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Novel UV assay for protein determination and the characterization of copper–protein complexes by mass spectrometry

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Abstract

A very simple, highly selective and sensitive assay of proteins based on the biuret absorption in the ultraviolet region has been developed. The well-known biuret assay is based on the reaction of proteins with copper ions under strong alkaline conditions to form a copper–protein complex. Yet, copper ions may seriously interfere with the determination if the measurement is made in the UV range. In the present approach, proteins mobilize copper ions from insoluble salts at different pH values, and the copper–protein complexes are investigated by UV spectrophotometry and mass spectrometry. Upon using copper phosphate, free copper ions do not interfere with the determination from 540 to 240 nm. Copper absorbance slowly increases from 240 to 190 nm where a blank with the reagents is recommended. A maximum absorbance for the copper–protein complex was found at 226 nm and high pH value. The stoichiometries of the copper–protein complexes measured directly with a mass spectrometer are pH dependent: half of the peptides without any histidine residue chelate just a single Cu^{2+} ion at pH 7.4 but each such peptide mobilizes from 1 to 6 Cu^{2+} ions at pH 10.3. To determine proteins, 1–1.5 ml of 1.8% KOH solution with 0–20 $\mu g\,ml^{-1}$ protein is treated with 25 mg of copper phosphate powder. The mixture is powerfully stirred, centrifuged, and the absorbance of the supernatant is measured at 226 nm in 1 cm quartz cuvettes against a blank of the reagents. The color system obeys Beer's law in the range 0.1–20 $\mu g\,ml^{-1}$ protein at this wavelength. The molar absorptivity value proved to be a characteristic of each protein being analyzed. Therefore, individual proteins should be used to plot calibration curves. This assay proved to be over 100 times more sensitive than the classical biuret procedure. The method is highly selective and the determination is little affected by the presence of other substances. All other important analytical parameters were studied and practical applicability of the method has been verified by the analysis of some biological materials.

Keywords: Protein determination; Biuret reaction; UV detection; Copper complex; Mass spectrometry

1. Introduction

Quantification of protein has been one of the most universal denominators and several methods have been devised to measure protein in microgram quantities [1–3]. Measurements of protein concentrations have been obtained by several different techniques such as micro-Kjeldahl method [4], near infrared spectroscopy [5], electrochemical procedures [6], Rayleigh light scattering [7], particle beam hollow cathode optical emission spectroscopy [8], turbidimetry [9], radio iodination [10], or spectroscopic techniques such as NMR [11]. Elemental analysis by X-ray fluorescence spectrometry [12], atomic absorption spectrophotometry [13], atomic fluorescence spectrometry [14], and optical emission spectroscopy [15] have also been used for detection of proteins by performing a digestion to dissociate them and then monitoring the metal elements that were bound to the protein molecules. Nevertheless, the widely used methods have their disadvantages [7,16]. Proteins in dilute solutions could also be determined by near infrared fluorescence recovery [17], pulse-polarography [18,19], and capillary gas chromatography [16], with electrospray ionization magnetic mass spectrometry [20] or by capillary zone electrophoresis at 214 nm [21]. Some of these analytical techniques are quite new and do not so far belong to the usual apparatus in biochemical laboratories. Protein concentrations of 0.10–15.3 $\mu g\,ml^{-1}$ could be determined by their enhancement effect on the Rayleigh light scattering of carboxyarsenazo [7]. This method is simple, rapid,

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and tolerant of most interfering substances. Unfortunately, it requires a spectrofluorometer and the protein-to-protein variability cannot be avoided with this technique. The spectrophotometric methods have great advantages over other procedures in simplicity, rapidity and sensitivity [1,22–24]. Nevertheless, the method based on the spectral absorption of the protein solution at 260–280 nm [24] has several major limitations. For example, proteins vary with respect to the concentrations of aromatic amino acids, which absorb at these two wavelengths and therefore vary with regard to their molar absorbencies, and the method is insensitive to protein concentrations of less than 100 $\mu g\,ml^{-1}$ [24]. The spectrophotometric determination of proteins at 210 nm [25] does not have this drawback, but it is not very selective. As the Lowry protein reaction [1] is susceptible to interference by various substances [26,27] purification of the protein sample by precipitation with acids prior to protein assay in frequently undertaken [28]. The Lowry procedure is also insensitive to protein concentrations of less than 10 $\mu g\,ml^{-1}$ [1]. The silver staining method involves multiple steps, high background and toxicity of formaldehyde [29]. The Coomassie brilliant blue assay shows a slight nonlinearity that compromises the sensitivity and accuracy, and only a narrow range of relatively high protein concentrations is used [7]. Bicinchoninic acid is also used as a highly specific chromogenic reagent for Cu(I) obtained by alkaline reduction of copper(II) ions by protein [30]. A very widely used reaction of the α-amino group, the ninhydrin reaction, is utilized to estimate amino acids quantitatively in very small amount [31]. As a consequence, proteins can easily be determined if they are hydrolyzed to amino acids, which are treated with ninhydrin [3,32,33]. One of the main difficulties inherent in protein assays arises from the presence of a wide variety of potentially interfering substances in sample solution [6,17,34,35]. The ninhydrin assay has the advantage to eliminate any interference and to increase the selectivity of protein determination [33]. The well-known biuret method is a very simple, selective, still less sensitive assay of proteins [36]. The two frequently employed methods for protein determination, the Lowry and bicinchoninic acid (BCA) procedures [1,30] are also derived from the biuret reaction. The biuret reaction is based on the formation of copper tetradentate coordination complexes when copper sulfate is added to proteins in alkaline medium. The coloration of biuret complex allows a spectrophotometric measurement at 540 nm. Unfortunately, the sensitivity of this method is limited to milligram per milliliter protein concentrations. In the Lowry method, biuret complexes reduce the Folin-Ciocalteu phenol reagent to increase the sensitivity of protein determination. In the BCA test, copper is further complexed with bicinchoninic acid. In that way, the detection limit can be enhanced to the microgram per millimeter range [6]. Another simple and rapid quantification method of proteins is based on the electrochemical detection of Cu^{2+} released from biuret complexes [6]. The measurement of the biuret absorbance in the ultraviolet region could increase the sensitivity of protein determination. Nevertheless, copper ions may seriously interfere with the determination in the UV range.

Therefore, this paper aims at presenting a new biuret procedure, in which proteins being analyzed mobilize copper ions from insoluble salts at high pH values. The absorbance of the copper–protein complex is then measured at 226 nm. UV spectrophotometry and mass spectrometry were also used to characterize copper binding to proteins at various pH values.

2. Experimental

2.1. Apparatus

An UVIKON 933-KONTRON double beam UV–vis spectrophotometer with 1 cm matched cells of quartz was used for the spectral measurements. The pH values were measured with a CG 837-Schott pH meter. Mass measurements were performed on a prototype hybrid RF/DC quadrupole-linear ion trap mass spectrometer with axial ejection (Q trap, Applied Biosystems/MDS SCIEX, Toronto, Canada). A TurboIonspray source (Ion Spray Voltage: 5000 V), a syringe pump (with 100 μl syringe) with a flow rate of 5 $\mu l\,min^{-1}$ were used. The instrument was working in positive mode and Analyst software from Applied Biosystems was used for data acquisition. Also, a laboratory centrifuge and usual laboratory glassware were used.

2.2. Reagent

All chemicals were analytical reagent grade and all solutions prepared with milliQ grade water with $R = 18.2\ \Omega$. Copper phosphate was obtained by the reaction of copper sulfate with sodium phosphate tribasic dodecahydrate. Peptide Code 1017 (with no histidine residue and the following sequence: KDSVSQEGLMNTLEQNVT from RAPP Polymere, Tübingen, Germany) was chosen to be investigated with the Q TRAP^{TM} LC/MS/MS mass spectrometer. The formation of a copper–peptide complex and the ability of the investigated peptides to mobilize copper ions from their precipitates were followed.

2.3. Procedure

Amounts of 50 mg of copper phosphate powder were added to each 1 ml solution of 20–100 $\mu g\,ml^{-1}$ of amino acid, peptide, and/or protein in capped Eppendorf vials. The vials were shaken for 1 h, centrifuged and filtrated, and then the absorbance of the supernatant was read in ultraviolet range from 190 to 350 nm in 1 cm quartz cuvettes. The pH values of all solutions were checked with a pH meter before and after the treatment with copper phosphate. The pH corrections were made with ammonium bicarbonate or ethanolamine, when necessary. Before mass spectrometric analysis, the peptide solutions were accordingly diluted with milliQ grade water up to a concentration of 10 $\mu g\,ml^{-1}$. After dilution, pH was measured as well.

To determine proteins in real samples, 1–1.5 ml of 1.8% KOH solution containing from 0 to 20 $\mu g\,ml^{-1}$ protein was treated with about 25 mg of copper phosphate powder. The mixture was powerfully stirred, centrifuged, and the absorbance of the supernatant was measured at 226 nm in 1 cm quartz cuvettes against a blank of the reagents. The calibration curves should be plotted with the proteins being analyzed in the range of concentrations from 0 to 20 $\mu g\,ml^{-1}$. Protein concentration expressed

as $\mu g\,ml^{-1}$ protein can simply be determined from the calibration curve. Lowry method [1] was applied as a standard method for protein determination.

2.4. Statistics

The standard deviation (s), standard deviation of the mean (s_x), t and F parameters, and the coefficient of variation, CV%, were calculated in order to compare the methods. In order to check for the accuracy and precision of the proposed method, a sample of protein containing 10 $\mu g\,ml^{-1}$ human albumin was analyzed by the proposed method and by the Lowry procedure. The result was expressed as the mean of six replicate analyses.

3. Result and discussion

3.1. UV measurement

Proteins bind copper ions at high pH values to yield a purple biuret complex, which can be quantitatively measured in a spectrophotometer. Under alkaline conditions, copper ions strongly interfere with the determination in the ultraviolet range. Therefore, the use of the biuret method or the investigation of copper binding to proteins at low wavelength proved to be almost impossible. Nevertheless, under neutral conditions, copper ions did not absorb in the ultraviolet range over 240 nm. However, copper absorbance slowly and proportionally increased below 230 nm (Fig. 1A). On treating albumin with copper phosphate at low pH, a copper–albumin complex with a maximum of absorbance at 193 nm occurred (Fig. 1B). Ratio of the albumin absorbance and that of copper ions (A_a/A_{Cu}) had a peak of 1.552 at 198 nm, while the difference between the two values (A_a/A_{Cu}) showed a maximum of 0.33 at 193 nm. Upon increasing pH, the absorbance of copper ions in the UV range increased dramatically and their maxima shifted towards higher wavelengths (Fig. 1C). Nevertheless, upon adding albumin to a mixture of copper phosphate and water at different pH values, the absorbance of the supernatant solutions proved to be much higher and the peaks shifted to much higher wavelengths. The effect of pH on the absorbance of copper–protein complex in the ultraviolet range was further investigated (Fig. 2). Upon increasing pH, the absorbance maxima shifted towards higher wavelength values, and their intensity dramatically increased. The highest absorbance was found to be at 226 nm at pH 12.74, while the lowest at 195 nm and pH 6.23. Also, a low peak was observed at 198 nm and pH 4.65. Therefore, UV measurements were able to evidence the formation of copper–protein complex and especially the feasibility of protein determination at 226 nm using a variant of the biuret method. By our opinion, such an investigation was not so far performed. Therefore, the interference of amino acids and other compounds was consequently studied. Thus, the absorbance of copper–albumin complex, amino acid-copper complex and copper ions was measured over pH 12 (Fig. 3A). Other amino acids gave similar absorbance in the ultraviolet range under the experimental conditions (curves not shown). On subtracting copper absorbance from that of the protein, a peak with a maximum at 226 nm was obtained (Fig. 3B). UV readings of copper–protein complex were not affected by amino acids and ammonia, because they absorbed more intense below 226 nm only. Therefore, proteins could be determined by the biuret procedure, on reading the absorbance at 226 nm. Spectrophotometric methods for protein analysis must take into account the absorbance of nucleic acid if wavelengths between 190 and 300 nm are to be used. We found that solutions of 20 $\mu g\,ml^{-1}$ DNA and guanidine, which are regularly present in the protein solutions, absorbed over 260 nm in the presence of copper ions at high pH (Fig. 3C). As a consequence, they do not interfere with the protein determination at 226 nm. In brief, proteins could be determined by a novel method based on the UV measurement of the copper–protein complex, formed by extracting copper ions from their insoluble precipitates under alkaline conditions.

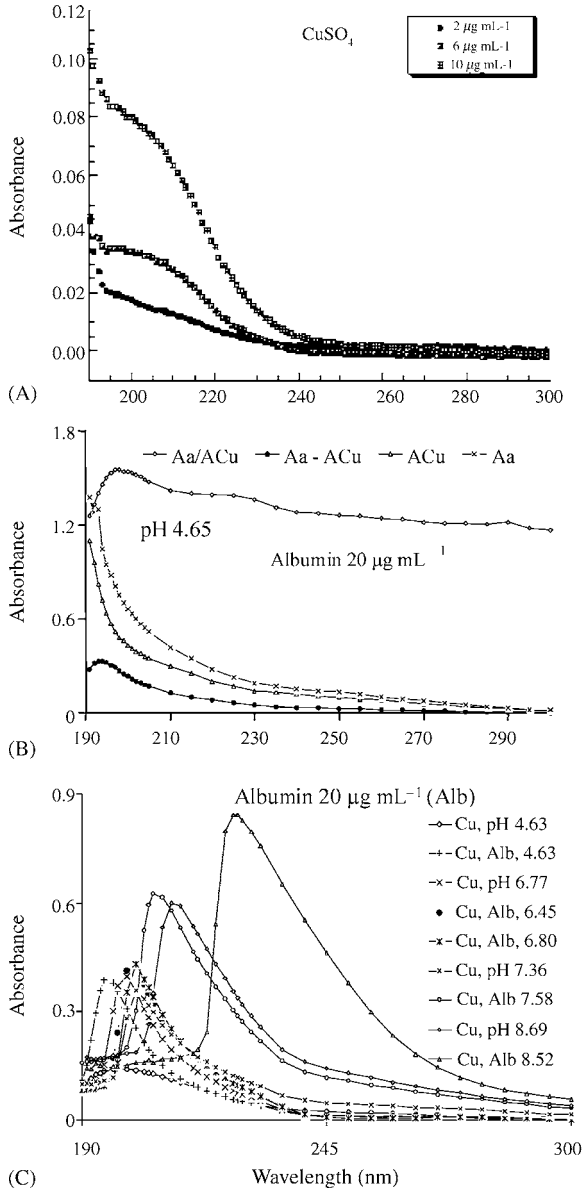

Fig. 1. UV spectra from 190 to 300 nm for copper sulfate (A), albumin (20 $\mu g\,ml^{-1}$) against copper phosphate at pH 4.65 (B), and for albumin in the presence of copper phosphate at various pH values (C).

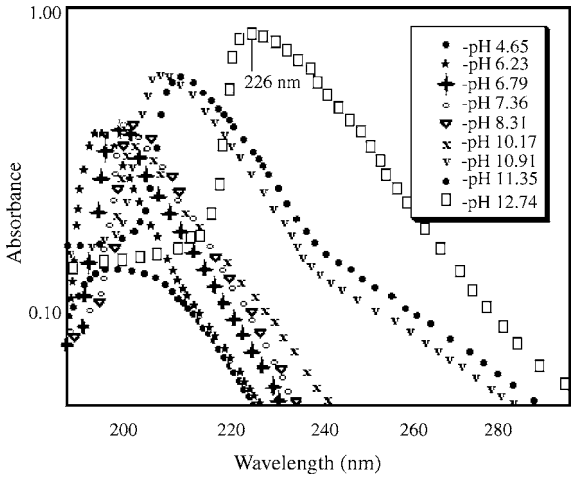

Fig. 2. UV spectra of human albumin (20 μg ml^{-1}) in the presence of copper phosphate at various pH values. Upon increasing pH, absorbance maxima shifted towards higher wavelengths. The highest absorbance was found to be at 226 nm for pH 12.74, while all the other pH values gave maxima at lower wavelengths (226 nm, pH 12.74; 221 nm, pH 11.35; 209 nm, pH 10.91; 205 nm, pH 10.17; 206 nm, pH 8.31; 201 nm, pH 7.36; 199 nm, pH 6.79; 195 nm, pH 6.23; 198 nm, pH 4.65).

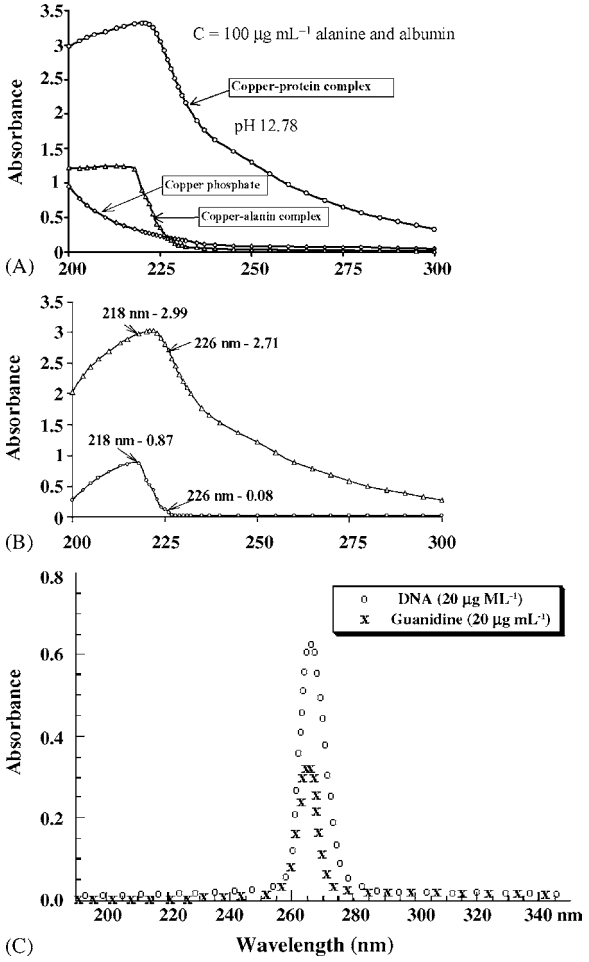

Fig. 3. UV spectra for (A) copper ions, alanine (100 μg ml^{-1}); and albumin (100 μg ml^{-1}) at high pH; (B) albumin and alanine against copper phosphate; and (C) DNA and guanidine (20 μg ml^{-1}) in the presence of copper phosphate at pH 12.78.

3.2. Mass spectra

The two new ionization methods in mass spectrometry, electrospray and matrix-assisted laser desorption ionization (MALDI), which have been introduced in the two last decades, have revolutionized protein chemistry [37–41]. They both allow the accurate molecular weight determination of proteins, peptides, as well as of nucleotides, sugars, etc. [42,43]. In the work reported here, the interactions between Cu^{2+} and synthetic peptides with no histidine residue were probed by Q TRAP^{TM} system under a variety of pH conditions. Thus, 1 mg ml^{-1} peptide was first treated with copper phosphate at pH 4.0, then with a solution of NH_4HCO_3 up to pH 6.70. Upon filtering and diluting, pH became 7.45. Similarly, another treatment with NH_4HCO_3 resulted in a solution with pH 7.32. Upon diluting 100 times with water, a solution with pH 8.00 and a concentration of 10 ng μL^{-1} resulted. The value of [H$^+$] of any buffer mixture does not change when this is moderately diluted with water. However, according to the well-known equation:

$$[H^+] = \frac{K_{acid}(C_{acid} - [H^+])}{(C_{salt} - [H^+])},$$

where [H$^+$] means the hydrogen ion concentration, C_{acid}, the acid concentration, and C_{salt}, the salt concentration of a buffered solution, highly diluting of a solution with acidic pH may result in a drastic increase in pH, even over the neutral point. By contrast, a higher pH (10.78) was obtained with ethanolamine. Upon diluting 100 times, a solution with an expected pH 10.30 was obtained.

Fig. 4 shows the original mass spectra as recorded without further processing. They are typical of ESI Q TRAP-MS in that they reveal an array of doubly charged ions. Thus, the peak at *m/z* 997.5 in the spectrum at pH 6 without metal ions (Fig. 4A) are attributable to the peptide with two protons attached being solved in milliQ grade water. Therefore, a molecular monoisotopic mass of 1993.0 atomic mass units (amu) was measured for the investigated peptide (average molecular weight 1994.0 amu), with a mass resolution $m/\Delta m$ 14,235.7, for which a molecular monoisotopic weight of 1993.14 amu was calculated. Upon adding copper phosphate to this peptide solution, the shape of the spectrum became more complicated and broader due to the changing in the isotopic composition.

Hence, a shift to higher *m/z* of each peak due to strongly binding the copper ions was observed only with the Cu^{2+} ions (Fig. 4B), for which the most abundant species corresponded to the attachment of a single Cu^{2+} ion, while the peak representing free peptide amounted to 24.35% of the total. Theoretical masses were calculated based on the assumption that two positive charges were derived from the addition of either: (1) a single Cu^{2+} ion with no additional protons involved (peptide·Cu)$^{2+}$, the mass/charge (*m/z*) value being $M = (1993 + 63)/2 = 1028.0$; (2) one Cu^+ plus one Cu^{2+} ion minus a single proton (peptide·(−H)·Cu$_2$)$^{2+}$; (3) two Cu^{2+} ions minus two protons (peptide·(−2H)·Cu$_2$)$^{2+}$, with monoisotopic mass $M = (1993 + 2 \times 63 - 2 \times 1)/2 = 1058.5$. However, copper-free peptide had a peak at 997.3 amu at pH 7.4, another one at 1005.2 amu corresponding to the fraction with

methionine-oxidized residue (Met → MetOx) and two signals at 1008.4 and 1020.2, respectively for a single Na^+ ion and two Na^+ ions bound. Furthermore, the observed isotope pattern closely matched the theoretical distribution for (1) with one single Cu^{2+} ion. In addition, the isotope pattern closely matched the theoretical distribution for (3) with two Cu^{2+} ions rather (2), which confirmed that, in some cases, there was no significant contribution from species containing copper in the lower state together with elimination of a proton. The ratio copper-bound peptide/copper-free peptide was calculated to be 1.528, where copper-free peptide means unchanged peptide, methionine-oxidized peptide, Na^+ bound peptide and copper-bound peptide the same species, which bound copper ions. Therefore, each signal in the spectrum was associated to a specific theoretical mass, the experimentally measured value being almost identical to the theoretical value for (1) free peptide (997.3: $[M+2H]^{2+}$); (2) peptide with a methionine-oxidized residue (1005.2: $[M+O+2H]^{2+}$); (3) Na^+ ions bound peptide (1008.2: $[M+Na+H]^{2+}$, average mass at 1020.2: $[M+2Na]^{2+}$, 1027.2: $[M+O+2Na]^{2+}$, and 1013.2: $[M+O+H+Na]^{2+}$; and (4) copper-bound peptide (monoisotopic peak at 1027.7: $[M+Cu]^{2+}$, average mass at 1028.7: $[M+Cu]^{2+}$, a monoisotopic mass at 1035.7 for $[[M+O+Cu]^{2+}$, 1039.7: $[M+Na+Cu-H]^{2+}$, 1047.6: $[M+O+Na+Cu-H]^{2+}$, 1050.2: $[M+O+2Na+Cu-2H]^{2+}$. The very small peak at 1059.3 amu might be attributed to a single Cu^+ bound peptide ($[M+Cu+H]^{2+}$). As a consequence, another peak at $997.3+65-2=1060.3$ amu, corresponding to $^{65}Cu^{2+}$ isotope could be found. Indeed, a small peak at 1060.6 amu was found. It was concluded that methionine residue was oxidized by Cu^{2+} ions, which generated Cu^+ ions bound to the peptide.

A small increase in pH, from 7.4 to 8.0, showed that the ratio mono-copper peptide/free peptide remained unchanged; its value was calculated to be 1.28. Nevertheless, the methionine-oxidized fraction was much abundant and some molecules of peptide with two Cu^{2+} ions bound appeared (Fig. 4C). However, the ratio copper-bound peptide/copper-free peptide decreased up to 1.34. Also, the experimentally measured value for each signal was almost identical to the theoretical value for (1) free peptide (997.3: $[M+2H]^{2+}$); (2) peptide with a methionine-oxidized residue (1005.2: $[M+O+2H]^{2+}$, where O

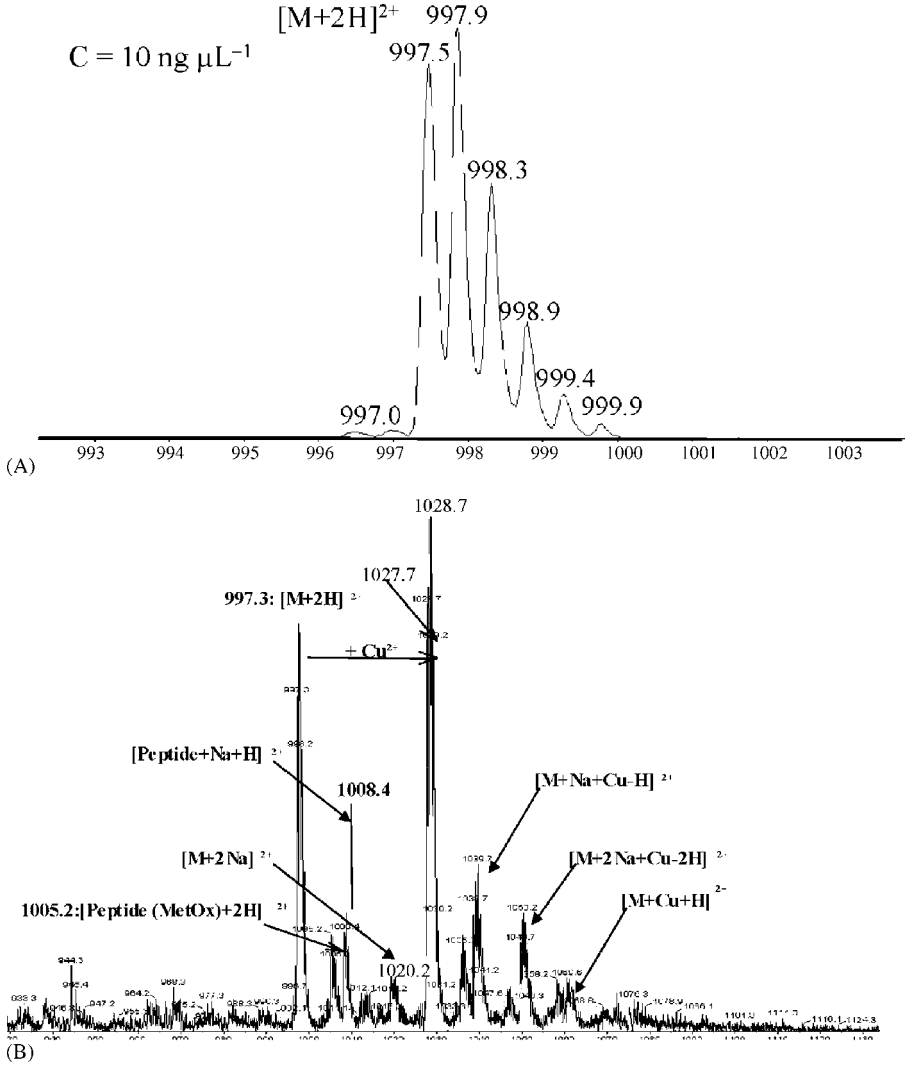

Fig. 4. ESI mass spectra of 10 ng μl^{-1} peptide with no histidine residue collected with a Q TRAPTM system (A) without added metal ions; (B) with copper phosphate, pH 7.40; (C) with copper ions at pH 8.00; and (D) with copper ions and etanolamine at pH 10.30.

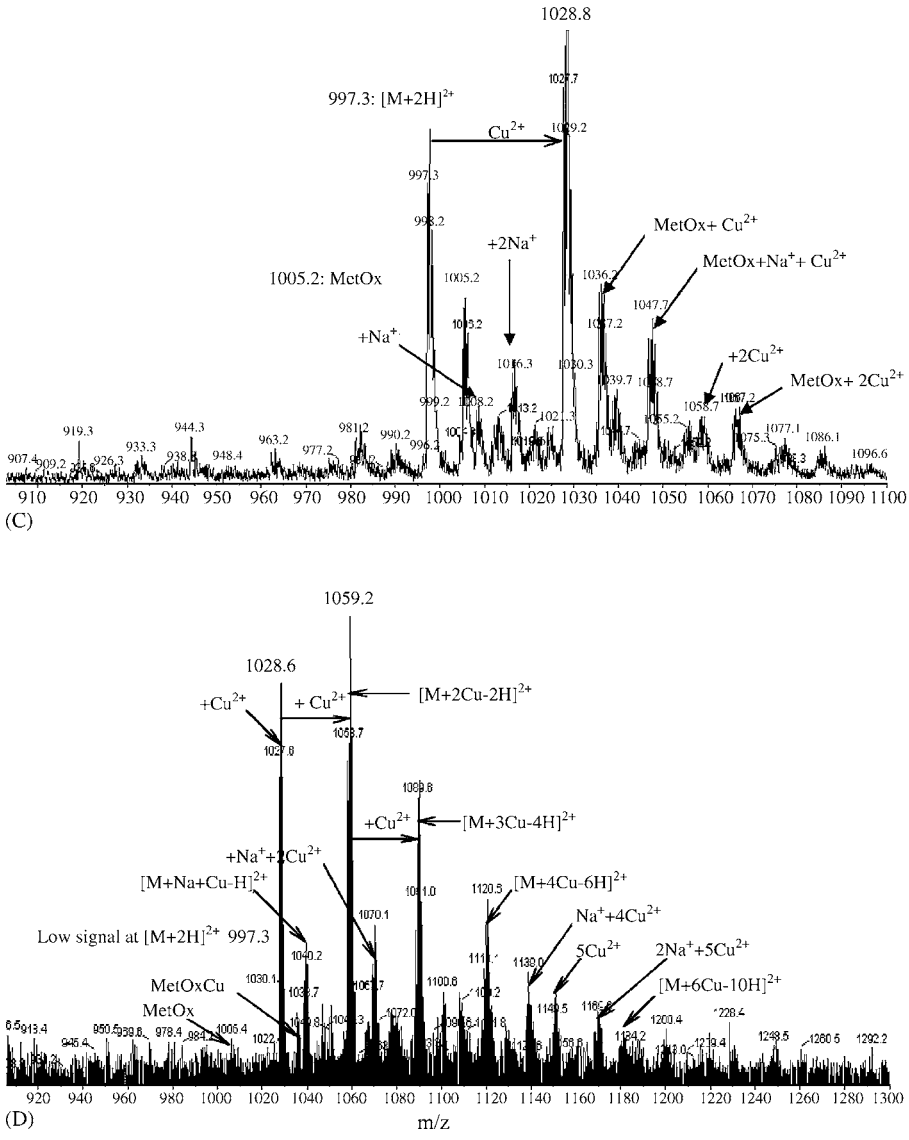

Fig. 4. (*Continued*).

means an oxygen atom); (3) Na^+ ions bound peptide (1008.2: $[M + Na + H]^{2+}$, 1019.3: $[M + 2Na]^{2+}$, 1027.2: $[M + O + 2Na]^{2+}$, and 1013.2: $[M + O + H + Na]^{2+}$; and (4) copper-bound peptide (monoisotopic peak at 1027.7: $[M + Cu]^{2+}$, average mass at 1028.8: $[M + Cu]^{2+}$, an average mass at 1036.2 for $[M + O + Cu]^{2+}$, 1039.7: $[M + Na + Cu–H]^{2+}$, 1047.7: $[M + O + Na + Cu - H]^{2+}$, 1058.7: $[M + 2Cu - 2H]^{2+}$, 1067.2: $[M + O + 2Cu - 2H]^{2+}$, 1077.1: $[M + O + Na + 2Cu - 3H]^{2+}$). The small peak at 1055.2 amu could be attributed to the average mass of double oxidized methionine residue of the peptide ($[M + 2O + Cu + Na - H]^{2+}$). These measurements established that the formation of these ions involved addition of Cu^{2+} and Na^+ ions and the elimination of a corresponding number of protons from peptide molecule, accounting for the overall charge of +2. This is consistent with the elimination of protons from the free COOH groups of the peptide at low pH value, and with the proposal that the backbone amides become deprotonated to act as copper binding sites at higher pH [8].

Adding of ethanolamine instead ammonium bicarbonate enhances the pH range of mass spectrometry analysis. Ethanolamine proved to be quite useful to obtain the Q TRAP spectra at pH 10.3 and to show the greater binding of copper (Fig. 4D). Contrary, when used ethanolamine in a MALDI-TOF experiment in the presence of α-cyano-4-hydroxy-cinnamic acid as matrix, we did not obtain any signal in the spectrum (results not shown). Na^+ and K^+ are currently observed to attach from the reagents to peptides examined by mass spectrometry, although this binding seems to be nonspecific. Methionine was also found to be partly oxidized. Thus, the signal from 997.3 or 997.5 amu corresponding to pure peptide disappeared, and new signals, which were attributed to up to five or even six copper ions bound to each molecule of peptide appeared.

An advantage of the mass spectrometric method is the ability to obtain speciation information directly. For example, at pH 10.3, there were on average 3.19 Cu^{2+} ions extracted by the peptide. Also, no peptide was free of complexation, 15.4% had a single Cu^{2+} bound, 18.0% had two Cu^{2+} ions bound,

Table 1
Absorbance of some possible interfering substances at 226 nm against water

Compound	Absorbance (226 nm; 10 mg ml^{-1})	Molar absorptivity (226 nm; 1 mol^{-1} cm^{-1})
Thiourea	3.300	25.12
Trizma base	1.040	12.59
Glycine	0.295	2.21
Guanidine	0.084	0.50
Ammonium sulphate	0.062	0.79
Urea	0.003	0.01

11.8% had three Cu^{2+} bound, and only 2.0% had six Cu^{2+} ions bound to the peptide. In addition, some peptide molecules proved to bind one or two Na^{+} ions and one to five copper ions to afford the 2+ ions. Therefore, one molecule of peptide, with an oxidized methionine residue or not, might contain from one to six Cu^{2+} bound. Under these circumstances, we recalculated Cu^{2+} percent bound to peptide molecules: 23.6% peptide molecules had one Cu^{2+} extracted, 30.4% had two Cu^{2+} ions bound, 20.8% three Cu^{2+} ions attached, 15.4% had four Cu^{2+} ions, 7.8% five Cu^{2+} ions bound, and 2.0% six such ions bound.

3.3. Interfering substances

Effect of co-existing species accompanying proteins in biological materials, such as amino acids, polysaccharides and substances often added to protein solutions was examined with a solution of 10 μg ml^{-1} human albumin. The method was found to be free from most of the interference due to the reading of the absorbance at 226 nm, where copper ions, amino acids, urea, nucleic acids, etc. do not absorb significantly. At lower wavelengths, ammonia as well as amino acids could interfere seriously with the determination yielding positive errors. Sodium dodecylsulfate did not interfere in the experimental conditions. Concentrations of 1 mg ml^{-1} of sulfate, nitrate, chloride, acetate, potassium, calcium, magnesium, manganese, chromium, lead, cadmium, zinc, nickel, sodium citrate, mercaptoethanol, nicotinic acid, sodium azide, starch, phenol, thymol, and urea did not affect the determination. A few highly concentrated solutions (10 mg ml^{-1} active substance) were measured at 226 nm against water (Table 1). Urea, guanidine, ammonium sulfate do not absorb under these conditions, thiourea and Trizma base have an intense absorption, while glycine over 0.5 mg ml^{-1} could interfere with protein determinations. Similar absorption behavior was observed when water was replaced by the working solution (under the method conditions). Upon adding ethyl alcohol to the working solution, the absorbance of the blank increased from 0.299 to 0.823. In addition, the usage of an alkaline-alcohol solution resulted in smaller absorbance values of the analyzed solutions (Table 2). Usually, alcohol is used in some variants of the biuret method to determine proteins in samples containing starch or other substances that can form turbid solutions. Such an alkaline-alcoholic solution proved to be not suitable to measure protein concentration at 226 nm (Table 2).

Table 3
Evaluation of the precision of the proposed method compared with the Lowry method [1] for the determination of human albumin

Parameter	Proposed method (μg ml^{-1})	Lowry method (μg ml^{-1})
Concentration	10.0 ± 0.2	10.0 ± 0.2
s	0.24	0.21
s_x	0.09	0.08
CV%	2.4	2.1

Mean of six replicate analyses.

3.4. The precision of the proposed method

Evaluation of the proposed method compared with the Lowry procedure showed they had similar precision when a sample of 10 μg ml^{-1} of human albumin was analyzed (Table 3). The coefficient of variation for replicate analyses was only 2.4%. The F parameter ($F = S_1/S_2 = 0.01928/0.01358 = 1.42$) also indicated that the two methods are similar.

Table 2
The absorbance values of some substances (50 μg ml^{-1}) treated with copper phosphate and basic solutions

Substance	Absorbance (226 nm)		Molar absorptivity (226 nm)			
	Basic solution	Alkaline-alcohol solution	Basic solution		Alkaline-alcohol solution	
			1 mg^{-1} cm^{-1}	1 mol^{-1} cm^{-1}	1 mg^{-1} cm^{-1}	1 mol^{-1} cm^{-1}
Ammonium sulfate	0.005	0.004	0.11	14.3	0.09	11.7
Glycine	0.004	0.003	0.08	6.0	0.06	4.5
Guanidine	0.005	0.004	0.10	6.0	0.08	4.8
Taurine	0.016	0.001	0.32	40.03	0.02	2.50
Thiourea	0.041	0.062	0.82	62.7	1.25	95.1
Trizma base	0.040	0.021	0.825	99.91	0.42	51.10
Urea	0	0	0	0	0	0
Albumine	1.326	0.424	26.52	–	8.48	–
Caseine	1.025	0.554	20.5	–	11.08	–
Gelatine	0.656	0.194	13.12	–	3.88	–

Table 4
Protein determination of some real samples

Sample	Proposed method (mg ml^{-1}) ($c \pm t$ s_x; CV%)	Lowry method (mg ml^{-1}) ($c \pm t$ s_x; CV%)
Human serum-1	72 ± 6; 2.3	71 ± 4; 1.86
Human serum-2	75 ± 5; 2.1	7.6 ± 5; 2.0
Human serum-3	78 ± 7; 2.4	77 ± 5; 1.8
Human serum-4	81 ± 6; 2.2	81 ± 5; 1.9
Human serum-5	84 ± 6; 2.1	83 ± 6; 2.1
Zein-1	0.21 ± 0.02; 2.4	0.23 ± 0.02; 2.70
Zein-2	0.30 ± 0.03; 2.3	0.30 ± 0.02; 2.2
Zein-3	0.38 ± 0.03; 2.8	0.37 ± 0.02; 1.90

Mean of three replicate analyses.

3.5. Copper binding and the biological processes

Many proteins might function as Cu^{2+} transporters by binding Cu^{2+} ions from the extra cellular medium under physiologic conditions and then releasing some of this metal upon exposure to acidic pH. Since Cu^{2+} ions might bind to peptides in a nonspecific manner, we sought to distinguish such artefactual complexes from peptide–metal ion complexes that might be meaningful biologically. Little is known on the mechanism by which Cu^{2+} ions are mobilized when copper insoluble salts are used. Cu^{2+} mobilization by the investigated peptide showed a strong dependence on the pH, which could be measured unambiguously and directly by mass spectrometry. Proteins proved to bind copper ions even at lower pH values. It was shown that high histidine content peptides as well as octarepeat synthetic peptides and PrP hexarepeat ones preferentially bind Cu^{2+} compared with nickel and zinc [44–48]. Nevertheless, it is possible that even nonhistidine proteins function as Cu^{2+} transporters by binding Cu^{2+} ions from the extracellular medium under physiologic conditions and then releasing some of this metal upon exposure to acidic pH. For example, upon decreasing pH of a copper–protein solution, a small amount of copper salt precipitated. When repeated the process of mobilizing and precipitating copper ions, the amount of precipitate copper increased dramatically. The same process might occur under physiological conditions within the body in the degenerative diseases, in which hypoxia creates rather acidic conditions.

3.6. Measurement of real samples

The ability to easily and reliably quantitate protein content is paramount to many biological assays. The proposed method proved to be a highly selective, accurate, reproducible, and, especially, very sensitive analytical procedure for protein determination. We analyzed a few protein samples both by the present and the Lowry method [1]. The original solutions were first diluted accordingly with milliQ grade water to reach the adequate concentration for each procedure. The calibration curves were plotted with protein standard from KIT (Sigma) and purified zein prepared in our laboratory. The results thus obtained for the real samples that were analyzed proved to be in good agreement (Table 4). In the case of amino acid-rich samples, a precipitation with trichloroacetic acid might be necessary *prior* protein determination. The results of the determinations made with the proposed method and the UV spectra indicate that the sensitivity of protein determination at 226 nm was over 100 times the sensitivity of the classical biuret method. A large variety of tissue samples can be successfully analyzed for protein content using this procedure, including proteins from polyacrylamide gels. A major strength of UV biuret assay for protein determination lies in the ability to analyze insoluble tissues as well as soluble proteins. In addition, the assay was precise, sensitive, and suitable for large numbers of samples. Nevertheless, various proteins have different molar absortivities, and, therefore, calibration curves must be plotted for each protein being analyzed. Also, to avoid the small interference of amino acids and free copper ions, the UV readings could also be done at higher wavelengths. Unfortunately, the absorbance proved to be less than maximum absorbance at 226 nm. Also, it was taken into account the absorbance of nucleic acid (DNA) if wavelengths between 190 and 300 nm are to be used. Examination of the absorbance spectrum of DNA revealed that its absorbance passes through a maximum around 260 nm, and thus to eliminate absorbance interference due to nucleic acid, wavelengths near 226 nm, where such absorbance is minimal, have been considered more thoroughly. The measurement of protein concentration at 226 nm, under the experimental conditions described here, resulted in an absorbance coefficient, which amounted to minimum 1.3×10^3 ml (mg cm)$^{-1}$. Detection limit was found to be 1.2 ng ml^{-1}.

4. Conclusions

A very simple, highly selective and sensitive assay of proteins based on the biuret absorption in the ultraviolet region has been developed. Treatment of peptides with $Cu_3(PO_4)_2$ at various pH values results in the formation of a copper–protein complex, which may be investigated by UV spectrophotometry and mass spectrometry. Copper ions are mobilized by the proteins from their insoluble precipitates. Even in the acidic or neutral solutions, peptides bind copper ions from the insoluble phosphate. On increasing pH, the amount of copper bound increases accordingly. It has been shown for the first time how the individual peptides mobilize copper ions. Addition of ethanolamine instead ammonium bicarbonate enhances the pH range of mass spectrometry analysis and could be of interest in the investigation of the proteins by such techniques. Ethanolamine does not interfere with the mass spectrometry analysis made with Q TRAP™ system.

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