



Short communication

Colorimetric assay of chitosan in presence of proteins and polyelectrolytes by using *o*-phthalaldehydeN.I. Larionova^{a,*}, D.K. Zubaerova^a, D.T. Guranda^b, M.A. Pechyonkin^a, N.G. Balabushevich^a^a Chemistry Department, Lomonosov Moscow State University, 119991, Moscow, Russia^b A.N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991, Moscow, Russia

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ABSTRACT

A novel approach of colorimetric quantification of chitosan based on the derivatization reaction of its primary amino groups with *o*-phthalaldehyde and a thiol – *N*-acetyl-L-cysteine has been developed. The reaction of equal volumes of sample solution and the reagent solution was allowed to proceed for 1 h, and then the absorbance values were measured at 340 nm against the reference solution. The procedure conditions have been optimized for chitosan assay in the presence of polyanionic electrolyte dextran sulphate (pH 8.9, the reagent solution: 4.0 mM *o*-phthalaldehyde, 2.6 mM *N*-acetyl-L-cysteine, 0.25 M NaCl). The method has proven to be convenient and reliable for quantitative determination of either the concentrations of chitosans of various molecular weights or their degree of deacetylation. The different reactivity of chitosans and proteins can be used in order to determine chitosan in presence of the protein. This approach ensured accurate assay within the chitosan concentrations ranging from 0.01 to 0.15 mg/ml and could be applied for quantitative analysis of chitosan in protein-loaded microparticles.

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1. Introduction

Chitosan is a family of cationic polysaccharides obtained from chitin by its deacetylation with molecular weights ranging from 10 to 1000 kDa, and containing about 5–25% of acetylated amino groups (Muzzarelli & Muzzarelli, 2002). The repeating units in chitosan are a 2-deoxy-2-(acetyl-amino) glucose and a 2-deoxy-2-amino glucose linked into linear polymers (Karrer & Hofmann, 1929).

Due to its biocompatibility and mucoadhesive properties chitosan becomes of great interest as a new functional material of high potential in various fields (Muzzarelli & Muzzarelli, 2002). One of the major challenges appears to be its application for development of safe and efficient drug delivery systems, especially for drugs of protein origin (Paul & Sharma, 2000). Therefore the development of methods of chitosan assay in protein-loaded microcapsules is imperative.

A method for determination of chitosan in wood and water samples has been developed based on acidic hydrolysis of chitosan to glucosamine followed by online derivatization by *o*-phthalaldehyde, chromatographic separation and fluorescent detection (Eikenes, Fongen, Roed, & Stenström, 2005). It appears to be rather complex and inaccurate. A method to assay the content of glucosamine using capillary electrophoresis procedure has also been developed (Chen,

Lee, Cheng, Hsiao, & Chen, 2006). More reliable analytical methods are colorimetric assay of chitosan with various anionic dyes, for instance C.I. Acid Orange 7, C.I. Acid Red 13, C.I. Acid Red 27, Orange II, Alizarin S, Alizarin GG, Congo Red (Wischke & Borchert, 2006), Cibacron Brilliant Red 3B-A (Muzzarelli, 1998). However, these techniques based on reactions between free amino groups of chitosan and acting agents are ineligible for determination of chitosan in presence of substances bearing primary amino groups, including peptides (Sėkmokienė, Speičienė, Šalašėvičienė, & Garmienė, 2005). In consequence the content of chitosan in the protein-loaded microparticles either could not be determined at all (Chen, Mohanraj, & Parkin, 2003; Cui, Zhang, Zheng, & Kawashima, 2004; Pan, Zheng, Zhao, Li, & Xu, 2002) or is indirectly estimated with serious errors (Balabushevich, Lebedeva, Vinogradova, & Larionova, 2006).

In this article we investigated the possibility of de novo application of the well known method of amino acids assay based on the derivatization of their amino groups with a mixture of *o*-phthalaldehyde and a thiol (Švedas, Galaev, Borisov, & Berezin, 1980) for quantitative colorimetric determination of macromolecules bearing primary amino groups like chitosan. The procedure conditions have been optimized for chitosan assay in the presence of model compounds used for protein-loaded microparticles preparation. Insulin was chosen as a pattern since chitosan and its derivatives are widely used in polymeric microparticulate peroral insulin-delivery systems (Paul & Sharma, 2000). Bovine serum albumin was also taken as a model protein with high content of primary amino groups (approximately 10% of amino acids are lysines).

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2. Materials and methods

2.1. Materials

Chitosan 22 kDa was kindly donated by Prof. Varlamov V.P. ("Bioengineering center" of Russian Academy of Sciences (RAS), Russia); chitosan 100 kDa with degree of deacetylation (DD) 84% was gently donated by Dr. Yatluk Y.G., (I.Ya. Postovsky Institute of Organic Synthesis of RAS, Russia); chitosans 150, 400, 600 kDa were purchased from Fluka (Switzerland); chitosan 460 kDa with DD 93% from Closed joint-stock company "Bioprogress" (Russia); dextran sulfate sodium salt 500 kDa, D-glucosamine hydrochloride, N-acetyl-L-cysteine (NAC), bovine serum albumin from Sigma (USA); o-phthalaldehyde from Koch Light (UK); human recombinant insulin, 29 U/mg, was obtained from Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of RAS (Russia). All other chemicals and solvents used were of analytical grade.

2.2. Methods

2.2.1. Preparation of solutions

All of the chitosan preparations were dissolved in 0.01 M HCl and pH value of the solutions was adjusted to 3.0. To prepare the model solutions exact amounts of proteins and dextran sulfate were dissolved in 0.2 M borate buffer, pH 8.9. To determine amount of chitosan in insulin-loaded microparticles composed of chitosan and dextran sulfate, the lyophilized samples were dissolved in 0.01 M NaOH solution, and diluted by 0.2 M borate buffer, pH 8.9, to final insulin concentration 0.1–0.2 mg/ml.

The reagent solution was freshly prepared immediately prior to the colorimetric assay by adding 200 μ l of each of the ethanol solutions 0.11 M o-phthalaldehyde and 0.071 M NAC to 5.0 ml of 0.2 M borate buffer, pH 8.9.

2.2.2. Spectrophotometric analysis

The formation of isoindoles was monitored continuously against the reference solution with a Shimadzu UV-265 FW spectrophotometer at 340 nm, 25 °C, pH 8.9, in 0.2 M borate buffer. The procedure was as follows: 500 μ l of the sample solution containing 0.25 M NaCl and 500 μ l of the reagent solution were introduced into the spectrophotometer cuvette and mixed, then the monitoring was started and the absorbance was continuously measured during 60 min.

Concentration of protein in samples was determined by Lowry routine as described in (Lowry, Rosebrough, Farr, & Randall, 1951).

2.2.3. Calculations

Experimental data were analyzed using the linear least-squares regression method. Relative standard deviations (RSD) were calculated for each sample from three measurements.

3. Results and discussion

3.1. Optimization of conditions of chitosan determination with o-phthalaldehyde

The method of assay of primary amines based on the derivatization of their amino groups with a mixture of o-phthalaldehyde and a thiol (Švedas et al., 1980) is well known and nowadays is used for quantitative and enantiomeric analysis of different low-molecular primary amino compounds (Guranda, Kudryavtsev, Khimiuk, & Švedas, 2005; Guranda, Shapovalova, & Švedas, 2004). Here we have newly applied this method to quantify macromolecules, bearing primary amino groups. Modification of primary amino groups of both the polyglucosamine and protein with the standard reagent

consisting of o-phthalaldehyde and NAC resulted in formation of isoindoles (Fig. 1) with a characteristic absorption maximum at 340 nm. The formation of isoindoles was monitored spectrophotometrically against the reference solution.

Initially the colorimetric reaction was carried out at the customary conditions for analysis of amino acids (pH 9.6, 25 °C, 4.0 mM o-phthalaldehyde, 2.6 mM NAC) (Švedas et al., 1980). Concentrations of chitosans were in the range 0.01–0.10 mg/ml, that corresponded to primary amino groups concentration 50–500 μ M. Protein concentrations were also in the range 0.01–0.10 mg/ml, that corresponded to primary amino groups concentration 5–52 μ M for insulin and 9–92 μ M for bovine serum albumin. In contrast to the derivatization reactions in case of low-molecular amino compounds, the reactions with proteins and chitosan proceeded very slowly, so their maximum absorbance had not been reached during 4 h. Both o-phthalaldehyde and NAC were in an excess over the amino groups and an additional 10-fold increase of the reagent concentration did not accelerate the reaction. As a whole the absorbance value in case of insulin was about three times higher than for chitosan–isoindoles at the same amino groups concentration, and the rates of two reactions could not be discriminated. Evidently, these conditions were not suitable for the quantification of chitosan in presence of proteins.

Bearing in mind that the pK values of primary amino groups in proteins and in chitosan differ, in next experiments we have varied the pH of reaction and the obtained results allowed to assume that the pH 8.9 is optimal for analysis of chitosan in mixtures with proteins. At pH > 9.5 the contribution of proteins into the absorbance significantly increase, while at pH < 8.5 the reaction rates with both chitosan and proteins dramatically decrease. So, choosing the pH 8.9 allowed to diminish the influence of proteins (pK of the ϵ -amino group of Lys is 10.54 (Dawson, Elliott, Elliott, & Jones, 1986)), while the reaction with chitosan proceeds identically as at pH 9.6 (pK of amino group of chitosan is 6.5).

It should be noted that chitosan is used for protein microencapsulation in tandem with anionic polyelectrolytes like dextran sulfate (Balabushevich et al., 2006; Pan et al., 2002), therefore the influence of dextran sulfate on the colorimetric reaction was also investigated. It was found that dextran sulfate in amount 0.04 mg/ml significantly lowered the absorbance in case of chitosan–insulin mixtures. When added to insulin sample, dextran sulfate caused an increase in absorbance of the reaction solution due to the turbidity apparently caused by formation of insoluble complexes between polycationic insulin and polyanionic dextran sulfate. A similar effect was stated between dextran sulfate and high-density lipoprotein (Olmos, Lasuncion, & Herrera, 1992). On the contrary, when added to chitosan sample, dextran sulfate caused a significant decrease in absorbance at 340 nm apparently due to interpolymer interactions resulting in steric screening of chitosan amino groups and their partial protonation. This is consistent with the fact that strong polyelectrolytes like dextran sulfate shift the potentiometric titration curve of weak oppositely charged polyelectrolytes by 2–3 pH units (Petrov, Antipov, & Sukhorukov, 2003).

In order to prevent undesirable interference of dextran sulfate, sodium chloride was dissolved in all samples to a final concentration 0.25 M (proteins could aggregate at higher ionic strengths). In

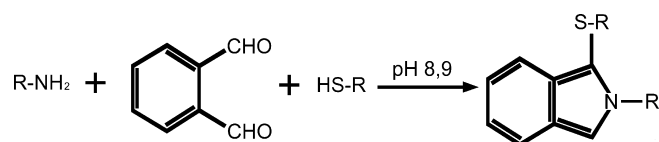


Fig. 1. Derivatization of primary amino groups by o-phthalaldehyde and a thiol.

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