter the human organism through food, water and antacid can affect activity of gastrointestinal enzymes and influence digestion and utilization of nutrients. Therefore, the objective of this study is to investigate the influence of Al³⁺ ions on pepsin and trypsin activity in vitro.

Materials and Methods

The influence of the concentration of Al^{3+} ions and substrate to enzyme ratio on enzymatic activity of pepsin and trypsin was studied in a 2 x 3 factorial design. Factor A was a substrate/enzyme ratio (10 and 100 for pepsin, and 100 and 1000 for trypsin) while factor B was the Al^{3+} ions concentration (0.25; 2.50 and 25.0 $\mu g Al^{3+}/ml$ of reaction solution). The combination of these factors as well as the reference samples made 16 experiments altogether. The reference samples consisted of respective amounts of reagents apart from the Al^{3+} ions added which were replaced by Na^+ ions (as nitrate). Preliminary tests showed the enzymatic activity was time-dependent and the highest activity of the studied enzymes was recorded 5 min. after the start of reaction. Therefore, the duration of enzymatic reaction of each experiment was 5 min.

The studied materials were:

- Enzymes: *Pepsin* (porcine gastric mucosa, Merck) and *Trypsin* (from hog pancreas, Flucka),
- Substrates: *Albumin* (from hen egg, GR, POCH) and *Casein* (light white, GR, BDH Chemicals Ltd. Poole England).
- Aluminium nitrate aqueous stock solution (1 mg Al/ml , ICP standard solution, Merck).
- Sodium nitrate aqueous stock solution (1mg Na/ml , ICP standard solution, Merck).

Activity of enzymes was determined using the colorimetric method based on the reaction of the casein or albumin degradation product complexed with the Folin-Ciocalteu phenol reagent (Merck) [14]. The reagent corresponds in its composition to molybdate-tung-

stenate reagent and to phenol reagent stock solution for the determination of pepsin activity. The reaction solutions were composed of the following reagents added to the test tubes in the following sequence: 1 ml of substrate (1% of casein solution in a phosphate buffer pH 7.4 or 1% albumin in redistilled water), 1 ml of redistilled water, and 1 ml of Al^{3+} ions solution (containing: 1.0; 10.0 and 100 $\mu g Al^{3+}$ ions/ml), and 1 ml of enzyme (pepsin solution in $In HCl$ or trypsin solution in 0.001 n HCl). The mixtures were incubated at 40°C for 5 min, then the reaction was discontinued through the addition of 2 ml of 5% (w/v) trichloroacetic acid (TCA). Subsequently the solutions were centrifuged to separate precipitate from supernatant. Then 1 ml of the supernatant was incubated with 5 ml of 0.2 mole Na_2CO_3/l of aqueous solution, and 1 ml of the Folin-Ciocalteu phenol reagent (diluted with redistilled water in the proportion 1:5. v/v) at 40°C for 20 min. Successively the absorbance was measured at $X = 625$ nm with the use of spectrometer Specol 20 (Zeiss).

The activity of enzymes was expressed in Proteolytic Activity Units (PAU) and calculated from the equation proposed by Namoto and Narahashi [15]:

$$PAU/ml = A \times V / A_1 \times t = 4.938 \times 10^{-4} \times A$$

where:

- A - absorbance of a sample
- A_1 - absorbance of 1 mEq of tyrosine (1620)
- V - sample volume (4 ml)
- t - reaction time (5 min)

Table 1. Activity of enzymes vs. concentration of Al^+ ions and substrate.

Exp. Nr	Enzyme	Substrate/Enzyme ratio (mg/mg)	Al^{3+} ions conc. ($\mu g/ml$)	PAU ($\times 10^{-6}/ml$) mean \pm sd	% of inhibition (-) or activation (+)	Difference vs. Control p
1	Pepsin	10	0	82.2 \pm 12.4	-	-
2			0.25	95.4 \pm 4.0	16.0 (+)	< 0.05
3			2.50	120.6 \pm 9.8	46.7 (+)	< 0.05
4			25.00	180.9 \pm 8.8	120.1 (+)	< 0.05
5		100	0	16.7 \pm 5.2	-	-
6			0.25	24.4 \pm 4.2	46.2 (+)	NS
7			2.50	34.4 \pm 4.0	106.5 (+)	< 0.05
8			25.00	48.6 \pm 10.5	191.0 (+)	< 0.05
9	Trypsin	100	0	33.2 \pm 12.9	-	-
10			0.25	28.5 \pm 5.9	14.2 (-)	NS
11			2.50	26.9 \pm 6.1	19.0 (-)	NS
12			25.00	26.8 \pm 10.4	19.3 (-)	NS
13		1000	0	20.6 \pm 8.3	-	-
14			0.25	14.0 \pm 3.1	32.1 (-)	< 0.05
15			2.50	15.9 \pm 2.4	22.9 (-)	NS
16			25.00	17.3 \pm 2.3	16.1 (-)	NS

Each experiment was performed in 11 replications to obtain reliable data and avoid errors. The results were expressed as means and standard deviation and verified statistically with the use of ANOVA and the Tukey tests at the significance level preset at $p < 0.05$, with the help of the Statgraphics 7.0 computer program.

Results and Discussion

Table 1 shows the mean and sd values for the activity of enzymes vs. concentration of Al ions and substrate while Table 2 presents the effect of substrate concentration on pepsin and trypsin activities irrespective of Al ion concentrations. Both the concentration of Al³⁺ ions and the substrate-to-enzyme ratio affected activity of pepsin in the established experimental conditions in vitro. The addition of Al³⁺ ions to the reaction milieu significantly activated pepsin. The degree of this activation depended on Al³⁺ ion concentrations and substrate vs. enzyme ratio, and increased with increasing metal ion concentration and higher reagents ratio. The highest activation of pepsin (by 191.0%) was observed in experiment 8. (S/E = 100, 25.0 µg Al³⁺ /ml of reaction mixture) in comparison with the control. Whereas in the lower substrate/enzyme ratio S/E=10, this elevation was markedly lower (experiment 4). As shown in Table 2 the activity of pepsin was significantly lower in experiments with the higher (S/E = 100) vs. lower substrate/enzyme ratio, irrespective of Al ion concentrations.

In contrast with pepsin, activity of trypsin was not affected by the concentration of Al³⁺ ions in the reaction milieu. However, there was one exception from this rule, when the addition of 0.25 µg Al³⁺ /ml of reaction solution to the higher substrate/enzyme ratio (1000) in experiment 14 distinctly decreased trypsin activity (by 32.1%) vs. the control. Moreover, slightly lower values were found for all experiments in the presence of Al³⁺ ions. On the other hand, activity of trypsin, like in the case of pepsin, depended on the substrate/enzyme ratio and was markedly lower in the higher (S/E = 1000) vs. lower proportion of these reagents (Table 2).

Table 2. Effect of substrate concentration (irrespective of the Al³⁺ ions concentration) on pepsin and trypsin activities in vitro.

Enzyme	Substrate/ Enzyme ratio (mg/mg)	PAU (x10 ⁻⁶ /ml) mean ± sd	Difference between means p
Pepsin	10	119.8 ± 43.8	< 0.05
	100	31.1 ± 13.6	
Trypsin	100	28.9 ± 3.0	< 0.05
	1000	17.0 ± 2.8	

In the available literature information about the influence of toxic metal ions on the activity of extracellular enzymes is scant. Most data have been obtained for intracellular enzymes affected by lead, cadmium and mer-

cury. For example, Sastry and Gupta [16-19] studied the effect of lead acetate on activity of alkaline and acidic phosphatase, maltase, lactase and aminotripeptidase and pepsin and trypsin in fish (*Heteropneustes Fossilis*). The Authors found that lead ions inhibited alkaline phosphatase and gluco-6-phosphatase in liver and intestine, while acidic phosphatase increased in these organs. Moreover, activity of pepsin slightly increased whereas trypsin significantly decreased.

It is known that Al interacts with Ca²⁺ and Mg²⁺ - dependent enzymes. Binding of Al³⁺ ion with ATP is stronger than with Mg²⁺, which is a natural activator of ATP-ase; therefore Al³⁺ ion is a strong inhibitor of all ATP-ases [20]. It has been documented that Al³⁺ inhibits activity of hexokinase [21], adenylate cyclase [22], calmodulin [23], NAD-kinase [24], plus other Mg-dependent enzymes, like alkaline phosphatase [24, 25], acetylcholinesterase [26], and forrooxidase [27].

The results of this study showed that Al³⁺ ions activated pepsin but not trypsin. The mechanism of its action is not known. Al³⁺ ions may influence binding of substrate with enzyme by various mechanisms. For example, binding of metal ions with enzyme or substrate, or both can lead to formation of active complexes easily degraded to final products. Probably Al³⁺ ions that occur mostly in a ionic form in the acidic milieu affect electric charge of the molecules of reagents, which can increase the "contact phase" between substrate and enzyme. As a result, the efficacy of proteolytic activity is increased in the presence of Al³⁺ ions. Likewise, Pb²⁺ and Cd²⁺ [14], Al³⁺ did not influence activity of trypsin in comparison with the control. This may be explained by the fact that the optimum activity of trypsin is shifted towards a weak acidic or neutral milieu (pH = 6-7). In such conditions Al occurs mostly in the form of insoluble polyhydroxy complexes with no electric charge. They precipitate and the concentration of free Al³⁺ ions is very low, thus the possibility of formation metal-enzyme-substrate active complexes is substantially reduced, which may explain no effect of Al³⁺ ions on trypsin activity.

Another phenomenon found in this study was the inverse correlation between substrate /enzyme ratio and activity of pepsin and trypsin. The mechanism of this interaction also is not fully known. Presumably the observed decrease of activity of enzymes may be caused by different reaction pathways in the case of high concentration of substrate in the reaction milieu. It is possible that high concentrations of substrates lead to the formation of different enzyme-substrate complexes (e.g. Substrate-Enzyme-Substrate), thus enzymatic reaction goes through additional intermediate stages that slow down the efficacy of protein breakdown. Moreover, unfavourable physicochemical properties, like increased density and viscosity of the reaction milieu, can inhibit diffusion of reagents towards/or products from the reaction phase.

Conclusions

1. The concentration of Al³⁺ ions influenced activity of pepsin that increased with the increasing metal ion concentration in the reaction milieu in vitro.
2. Al³⁺ ions did not affect activity of trypsin in vitro.

3. Proteolytic activity of pepsin and trypsin depended on substrate/enzyme ratio. Higher concentration of substrate decreased efficacy of enzymatic protein breakdown in vitro.

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