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Preparation and Characterization of a Novel Ala-Gln Dipeptide-Zn^{2+} Complex

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Peptide-trace elements complex with constant coordination ratio and high biological activity has drawn wide attention around the world. The aim of the present study was to synthesize and characterizes Ala-Gln dipeptide-Zn^{2+} complex as an oral medicine. A freeze-dry powder containing Ala-Gln dipeptide and Zn^{2+}, of which a chelating ratio of the dipeptide to Zn^{2+} is 2 to 1, was obtained in this study. Cyclic voltammetry analysis showed that $ZnSO_4$ has only cathodic peak, while the dipeptide and the complex have both cathodic and anodic ones. The analysis of the complex by differential thermal analyzer showed that a new endothermic peak at 537.3 ºC appeared. It may be deduced that the coordination bond between carbonyl oxygen and Zn^{2+} broke. The endothermic peak at 279.5 ºC belonged to ionic bond fission between the carboxyl oxygen and Zn^{2+}. Infrared analysis of the complex showed that absorption at 3334 cm^{-1} disappeared, which belonged to hydroxyl hydrogen of carboxylic acid. These characteristics suggested that an ionic bond formed between the carboxyl group of C_8 and Zn^{2+}, and the carbonyl oxygen in amide plane of C_4 and carbonyl oxygen of carboxyl of C_8 formed coordinate bond with Zn^{2+}, respectively, providing lone pair electrons to the *p* empty orbital of Zn^{2+}. Quantitative and biological qualitative evaluations showed that this Ala-Gln dipeptide-Zn^{2+} complex may serve as an effective oral medication for adsorption of the peptide and Zn^{2+} simultaneously *in vivo*.

Key Words: Ala-Gln dipeptide, Zn^{2+}, Chelate, Coordinate bond, Hydrolysis yield.

INTRODUCTION

As a cofactor of more than 80 kinds of enzyme in bodies, zinc is not only directly involved in metabolism of carbohydrate, protein, fat, nucleotide, but also participate in the promotion of cell division, the skeletal growth and calcification, the protection of myocardial cells *etc.*[1-3]. In the small intestine, Zn^{2+} form complex compound with ligand at first and the compound are absorbed into the intestinal epithelial cells. Then Zn^{2+} is transported into tissues by blood venous circulation in succession[4-6]. However, the pH of the bottom segment of small intestine is weak alkaline and Zn^{2+} can easily form $Zn(OH)_2$ precipitate. Therefore, it is crucial to maintain solubility of Zn^{2+} for the absorption and utilization of the bodies. Organic acids, amino acids and others are frequently used as ligands[7], yet their application is seriously affected for strong flavor or high osmotic pressure[8]. Thus, it is necessary to develop new ligands that could chelate trace elements with non-odor and low osmotic pressure for nutrition supplementation.

Compared with organic acids and amino acids, short peptides-the condensation compound of the several amino acid residues with no peculiar smell and low osmotic pressure, are widely used in food, health products, pharmaceutical and feed industries[9,10]. The preparation of short peptides-trace element complexes have also attracted people's attention in recent years[11,12]. There are many methods for the preparation of the complexes, such as phase equilibrium, microwave solid-phase synthesis, *etc.*[13-18]. However, the former method could not effectively remove surplus ion and ligand non-chelated. A variety of side reactions may occur with the latter method at a high temperature, which may cause some negative effects in body. Therefore, it is necessary to develop a novel method to prepare peptide-trace elements complex with constant coordination ratio and high biological activity.

L-Alanyl-L-glutamine (Ala-Gln) is a dipeptide composed by L-alanine and L-glutamine, which has 16 times solubility of Gln, high stability and bioavailability. This dipeptide has often been used for the preparation of intravenous transfusional amino acid as an alternative of L-Gln[19]. Intravenous administration is one of the most important methods for the administration of Ala-Gln[20]. However, it makes patient feel body pain and a sense of fear. Furthermore, it may bring the cross-infection due to contaminated syringes. Due to the advantages of convenience, safety, no pain and no chance of cross-infection, oral drugs become the most widely used route of administration[21]. The lone pair electrons in free amino nitrogen, carbonyl or

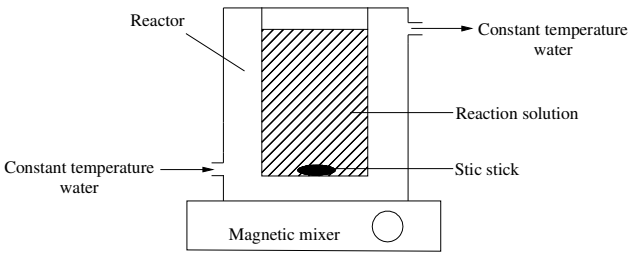
carboxyl oxygen of Ala-Gln dipeptide and unoccupied *p* orbital in Zn^{2+} make it easy to form a coordination compound. So a kind of oral nutritional medicine could be prepared, with active Ala-Gln peptides and trace element Zn^{2+}. Furthermore, the chelate effect can increase the hydrolysis resistance of proteolytic enzymes of the Ala-Gln in intestinal. However, to our best of knowledge there has not been any report about the preparation and charac-terization of Ala-Gln dipeptide-Zn^{2+} complex.

In this paper, Ala-Gln dipeptide-Zn^{2+} complex were prepared by a novel method using dialysis equilibrium[22,23]. The main physical, chemical and biological characteristics of this complex were examined, providing basic data series for its potential application as an oral medicine.

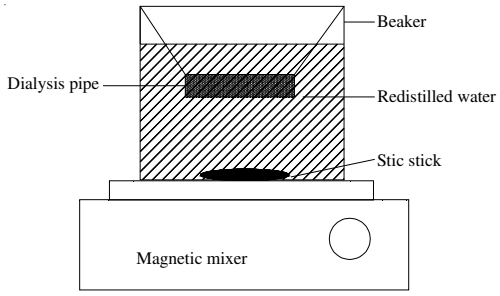
EXPERIMENTAL

L-Alanyl-L-glutamine, A.R. was purchased from Shanghai Qiude Biochemical Engineering Company Ltd., China. $ZnSO_4 \cdot 7H_2O$, A.R. was purchased from Guoyao Shanghai Chemical Reagent Plant, China. Detective reagent boxes of anti-superoxide radical, Superoxide dismutase (6300 U/mg) and carboxypeptidase A (720 U/mg) were purchased from Merk. All reagents used in this work were analytical grade.

Preparation of the Ala-Gln-Zn^{2+} complex: Quantitative Ala-Gln powder was dissolved with redistilled water, which was 100 times the weight of the powder. $ZnSO_4 \cdot 7H_2O$ was added in the solution and the mol ratio of Zn^{2+} was from 1 to 5 times of the mol of Ala-Gln. Then the solution was transferred in a sandwich glass reactor, which was airproofed by a rubber plug. The reaction equipment was shown in Fig. 1a. A chelate compound solution of Ala-Gln dipeptide and Zn^{2+} was obtained after the reaction was lasted for 1.5 h at 60 ºC with magnetic stirring at 60 rpm.



(a) Reaction equipment



(b) Dialysis equipment

Fig. 1. Preparation equipment of Ala-Gln-Zn^{2+} complex

The chelate compound solution obtained above was then encapsulated in dialysis pipe, where complex of Ala-Gln dipeptide and Zn^{2+} with molecule weight higher than 100 Da would be trapped. The volume of the redistilled water was 100 times of the liquor. The dialysis was conducted with magnetic mixing at 200 rpm and the equipment was shown in Fig. 1b. Dialysis extra liquor was collected on schedule and determined by EDTA method intermittently. The results were exchanged to dialysis curve until Zn^{2+} was exhausted. While the equilibrium was arrived, mol ratio of Ala-Gln to Zn^{2+} was definite in Ala-Gln-Zn^{2+} complex solution. The solution was freeze-dried at subzero -58 ºC and 40 Pa vacuum with FLEXI-DRY (FD-1-84) (FTS. Systems, New York) for 24 h to obtain the freeze-dried powder.

Characterization of the Ala-Gln-Zn^{2+} complex

Crystal shape of Ala-Gln -Zn^{2+} complex: Ala-Gln-Zn^{2+} freeze-dried powder, Ala-Gln dipeptide and $ZnSO_4 \cdot 7H_2O$ powder were dispersed on the glass slide, respectively and then observed by TH4-200 bioluminescence inverted microscope (Olympus, Japan). Their shapes were shown in Fig. 2.

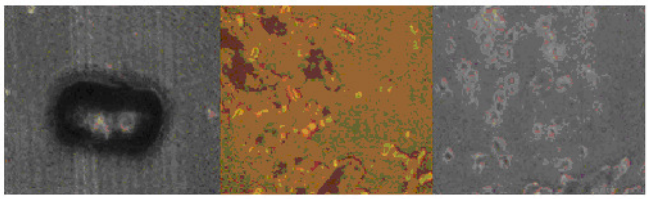


Fig. 2. Crystal morphology of three samples, left $ZnSO_4 \cdot 7H_2O$, middle Ala-Gln dipeptide, right Ala-Gln-Zn^{2+} complex

Melting point of Ala-Gln-Zn^{2+} complex: The melting points of Ala-Gln-Zn^{2+} freeze-dried powder, Ala-Gln dipeptide and $ZnSO_4 \cdot 7H_2O$ powder were determined, respectively by micro-melting point apparatus (Cole-Parmer, USA). The results were shown in Table-1.

X-Ray diffraction: Ala-Gln or Ala-Gln-Zn^{2+} complex were measured, respectively by XRD6000 X-ray powder diffractometer (Shimadzu, Japan). The results were shown in Fig. 3a-b, in which the abscissa was diffraction peak angle (θ) and ordinate was diffraction peak intensity.

Analysis of TG-DTA: Ala-Gln-Zn^{2+} freeze-dried powder, Ala-Gln dipeptide and $ZnSO_4 \cdot 7H_2O$ powder were analyzed, respectively by DTG-6H differential thermal analyzer (Shimadzu, Japan)[24]. The Fig. 4a-b were obtained at temperatures of 0-600 ºC with the heating rate of 10 ºC/min.

Oxidation-reduction potential: Quantitative Ala-Gln dipeptide, $ZnSO_4 \cdot 7H_2O$ or Ala-Gln-Zn^{2+} complex were, respectively dissolved in 0.2 mol/L CH_3COOH-CH_3COONa (pH 6.0) and measured by CHI660B electrochemical workstation (Bank Elektronik, Germany)[25]. The results were shown in Fig. 5, in which abscissa was voltage (V) and ordinate was current (A).

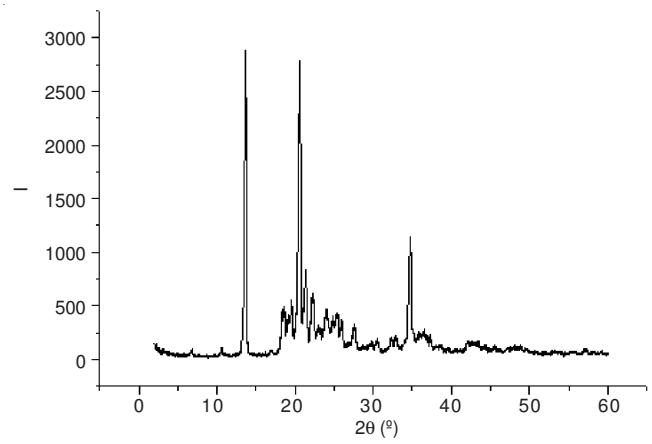
Infrared spectrum: Ala-Gln dipeptide, Ala-Gln-Zn^{2+} complex or $ZnSO_4 \cdot 7H_2O$ were mixed with KBr and pellet and were analyzed with 550 II infrared spectrum (Nicolet, USA). In Fig. 6, abscissa was wavelength (cm^{-1}) and ordinate was transmittance (T).

Analysis of Zn^{2+} concentration: Zn^{2+} concentration was determined by EDTA complexometry[26].

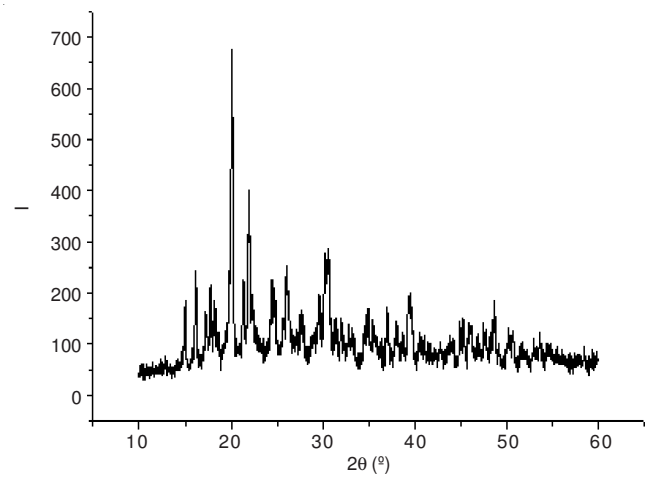
Mensuration of stability constants: 0.01 mol/L Ala-Gln dipeptide or 0.01 mol/L Ala-Gln-Zn^{2+} complex liquor was

TABLE-1
MELTING POINTS OF THREE SAMPLES

Sample	$ZnSO_4 \cdot 7H_2O$	Ala-Gln dipeptide	Ala-Gln-Zn complex	Ala-Gln-Zn^{2+}complex
m.p. (ºC)	104~105	209~210	183~184	167~172
log K	–	2.56	4.46	–



(a)



(b)

Fig. 3. X-Ray diffractogram, upper X-ray diffractogram of Ala-Gln-Zn^{2+} (a), below X-ray diffractogram of Ala-Gln (b)

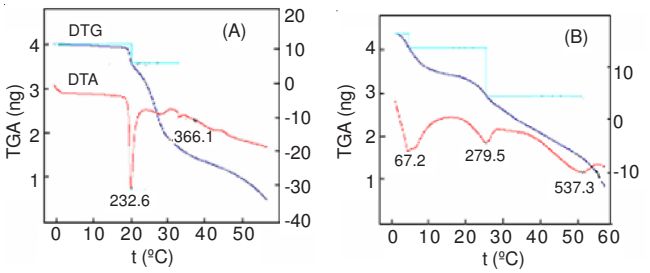


Fig. 4. Differential thermal curve of Ala-Gln dipeptide and complex of Ala-Gln, left Ala-Gln dipeptide, right Ala-Gln-Zn^{2+} complex

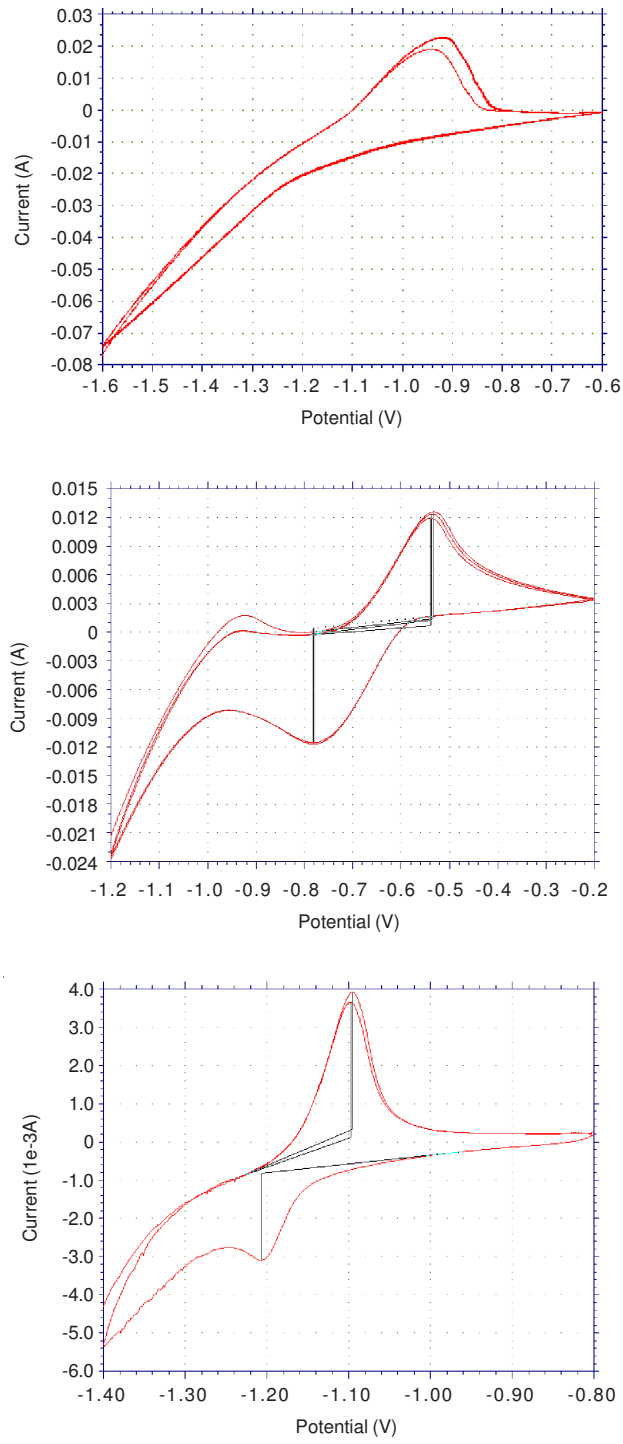


Fig. 5. Cyclic voltammetry plot of $ZnSO_4$, Ala-Gln dipeptide and Ala-Gln-Zn^{2+} complex, left $ZnSO_4$, middle Ala-Gln dipeptide, right Ala-Gln-Zn^{2+} complex

dissolved with 0.1 mol/L $NaNO_3$, then they were titrated with standard NaOH liquor, respectively until voltage measured by automatic potentiometric titrator (Mettler Toledo,Swiss) was stable. The results were converted into stability constants[27].

Biological stability: Biological stability of peptide or protein is usually characterized by hydrolysis yield by carboxypeptidase A[28]. Ala-Gln or Ala-Gln-Zn^{2+} complex liquor contained 0.05 mol/L Ala-Gln dipeptide and 0.2 mol/L (pH 7.60) phosphate buffer was, respectively mixed with 50 U carboxypeptidase A by magnetic stirring. It was hydrolyzed for 1 h at 50 ºC. The enzyme was separated by ultrafiltration membrane

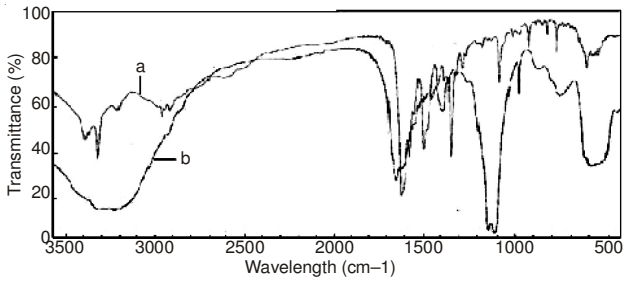


Fig. 6. Infrared spectrum of Ala-Gln dipeptide and Ala-Gln-Zn^{2+} complex, a Ala-Gln dipeptide, b Ala-Gln-Zn^{2+} complex

trapping molecule weight of 6000 Da. The amino nitrogen in the filtrate was determined by formaldehyde method[29] and the hydrolysis yield was analyzed as follows.

Hydrolyzed percent (%) = (amino acid nitrogen before hydrolyzed -amino acid nitrogen after hydrolyzed)/amino acid nitrogen before hydrolyzed.

Antibacterial property[30]: Quantitative $ZnSO_4 \cdot 7H_2O$, Ala-Gln dipeptide and Ala-Gln-Zn^{2+} complex were, respectively dissolved in sterile distilled water and then filtered with sterilized filter membrane for reserve. Above solutions were then added into peptone beef extract culture medium, which had been sterilized for 0.5 h at 0.14 MPa and cooled below 60 ºC. Then the culture was stirring for 10 min and prepared as culture plate.

Small amount of *Escherichia coli* and *Staphylococcus aureus* was dispersed in sterilized distilled water and was inoculated on the surface of the culture with 0.2 mL in each plate. The above plates were located in an incubator at 37 ºC for 12-24 h. Finally, the inhibitory efficiency was characterized by quantitative analysis of residual colonies.

RESULTS AND DISCUSSION

Crystal shape and Melting point of Ala-Gln-Zn^{2+} complex: The microscopic pictures (Fig. 2) showed that the crystal morphology of $ZnSO_4 \cdot 7H_2O$ resembled short rod, compared with long rod for Ala-Gln dipeptide and spherical for Ala-Gln-Zn^{2+} complex, respectively. As it could be seen from Table-1, the melting range of Ala-Gla-Zn^{2+} complex is 183-184 and 104-105 ºC for $ZnSO_4 \cdot 7H_2O$ and 209-210 ºC for Ala-Gln dipeptide. The differences in structure and melting point confirmed that a novel Ala-Gln dipeptide-Zn^{2+} complex were prepared by dialysis equilibrium method in this study.

X-Ray diffraction: In Fig. 3, the most intensive X-ray diffraction peaks of Ala-Gln-Zn^{2+} appeared at 20.07, 21.93 and 30.62º, yet it displayed at 13.64, 20.56 and 34.66º for Ala-Gln dipeptide. It suggested that Ala-Gln-Zn^{2+} complex was a kind of new material different from Ala-Gln dipeptide and Zn^{2+}.

TG-DTA analysis: The results of differential thermal analysis of Ala-Gln dipeptide was shown in Fig. 4a. The peak apex at 232.6 ºC, of which the weight was decreased of 10.54 %, was caused by the salt bond breakage between free amidic and adjacent carboxyl oxygen of the peptide. The peak apex appeared at 366.1 ºC, of which the weight was decreased of 46.81 %, was caused by the C-N bond breakage between N and C_2, C_7 or C_4. The result of the thermal analysis of Ala-Gln-Zn^{2+} complex was shown in Fig. 4b. There were three peak apexes. The peak apex at 67.2 ºC, having a weight decrease of 4.97 %, was due to loss of water adsorbed by freeze-dry powder. The apex at 297.5 ºC with a weight decrease of 17.49 % was caused by breakage of salt linkage and ionic bond of the complex between oxygen anion of C_8 carboxyl and Zn^{2+}. The peak apex was higher than the apex of Ala-Gln dipeptide at 232.6 ºC. The peak apex at 537.3 ºC, of which the weight was decreased of 22.61 %, was caused by the breakage of coordination bond between carbonyl oxygen and Zn^{2+}. It could be deduced that certain chemical interactions occurred between the center ion and the ligands, which affected electronic cloud distribution of the molecular.

Oxidation-reduction potential: Stability of center ion in complex was characterized by oxidation-reduction potential analysis with cyclic voltammetry method. As it could be seen from Fig. 5, $ZnSO_4$ had only cathodic peak, but Ala-Gln dipeptide and Ala-Gln-Zn^{2+} complex had both cathodic and anodic peaks. It showed that Zn^{2+} could only be reduced by accepting electron, but the dipeptide and the complex both could obtain and lose electron. Though peak shapes and locations of the complex were similar to those of Ala-Gln dipeptide, the peak areas reduced evidently. The current of anodic peak (i_{pa}) changed from -(1.21e + 2 A) to -(2.26e + 3 A) and the corresponding potential (E_{pa}) changed from -0.78 V to -1.207 V. Meanwhile, the current of cathodic peak (i_{pa}) changed from (1.261e-2 A) to (3.599e-3 A) and the corresponding potential (E_{pa}) changed from -0.539 V to -1.095 V. Further more, cathodic peak of Zn^{2+} disappeared in Fig. 5. Those changes showed that electrons from Ala-Gln dipeptide were accepted by Zn^{2+} and a new compound was formed. This compound was likely to be chelate complex of Ala-Gln-Zn^{2+} and thereby Zn^{2+} was stabilized.

Infrared spectrum: The infrared spectra of Ala-Gln dipeptide and complex of Ala-Gln-Zn^{2+} was shown in Fig. 6. It could be seen that in complex of Ala-Gln-Zn^{2+}, the characteristic absorption of the hydroxyl hydrogen at 3334 cm^{-1} disappeared, which meant that carboxylic acid is changed into carboxyl. This may be caused by the formed ionic bond between oxygen iron and adjacent Zn^{2+} and thereby the positive charge of Zn^{2+} was neutralized. There was a single peak of

TABLE-2
MAIN PROPERTIES OF Ala-Gln-Zn^{2+} COMPLEX

Samples	Stability constant (log K)	Hydrolysis rate by carboxypeptidase A (%)	Feudal number of bacteria/Petri dish	
			Staphylococcus aureus	*Escherichia coli*
Ala-Gln-Zn^{2+} complex	4.46	4.37	31	21
Ala-Gln dipeptide	2.56	18.14	148	91
$ZnSO_4 \cdot 7H_2O$	–	–	28	15

Zn^{2+} concentration is 50 mmol/L; Ala-Gln dipeptide concentration is 100 mmol/L; number ofbacteria of *Staphylococcus Aureus* is 150 strains/Petri dish; number of *Escherichia coli* is 100 strains/Petri dish.

amide (V_{N-H}) at 3334 cm^{-1} in the complex and the intensity was distinctly increased, compared with Ala-Gln dipeptide. Ala-Gln dipeptide had multi vibration peaks of amide (V_{N-H}) from 3450-3200 cm^{-1}. Double peaks of the complex, which was caused by amide ($\delta_{NH} + V_{CN}$) in region of 1510-1370 cm^{-1} and peaks of carboxyl in 1150-1100 cm^{-1} were significantly strengthened. These characteristics indicated that the peptide bond and its free carboxyl both took part in coordination with Zn^{2+} in the complex.

Capability of providing lone pair electrons by nitrogen atom was obviously reduced in the complex because of conjugation effect between carbonyl oxygen and imino nitrogen in peptide bond planar. Thus, lone pair electrons were provided by carboxyl oxygen in the chelate compound. Because of orientating function of ionic bond between the carbonyl oxygen and Zn^{2+}, carboxyl oxygen got close to the center ion and then coordinated with its empty orbit. Therefore, carbonyl oxygen of the peptide bond, the carboxyl oxygen and its oxygen anion might all take part in coordinating reaction with Zn^{2+}, with the former two offering one pair electrons, respectively and the latter neutralizing positive charge of Zn^{2+}.

Structure of the complex: Results of analysis of the Ala-Gln-Zn^{2+} complex above showed that dialysis equlibrium was arrived *ca.* 26.5-29.0 h and the mol ratio of Ala-Gln dipeptide and Zn^{2+} was 2:1 at that time. A conclusion could be drawn from above-mentioned researches: carbonyl oxygen of carboxyl of C_8 and carbonyl oxygen atom on C_4 amide plane of dipeptide formed coordination bonds with Zn^{2+}, respectively and oxygen iron of C_8 carboxyl formed ionic bond with Zn^{2+}. Therefore, a stable Ala-Gln-Zn^{2+} complex was obtained by the chelating reaction between two Ala-Gln dipeptide and one Zn^{2+}. Its possible structural formula was shown in Fig. 7.

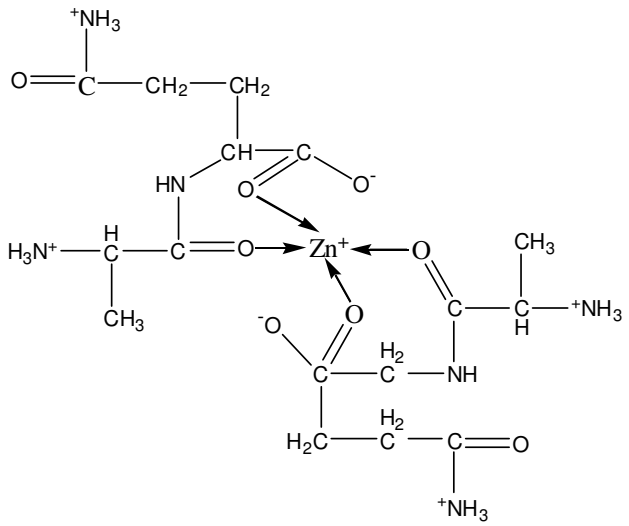


Fig. 7. Molecular structure of Ala-Gln-Zn^{2+} complex

Main properties of Ala-Gln-Zn^{2+} complex: From Table-2, it could be seen that stability constant of the complex was 4.46, which was 174 % times of the dipeptide's, suggesting that Zn^{2+} was restrained by forming coordination bond with Ala-Gln dipeptide. Santos *et al.*[31] indicated that the complex with stability constants between 4 to 7 was in favour of bio-absorption. Therefore, Ala-Gln-Zn^{2+} complex could be utilized by human body easily. Hydrolysis yield of Ala-Gln dipeptide by carboxypeptidase A was 18.14 %, while it was just 4.37 % for the complex. According to biocatalysis theory, the possible reason was that the stable complex increased combining resistance with the enzyme, which leaded to the difficult location on the active center for carboxypeptidase-A accurately and the lower hydrolysis yield. Short-peptide is a important nitrogen nutrition absorbed in small intestine[6], where the value of pH is more than 7.0. The high pH caused free Zn^{2+} to deposit as $Zn(OH)_2$. However, Ala-Gln-Zn^{2+} complex could stabilize Zn^{2+}, while at the same time decrease the hydrolysis of carboxypeptidase-A. So, it is favourable for the absorption of the peptide and Zn^{2+} simultaneously *in vivo*. The results in Table-2 also showed that inhibition ability of Ala-Gln-Zn^{2+} complex to *Staphylococcus aureus*[32] and *Escherichia coli*[33] was, respectively reduced about 10 and 28.5 % compared with Zn^{2+} at the same concentration, but 4.77 and 4.33 times higher than Ala-Gln dipeptide, respectively. Hence, the complex also could serve as a kind of antibiotic active substance and can inhibit growth of certain microorganisms in appropriate conditions.

Conclusion

Ala-Gln-Zn^{2+} complex synthesized in this study had a coordination ratio of 2:1. The carbonyl oxygen of carboxyl of C_8 and carbonyl oxygen atom on C_4 amide plane of dipeptide formed coordination bonds with Zn^{2+}, respectively and oxygen iron of C_8 carboxyl formed ionic bond with Zn^{2+}. The Ala-Gln-Zn^{2+} complex could be used as nutrition drugs or health products by supplying Ala-Gln dipeptide and Zn^{2+} simultaneously.

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