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CONTRIBUTION TO CASEIN DETERMINATION BY UV SPECTROPHOTOMETRY

Raluca Stefanescu[a], Stefania Brebu[a], Mihaela Matei[a], Ioan Marian Risca[b], Andriana Surleva[c] and Gabi Drochioiu[a*]

[a]*Faculty of Chemistry, "Al. I. Cuza" University Iasi, 11 Carol I, Iasi 700506, Romania*

[b]*Faculty of Forestry, Stefan cel Mare University, 13 Universitatii, Suceava, Romania*

[c]*Analytical Chemistry Department, University of Chemical Technology and Metallurgy,8 St. Kl. Ohridski av., 1756 Sofia, Bulgaria*

Abstract: In the present paper, the interaction between copper ions and proteins is presented, in order to elaborate a simple and rapid spectrophotometric assay of casein in milk. Under alkaline conditions, copper ions form the biuret complex with the proteins, which can be used in protein determination. Although very specific, the biuret method is less sensitive. Using insoluble copper phosphate, casein is able to extract copper ions, with which it forms the biuret complex, while either the complex or copper ions could be determined in the ultraviolet range. Indeed, an increased absorbance of biuret complex at 215 nm was found. Nevertheless, copper ions can be determined in UV as well, their concentration being proportional to that of casein. When used tetraglycine instead casein, mass spectrometric measurements at pH higher than 11 revealed the formation of complexes with many copper ions bound to each peptide bond-containing molecule. Nevertheless, on diluting the biuret solution the complex may dissociate leading to very complex UV spectra that should be further studied.

***Keywords*:** Protein analysis; Casein; Spectrophotometry; Copper ions

* Gabi Drochioiu, *e-mail:* gabidr@uaic.ro

Introduction

Many spectrophotometric methods have been evaluated for total protein determination in milk samples. The description of all previous protein assays will not be duplicated here. However, among them, the Bradford assay was used for the determination of total proteins in skim milk powder and whole milk powder instead of the Kjeldahl method.[1] Other authors reported the determination of crude protein and casein in cow milks by near-infrared transmission spectroscopy.[2,3] In another paper, protein was estimated from the absorbance at 280 nm.[4] Indeed, most proteins strongly absorb around 280 nm due to their tryptophan content, which shows an absorption maximum at this wavelength. The determination of protein concentration in milk was also based on their characteristic absorbance, which includes two bands in the 1500 to 1700 cm^{-1} range.[5,6] Previously, based on the property of proteins to bind Orange G dye, the milk proteins were determined by measuring the concentration of residual dye in their solutions.[7] Another spectrophotometric method was based on the alkaline solution-induced changing the spectrum of tyrosine to higher wavelength values in the UV region. In the range between 248 and 256 nm the absorbance was found to be a linear function of the wavelength and the slope coefficient is directly proportional to the protein concentration.[8] During the protein determination procedure, the only preservative found suitable for use in raw milk was mercuric chloride, whereas a completely clear solution was obtained by adding 5 ml of 97% acetic acid to 0.05 ml of whole milk. The turbidity depending on fat content was developed thereafter by adding 2.5 ml of a solution containing 20% urea and 0.2% imidazole. Generally, the results of spectrophotometric methods proved to be similar to those by an established method for the determination of total

nitrogen (micro-Kjeldahl). Thus, for the biuret assay, Lowry-Peterson assay, Bio-Rad Coomassie Blue assay, and Pierce BCA assay, the correlation coefficients were 0.96, 0.97, 0.89, and 0.99, respectively. When assessed the sensitivity of each assay, the Pierce BCA assay showed the least difference in values among different types of protein. Due to its great precision, the BCA method was recommend by Keller & Neville (1986),[9] while the biuret method was found less sensitive. Some other assays for proteins in milk or casein have been advanced and evaluated.[10-13]

In the present paper, starting from the largely used biuret method in protein determination,[14] we investigated the relationship between casein and copper ions under alkaline conditions. We have hypothesized that casein may form a biuret complex with copper ions extracted from the insoluble copper phosphate, which can be measured in the ultraviolet region where either biuret or copper ions absorb. However, during the measurements we have observed that the relationship of casein with copper ions is more complex and should be investigated in detail. Therefore, herein some results which suggest that UV determination of casein could be much improved are presented.

Results and Discussion

Experiment 1. Proteins can be easily determined in UV around 280 nm at concentrations less than 1 mg/mL or below 230 nm at concentrations less than 50-100 μg/mL, depending on the amino acid composition of each protein (Fig. 1). The reference were those used as solvents for the three proteins, namely water for papain, 70% ethyl alcohol for zein, and 60/1/39 acetonitrile/KOH solution/water system for casein. The absorbance values and maxima (nm) for 1 mg/mL protein solution are $A_{casein,\ 290}$ = 0,972,

$A_{zein,282}$ = 0.939, and $A_{papain,278}$ = 1.008, respectively. Although higher, the maxima and the individual absorbencies were found quite different one from another (A_{casein} = 1,142 at 217 nm; A_{zein} = 1.960 at 207 nm, and A_{papain} = 2.416 at 194 nm, respectively). The spectra acquired show that the individual proteins should be measured at different wavelengths, and not only at 280 nm.

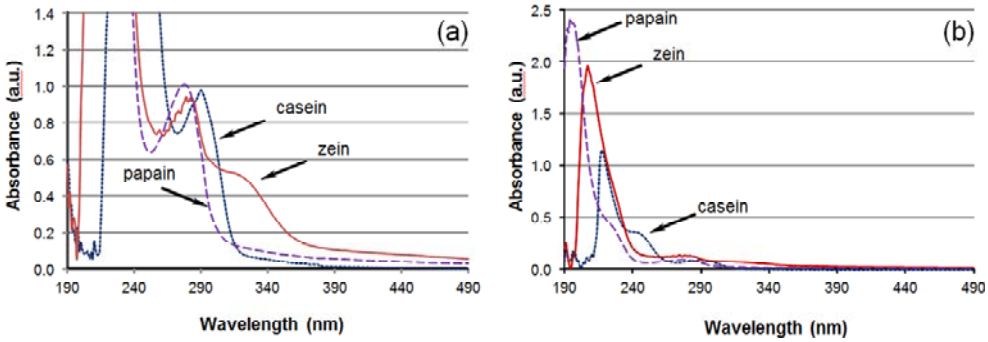

Figure 1. UV-*vis* spectra of three different proteins, namely casein, zein and papain: (a) 1 mg/mL protein concentration and (b) 100 μg/mL protein concentration.

Copper phosphate supernatant displays an absorption band at 256 nm in the ultraviolet range (Fig. 2). When diluted three times with water, it showed an absorbance value of A = 0.621. It is logically to assume that if casein or other protein can extract copper ions under alkaline conditions, they could be measured at 256 nm, being proportional with the protein concentration. In such case, small volumes of casein solutions would react with copper phosphate, and the measurements should be made after appropriate dilution.

However, KOH solution also absorbs in the UV range, and, therefore, on diluting the KOH-containing solutions of proteins and copper ions pH is expected to change and the biuret complex to breakdown.

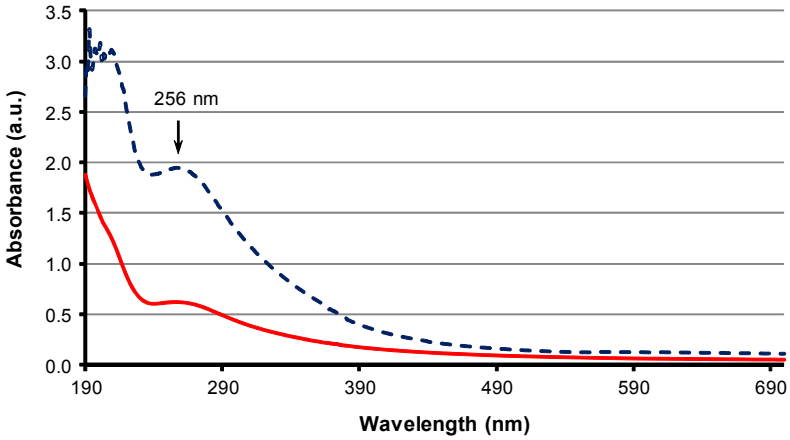

Figure 2. UV-*vis* spectrum of copper phosphate supernatant (undiluted at pH 11, and diluted three times with milliQ water, respectively).

Experiment 2. It was also found that the protein (casein) in the presence of copper ions forms a complex that can be measured at 215 nm (Fig. 3). On decreasing the casein concentration the maximum found at 215 nm tends to appear at lower wavelength values. Hence, we made a calibration curve with the absorbance values at these maxima and calculated the correlation coefficient, r ($r = 0.974$; $r^2 = 0.948$) and the equation, $y = 0.0423 \cdot x$, where y = absorbance and x = casein concentration (μg/mL).

However, on reading the absorbance values at 215 nm for all casein concentration range, some negative values were observed, suggesting either the decomposition of the biuret complex or the presence of many species which precipitate the free copper ions of the copper phosphate supernatant. Nevertheless, the correlation coefficient corresponding to the 215-nm reading was higher then that determined above ($r = 0.996$). Therefore casein could be determined at 215 nm, but the spectra are very complex and further investigations should be made to understand the phenomena that take place in the presence of copper ions.

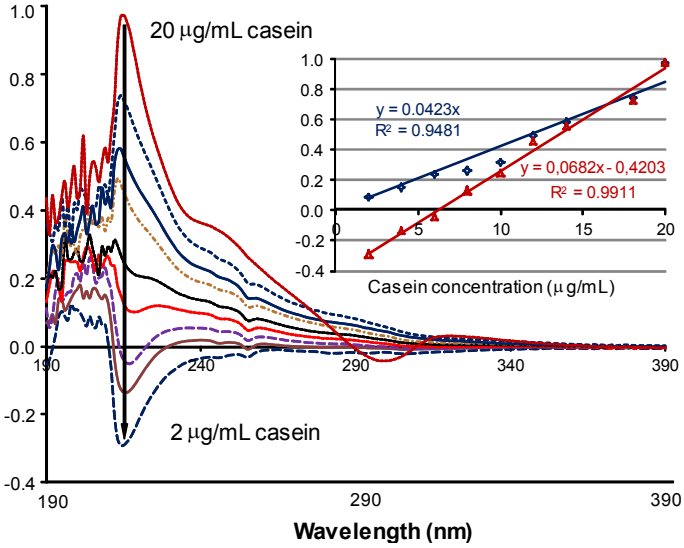

Figure 3. UV spectra of casein in copper phosphate supernatant and in the presence of KOH. A linear relationship was found between casein concentration (2-20 µg/mL) and 215-nm absorbance (r = 0.996).

When copper phosphate supernatant was treated with 0.1 N KOH, a very specific UV spectrum was generated (Fig. 4). Casein (20 µg/mL) had a strong absorption at 230 nm (A = 0.931) and a shoulder at 243 nm (A = 0.816). Since 1 mL of 20 µg/mL casein solution was treated with 0.350 mL supernatant and 0.050 mL KOH, the absorbance values should multiplied by 1.4. On the contrary, the 100 µg/mL casein solution presented A = 1.142 at 217 nm, and A = 0.356 at 240 nm, respectively, as Fig. 1 shows. Therefore, casein determination as a complex at 230 nm was found (100/20)·1.303/1.142 = 5.7 times more sensitive than casein reading at 217 nm. Following the same calculation, reading at 243 nm in the presence of copper ions would be 11.5 more sensitive than that of pure casein at 240 nm. However, further research is expected to use more diluted copper phosphate extracts, in order to decrease the absorbance values of the background, and to calculate new calibration curves.

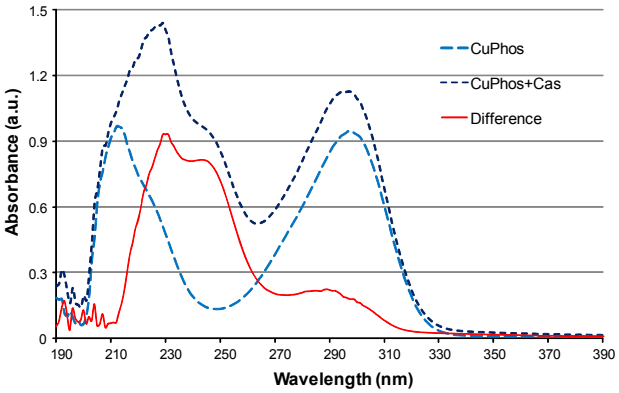

Figure 4. UV spectrum of 1 mL of casein solution (cas, 20 μg/mL), containing 350 μL of copper phosphate supernatant (CuPhos) and 50 μL of 0.1 N KOH.

Mass spectrometry. ESI-MS spectra showed that copper binding is low at acidic pH, but is much increased with increasing the pH value (Fig. 5). First of all, the model peptide Gly4 (Fig. 5a; here referred as M) was found as monomer at m/z 247.0 (as $[M+H]^+$ molecular ion), at 493.2 and 739.2 as dimer and trimer ($[2M+H]^+$ and $[3M+H]^+$, respectively). In the presence of copper ions ($CuCl_2 \cdot 2H_2O$) in the 0.1: 1 copper: peptide molecular ratio, at pH 11.0, a complex containing two tetraglycines and one copper ion is found at m/z 554.1, but not at m/z 308.0 as expected. We explained this phenomenon by copper tendency to induce dimerization and β-sheet structures of peptides. However, smaller signals were observed at m/z 800.1 and m/z 1046.2, being assigned to $[3M+Cu-H]^+$ and $[3M+Cu-H]^+$, respectively. Moreover, the tendency of tetraglycine to form oligomers was increased under such conditions ($[4M+H]^+$ at m/z 985.2 (Fig. 5b). Besides, copper complexes of this last molecular ion were also found ($[4M+Cu-H]^+$ at m/z 1046.2, and, especially, $[4M+Cu+K-2H]^+$ at m/z 1046.2).

The isotopic distribution of complexes at m/z 554.1 and 800.1 showed the presence of copper ion (^{63}Cu: ^{65}Cu in 3: 1 ratio). Similar

isotopic distribution was observed for complexes of Gly4 and its oligomers with more than one copper ion. At m/z 861.1 (Fig. 5c), the 1: 1 peptide: copper ion molar ratio mixture displayed the $[3M+2Cu-3H]^+$ molecular ion, when a superposition of ^{63}Cu and ^{65}Cu can be observed at m/z 863.1. Hence, when more than one copper ion is bound to a protein or peptide, the shape of isotopic distribution is changing.

Finally, the alkaline solution of Gly4 (1 mg/mL) was sonicated for 10 min. in eppendorf vials with 20 mg of insoluble copper phosphate powder, then centrifuged at 15,000 rpm and diluted accordingly.

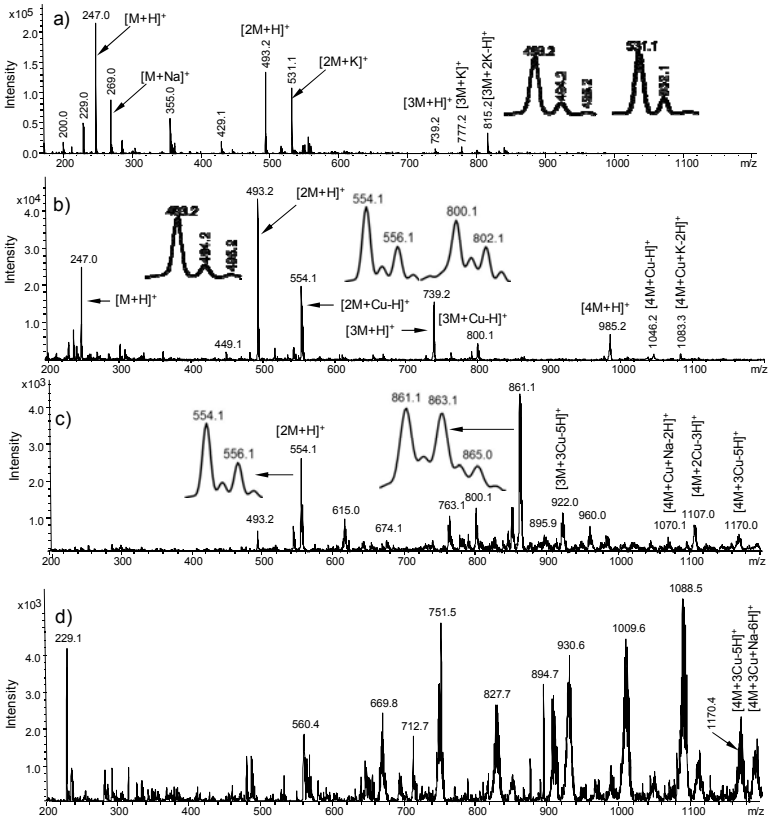

Figure 5. ESI ion trap MS spectra of tetraglycine (Gly4, M) in the presence of copper ions at various pH values. Spectra: a) Gly4 acidic pH, and isotopic distributions of peptide at m/z 492.2 and m/z 531.1; b) 0.1: 1 Cu: Gly4 molar ratio, pH 11 with ethanolamine; c) 1: 1 Cu: Gly4 molar ratio, pH 11 with ethanolamine; d) Gly4 at pH 11 reacted with insoluble copper phosphate.

The resulted spectrum was more complex, but still understandable. For example, at m/z 1170, the $[4M+3Cu-5H]^+$ molecular ion was found in both spectra (Fig. 5c & 5d), since their isotopic distribution is similar. However, some peaks and bands suggested that ethanolamine may interfere to form complexes with copper ions, as well. In brief, we demonstrated here that peptide bond in proteins, can extract copper ions from insoluble phosphates to form biuret-like complexes, as peptide Gly4 did.

Experimental

Materials. All reagents were of analytical grade and used without any further purification. MilliQ grade water (18.2 M Ω·cm) was employed for solution preparation. Casein, zein, pepsin were purchased from Sigma Aldrich (Taufkirchen, Germany), whereas acetonitrile, copper chloride, acetic acid, diethanolamine, and ethyl alcohol were from Merck (Darmstadt, Germany). Tetraglycine, H_2N–Gly–Gly–Gly–Gly–COOH (Gly4), was purchased from EGA Chemie (Steinheim, Albuch, Germany). Copper phosphate was obtained in the reaction of trisodium phosphate (Merck) with copper sulfate (Merck), washed many times with milliQ grade water, dried at 105 ºC and milled up to a fine powder.

The best casein solvent was found to be a mixture of 60 mL acetonitrile, 1 mL of 0.1 N KOH solution and 39 mL water. Zein was dissolved in an aqueous 70% (v/v) ethanol solution, whereas papain in water. From 1 mg/mL stock solution, differently diluted solutions were prepared.

Instruments. UV-vis measurements were done using a LIBRA S35 spectrophotometer (Biochrom, Cambridge, England) in 1 cm quartz cuvette against each control solution containing only the reagents. The electrospray

ionization mass spectrometric (ESI-MS) measurements were carried out on a Bruker Daltonics Esquire 3000 Plus (Bremen, Germany) ion trap mass spectrometer. The eppendorf vials were centrifuged for 10 min at 15,000 rpm and 20 °C using a Hettich centrifuge (Germany). Ultrasonic stirring was performed on an ultrasound bath cleaner (J.P. Selecta Ultrasons system, 40 kHz; Barcelona, Spain). The pH values of all solutions were measured with a Hanna pH 211 microprocessor pH meter (Hanna Instruments) before and after the treatment with copper phosphate.

Procedure. We have investigated the formation of the biuret complex between casein and copper ions. According to our hypothesis, proteins extract copper ions from the insoluble copper phosphate under alkaline conditions and these ions can be determined by spectrophotometry in the ultraviolet range either as such or as the biuret complex.[15,16] Duplicate samples were measured throughout of this work.

Experiment 1 consisted in absorbance measurements of three different proteins *prior to* copper-protein complex formation. Separately, copper phosphate was sonicated in a 0.1 N KOH solution, centrifuged at 15,000 rpm and the supernatant measured in the UV range.

Experiment 2 aimed at demonstrating copper-casein complex formation.

Sample 1. In each of the two eppendorf vials, 100 μL of solvent consisting of acetonitrile (60%), milliQ grade water (39%), and 0.1 N KOH (1%) were pipetted. Then, 50 μL of 0.1 N KOH (for advanced alkalization to pH > 12) and about 20 mg of insoluble copper phosphate were added. The mixtures were sonicated for 10 min on an ultrasonic bath, then the eppendorf vials were centrifuged at 15,000 rpm for 10 min.

Sample 2. 100 μL of milliQ distilled water, 50 μL of 0.1 N KOH, and about 20 milligrams of insoluble copper phosphate were mixed. We proceeded as in the case of sample 1.

Sample 3. 100 μL of 1 mg/mL casein dissolved in the solvent of sample 1, 50 μL of 0.1 N KOH and about 20 mg of insoluble copper phosphate were added. We proceeded as in the case of sample 1.

Sample 4. 100 μL of casein dissolved in the solvent of sample 1, 50 μL of 0.1 N KOH, but no insoluble copper phosphate were added. Then, we acted similar to sample 1.

The supernatant (100 μL) of each of the 8 eppendorf vials was diluted with distilled water (1 mL) and the resulting clear solution was read on a spectrophotometer in a 1 cm quartz optical cuvette in the 190-390 nm range against milliQ grade water. The spectra were presented as such or in the form of a difference between two different samples.

Experiment 3 consisted in measuring the ESI ion trap MS spectra of tetraglycine (Gly4) in the presence of copper ions ($CuCl_2$) or insoluble copper phosphate and ethanolamine as alkaline agent. The molar ratios and concentrations are mentioned in Fig. 5 and in the text.

Mass Spectrometry. The acquisition parameters were as follows: capillary voltage: 4000 V, skimmer 40 V, nebulizer gas pressure: 10 psi, drying gas flow: 9 L/min, heated capillary temperature: 300 ºC. Argon was used as target gas for collision induced dissociation. The stock solution of tetraglycine (1 mg/mL) was diluted in water to obtain a 4 μM final concentration. ESI-MS spectra (average of 15 individual spectra) were recorded in the positive ion mode in the 200–1200 m/z range by direct injection, using a syringe pump with a flow rate of 4 μL/min.[17]

Discussion

The spectrophotometric method for the determination of copper in water samples is advantageous because it is easily operated and inexpensive.[15,16] Furthermore, we showed here that that the absorbance at 215 nm is proportional to copper-casein complex, and that casein can be determined in highly diluted samples. Some authors claim that the Bradford method shows the highest sensitivity of the spectrophotometric methods.[1,18] However, our results indicate that a micromethod based on absorbance reading at 215 nm could be 10 times more sensitive that Bradford assay. Using casein as standard protein, the micromethod would show the lowest variation of specific absorbance. The UV-220 nm method with previous extraction of lipids showed the best results for the determination of total proteins in all the samples.[1] However, there is much variation between UV spectra of proteins, which requires that each protein should be measured at specific UV maxima. Here, we did not measure the real samples, but literature suggests that a control without copper phosphate could be read to overcome the turbidity of solutions due to the fat material in the samples.

Conclusions

Interaction between casein and copper ions was investigated by UV-vis spectrophotometry. Casein easily forms a biuret complex with copper ions extracted from the insoluble copper phosphate, which is an opportunity to determine it in the ultraviolet region either as biuret complex or indirectly, based on the absorption of extracted copper ions. Nevertheless, during the measurements we have observed that the relationship of casein with copper ions is more complex and should be studied. Tetraglycine peptide was used to prove the copper ion extraction by the peptide bonds,

and mass spectrometry revealed copper binding to this tetrapeptide as a function of pH. Further research is still necessary to complete understanding of the relationship between casein and copper ions and the opportunity to determine easily this protein in diluted samples.

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