IMMUNOSTIMULANT ACTIVITY OF THE COORDINATION COMPOUNDS OF ISOLEUCINE – TRYPTOPHAN DIPEPTIDES WITH ZINC IONS

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Secondary immunodeficiency states related to T-cell immunity disorders are treated by immunomodulants such as tactivin (T-activin) and thymogen [1]. Tactivin is a mixture of peptides isolated from thymus [2], while thymogen is based on a synthetic glutamyl – tryptophan dipeptide [3]. Hyperimmunity and immunodeficiency states are usually treated with a composition comprising a dipeptide mixture with the general formula X-Trp, where X is glutamic acid, glutamine, leucine, and isoleucine [4]. It is known that complexation with metal (zinc, iron, platinum) ions leads to an increase in the specific activity of medicinal preparations [5]. In this context, we have studied the effect of complex formation with zinc ions on the immunostimulant activity of an isoleucine – tryptophan dipeptide.

The dipeptide was obtained by condensation of carbobenzoxy-*L*-isoleucine with *L*-tryptophan using dicyclohexylcarbodiimide as a condensing agent and 1-hydroxybenzotriazole as a nucleophilic additive. The carboxy group of tryptophan was protected by forming a salt with triethylamine. Upon purification, the protected peptide was unblocked by catalytic hydrogenation in the presence of palladium (10% Pd on activated charcoal). Preliminary purification of the free dipeptide was performed by extraction with *n*-butanol, while the final purification was effected by HPLC. The free dipeptide yield after lyophilization amounted to 98%.

Before the synthesis of coordination compounds, it was necessary to determine the pH range in which the target dipeptide occurs in the zwitterion form. For this purpose, we performed the pH titration of isoleucine, tryptophan, and the isoleucine – tryptophan dipeptide and constructed the corresponding distribution diagrams using a method described in [6]. According to these data (Fig. 1), isoleucine, tryptophan, and the isoleucine – tryptophan dipeptide occur in the zwitterion form in the pH range 4.0 – 9.0, 3.0 – 10.0, and 6.0 – 8.0, respectively. The experimental dissociation constants are as follows: isoleucine, $K_1 = 7.41 \times 10^{-4}$ ($pK_1 = 3.13$) and $K_2 = 2.45 \times 10^{-10}$ ($pK_2 = 9.61$); tryptophan, $K_1 = 1.12 \times 10^{-2}$ ($pK_1 = 1.95$) and $K_2 = 3.63 \times 10^{-11}$ ($pK_2 = 10.44$); isoleucine – tryptophan dipeptide, $K_1 = 7.4 \times 10^{-6}$ ($pK_1 = 5.13$) and $K_2 = 1.45 \times 10^{-11}$ ($pK_2 = 10.84$). According to the available published data [7], the dissociation constants of amino acids are $pK_1 = 2.36$ and $pK_2 = 9.68$ for isoleucine and $pK_1 = 2.38$ and $pK_2 = 9.39$ for tryptophan. The difference between our values and the published data can be explained by the fact that our pH titration procedure was performed in aqueous amino acid solutions without maintaining constant ionic strength.

Solutions of the zinc-containing coordination compounds were obtained by interaction of dilute aqueous solutions of dipeptide and zinc acetate at at a reagent concentration of 3.133×10^{-4} mole/liter, pH 6.0, and a temperature of 110 – 120°C. The reaction was conduced in the dark without access to air in order to eliminate oxidation of the indole group of tryptophan. The formation of a target coordination compound was confirmed by pH titration measurement, the results of which are presented in Fig. 2. The presence of an equivalence point in the titration curve confirms the coordination of dipeptide to zinc ion.

We also attempted to determine the composition of the coordination compound using the method of isomolar series [8]. As can be seen from the results of these experiments (Figs. 3 and 4), an increase in the optical density of the isomolar solution with decreasing dipeptide concentration takes place at a wavelength of 310 – 345 nm. The calculation conducted for various wavelengths showed that the coordination compounds are characterized by a zinc to dipeptide ratio of 1 : 2 and 1 : 6, which corresponds to the possible complex ion compositions of $[ZnL_2(H_2O)_4]^{2+}$ and $[ZnL_6]^{2+}$, respectively (here, L denotes the isoleucyl – tryptophan fragment).

The immunostimulant activity of dipeptide and the coordination compounds was evaluated *in vivo* by determining the titer of specific antibodies in the blood serum of calves

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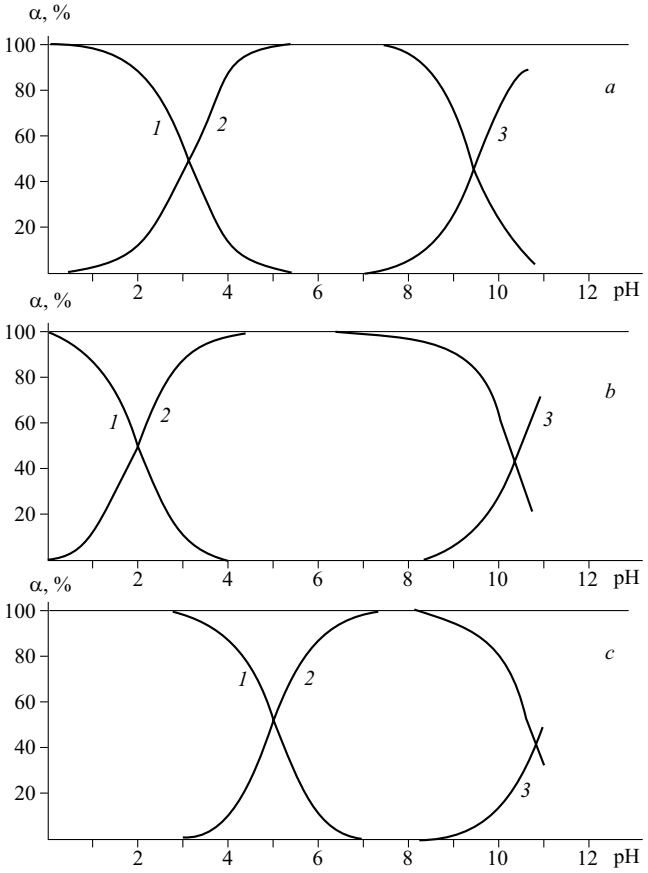

Fig. 1. Distribution of the degree of dissociation α for (*a*) isoleucine, (*b*) tryptophan, and (*c*) isoleucine – tryptophan dipeptide in (*1*) H_2L^+ cation form, (*2*) $HL^{\pm}$ zwitterion form, and (*3*) L^- anion form.

immunized with a living antitheileriasis vaccine (ATV) culture [9]. In the blood serum of animals immunized with ATV in combination with dipeptide, the antibody titer determined by immunoenzymometric assay ranged within 1 : 100 – 1 : 1600 (against 1 : 50 – 1 : 800 in the control group), while the analogous titer in animals immunized with ATV in combination with the synthesized coordination compounds was 1 : 200 – 1 : 1600 (against 1 : 50 – 1 : 200 in the control group). As can be seen from these data, the use of the free dipeptide produces a twofold increase in average specific antibody titer, while the zinc-containing coordination compound increases this titer on the average by a factor of 4 – 8. Thus, the coordination to zinc ions results in a 2 – 4-fold increase in the immunostimulant activity of the initial dipeptide.

Based on the zinc coordination compounds of isoleucine – tryptophan dipeptide, a new immunostimulant drug thymocin was developed [10], which is certified and allowed for use in the Republic of Tajikistan as a veterinary immunostimulant preparation intended for the prophylaxis and treatment of respiratory-intestinal infectious (viral and bacterial) disorders in horned and small cattle and for increasing the general resistance of the animal in immunodeficiency states.

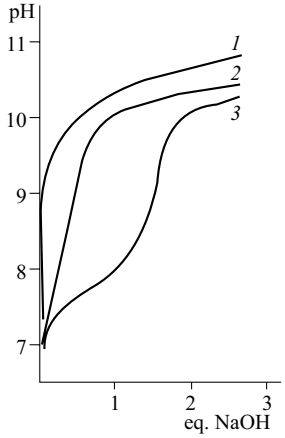

Fig. 2. pH titration curves of solutions of (*1*) isoleucine – tryptophan dipeptide and coordination compounds with zinc – dipeptide ratios (*2*) 1 : 6 and (*3*) 1 : 2.

EXPERIMENTAL CHEMICAL PART

The purity of the synthesized compounds was checked by TLC on Silufol UV-254 plates (Kavalier, Czech Republic) developed by sequentially treating with chlorine and saturated benzidine solution in a 2% acetic acid. The chromatographic mobilities (R_f) of the dipeptide – metal complex studied was 0.81 in the chloroform – methanol – 32% acetic acid (60 : 45 : 20) system and 0.65 in the chloroform – ethyl acetate – methanol – acetic acid (90 : 30 : 10 : 3) system.

***L*-Isoleucine-*L*-tryptophan dipeptide**. To a solution of 2.65 g (10 mmole) of carbobenzoxy-*L*-isoleucine (Reanal, Hungary) in 25 ml of DMF cooled to –5 – 0°C were added 2.06 g (10 mmole) of dicyclohexylcarbodiimide (Merck, Germany) and 1.265 g (11 mmole) of 1-hydroxybenzotriazole. Upon stirring the mixture at –5 – 0°C for 1 h, 2.04 g (10 mmole) of *L*-tryptophan (Reanal, Hungary), and 1.2 ml (10 mmole) of triethylamine were added and the mixture was stirred at room temperature for 16 h. Then the DMF was evaporated and the residue was dissolved in 200 ml of ethyl acetate. The solution was washed with 2% aqueous sulfuric acid and then with water until neutral reaction in the wash. The neutral washed solution was dried over anhydrous sodium sulfate and ethyl acetate was evaporated. The residue was dissolved in 200 ml of methanol and hydrogenated over a 10% Pd/activated charcoal catalyst (Fluka, Germany). After a 4-h treatment, the catalyst was separated by filtration, the solvent evaporated in vacuum, and the residue dissolved in an *n*-butanol – water mixture. Upon layer separation, the aqueous layer is decanted and *n*-butanol was evaporated. The residue was dissolved in 0.05 M ammonium acetate buffer (pH 6.8) and purified by HPLC on an Altex-334 chromatograph (USA) using a 1.65 × 25 cm column filled with Silasorb C_{18}. The target compound was eluted with acetonitrile gradient (10 to 30%) at a flow rate of 14 ml/min and detected at wavelength of 220 nm. The yield of free dipeptide was 3.1 g

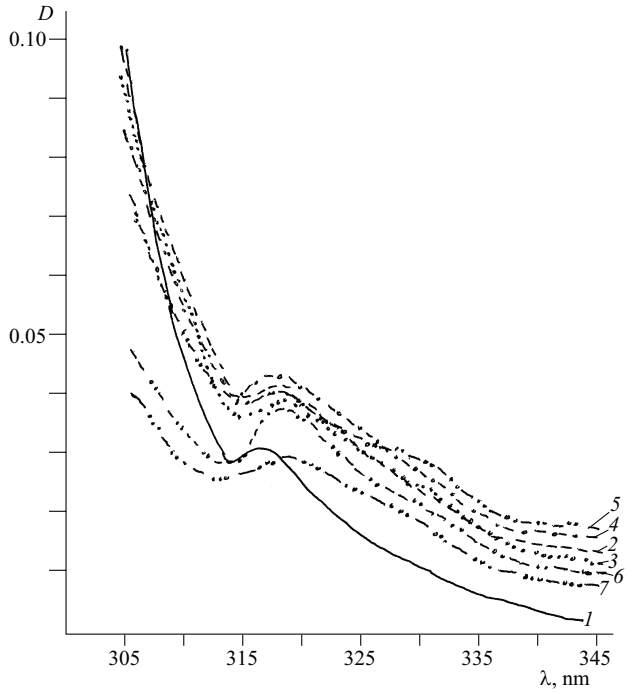

Fig. 3. UV absorption spectra of a series of isomolar solutions featuring the interaction of Zn^{2+} ions with isoleucine – tryptophan dipeptide: (*1*) pure isoleucine – tryptophan dipeptide solution; (*2 – 7*) solutions with zinc – dipeptide ratio 1 : 6 (*2*), 1 : 4 (*3*), 1 : 2 (*4*), 1 : 1 (*5*), 2 : 1 (*6*), and 4 : 1 (*7*).

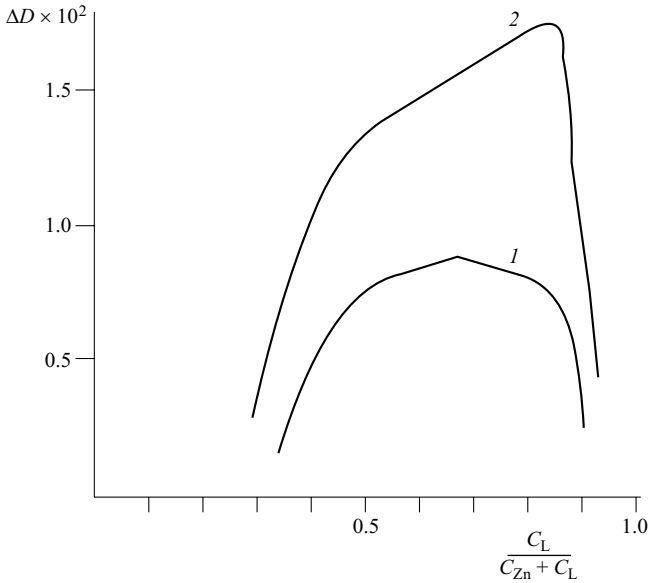

Fig. 4. Graphical determination of the composition of coordination compounds of zinc and isoleucine – tryptophan dipeptide (L) by the method of isomolar series [8]: (*1*) $[ZnL_2(H_2O)_4]^{2+}$ (λ = 318 nm); (*2*) $[ZnL_6]^{2+}$ (λ = 310 nm).

(98%); R_f, 0.44 (*n*-butanol – pyridine – acetic acid – water, 30 : 20 : 6 : 24), 0.71 (acetic acid – water – methanol – chloroform, 7 : 3 : 1 1); $[\alpha]_D^{20}$, – 31.14 (c, 1; MeOH); amino acid analysis: Ile, 1.0 (1.0); Trp, 0.99 (1.0).

Dipeptide – zinc coordination compound solutions. To a solution of 31.7 mg (0.1 mmole) of *L*-isoleucine-*L*-tryptophan dipeptide in 40 ml of sterile distilled water at 100°C was added at pH.6.0 with stirring 17.3 mg (0.1 mmole) of zinc acetate. Then the reaction vessel was hermetically closed and heated to 110 – 120°C, treated at this temperature for 1 h in the dark, and cooled to room temperature.

EXPERIMENTAL BIOLOGICAL PART

The dipeptide and coordination compounds were introduced in the form of 0.01% and 0.02% solutions, respectively, by intramuscular injections in a dose of 1 ml per 100 kg animal body weight. The test vaccine was used as prescribed by the instruction [9]. The peptide preparations were administered 3 – 5 days after the postvaccinal reaction (15 – 17 days upon immunization) The antitheileriasis antibody titer in the blood serum was determined by immunoenzymometric assay [11] in both test and control animals 35 days after immunization.

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