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Short communication

Colorimetric assay of chitosan in presence of proteins and polyelectrolytes by using *o*-phthalaldehyde

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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.

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, & Stenstrøm, 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 (Sekmokienė, Speičienė, Šalaševičienė, & 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 μl 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 μl of the sample solution containing 0.25 M NaCl and 500 μl 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 μM. Protein concentrations were also in the range 0.01÷0.10 mg/ml, that corresponded to primary amino groups concentration 5÷52 μM for insulin and 9÷92 μM 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 p*K* 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 (p*K* 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 (p*K* 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

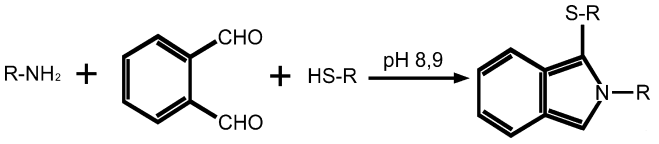

Fig. 1. Derivatization of primary amino groups by *o*-phthalaldehyde and a thiol.

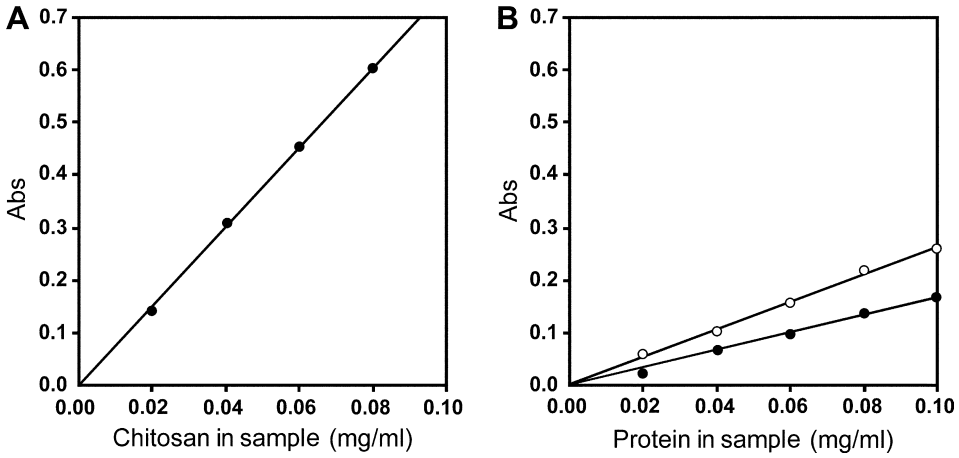

Fig. 2. Calibration plots for chitosan M_w 400 kDa (A) and proteins: bovine serum albumin (○) and insulin (●) (B). Conditions: 0.2 M borate buffer, pH 8.9, 4.0 mM *o*-phthalaldehyde, 2.6 mM N-acetyl-L-cysteine, 0.25 M NaCl, reaction time 60 min.

these conditions calibration plots of proteins and chitosan 400 kDa have been obtained (Fig. 2) with the correlation coefficient (R^2) higher than 0.99. The reactivity of chitosan turned out to be significantly higher than that of proteins, including albumin with high content primary amino groups. This can be used in order to determine chitosan in the presence of the protein. Thus, the quantitative analysis of chitosan in real mixtures with insulin and dextran sulfate became accessible. Chitosan concentration in such mixtures could be calculated according to the equation:

$$[\mathrm{Ch}] = \frac{A_{340} - [\mathrm{Prot}] \times tg_{\mathrm{Prot}}}{tg_{\mathrm{Ch}}},$$

where tg_{Ch} and tg_{Prot} are the slopes of calibration plots for chitosan and protein respectively, A_{340} – the absorbance value at 340 nm against the reference solution, [Prot] – protein concentration.

The results of quantitative determination of chitosan in several model mixtures containing insulin and dextran sulfate (Table 1) demonstrated high accuracy and trustworthiness of the developed approach.

3.2. Assay of chitosans of various molecular weights and determination their degree of deacetylation

It should be emphasized that this method is sensitive to the molecular weight as well as DD of chitosan. This means that for each form of chitosan corresponding calibration plot should be obtained. On the other hand, this allows to determine the DD of each chitosan form based on the calibration plots for D-glucosamine, chitosan with known DD considered as a standard, and tested chitosan. The value of DD (%) could be calculated according to the equation:

$$\mathrm{DD} = 100\% - \frac{(\mathrm{tg}_{\mathrm{Gluc}} - \mathrm{tg}_{\mathrm{Ch}}) \times (100 - \mathrm{DD}_{\mathrm{st}})}{\mathrm{tg}_{\mathrm{Gluc}} - tg_{\mathrm{st}}}$$

Table 1
Assay of chitosan 400 kDa in the model mixtures containing 0.125 mg/ml insulin and 0.050 mg/ml dextran sulfate ($n = 3$, $P = 0.95$).

Nominal concentration, mg/ml	Back-calculated concentration, mg/ml	Recovery, %	RSDA[a], %
0.020	0.019 ± 0.003	95	13
0.040	0.041 ± 0.005	103	12
0.060	0.056 ± 0.004	93	8
0.080	0.086 ± 0.004	107	5

[a] Relative standard deviation.

Table 2
Determination of the deacetylation degree (DD) of different chitosans.

Chitosan, M_W, kDa	DD, %		Slope of calibration plot, Abs/M	Correlation coefficient (R^2)
	Declared	Experimental		
22		98	11.3	0.9982
100	84[a]	84	8.38	0.9925
150	75–85	87	8.11	0.9958
400	75–85	85	7.80	0.9969
460	93	92	9.38	0.9948
600	75–85	84	7.81	0.9916
D-glucosamine	100	100	12.3	0.9727

[a] Standard for calculation of DD.

where tg_{Ch} and tg_{Gluc} are the slopes of calibration plots for chitosan and D-glucosamine respectively, tg_{st} and DD_{st} are the slope of calibration plot and the reported DD value for chitosan considered as a standard.

The possibility to determine the value of DD of various molecular weights chitosans has been validated by the example of six different chitosans (Table 2). The correlation coefficients (R^2) of calibration plots were higher than 0.99 for all chitosans tested. The calculated values of DD were close to the data obtained by NMR with the error 1–2%.

4. Conclusions

A new application of the well known derivatization reaction of primary amines with *o*-phthalaldehyde and a thiol allowed to develop an approach of colorimetric assay of chitosan. The method has proven to be convenient and reproducible assay for quantification of chitosans of various molecular weights. The technique is suitable for quantitative determination of either the concentration or degree of deacetylation of chitosan in the presence of proteins and strong polyanions. The *o*-phthalaldehyde assay may be considered as an essential aid in characterization of protein-loaded microparticles composed of chitosan and polyanion.

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References

Balabushevich, N. G., Lebedeva, O. V., Vinogradova, O. I., & Larionova, N. I. (2006). Polyelectrolyte assembling for protein microencapsulation. *Journal of Drug Delivery Science and Technology, 16*(4), 315–319.

Chen, J. J., Lee, Y. C., Cheng, T. J., Hsiao, H. Y., & Chen, R. L. C. (2006). Determination of glucosamine content in nutraceuticals by capillary electrophoresis using in-capillary OPA labeling techniques. *Journal of Food and Drug Analysis, 14*(2), 203–206.

Chen, Y., Mohanraj, V. J., & Parkin, J. E. (2003). Chitosan-dextran sulfate nanoparticles for delivery of an anti-angiogenesis peptide. *Letters in Peptide Science, 10*(5–6), 621–629.

Cui, F., Zhang, L., Zheng, J., & Kawashima, Y. (2004). A study of insulin–chitosan complex nanoparticles used for oral administration. *Journal of Drug Delivery Science and Technology, 14*(6), 435–439.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1986). *Data for biochemical research*. Oxford: Clarendon Press.

Eikenes, M., Fongen, M., Roed, L., & Stenstrøm, Y. (2005). Determination of chitosan in wood and water samples by acidic hydrolysis and liquid chromatography with online fluorescence derivatization. *Carbohydrate Polymers, 61*(1), 29–38.

Guranda, D. T., Kudryavtsev, P. A., Khimiuk, A. Y., & Švedas, V. K. (2005). Efficient enantiomeric analysis of primary amines and amino alcohols by high-performance liquid chromatography with precolumn derivatization using novel chiral SH-reagent *N*-(*R*)-mandelyl-(*S*)-cysteine. *Journal of Chromatography A, 1095*(1–2), 89–93.

Guranda, D. T., Shapovalova, I. V., & Švedas, V. K. (2004). A new *N*-acyl derivative of (*S*)-cysteine for quantitative determination of enantiomers of amino compounds by HPLC with a precolumn modification with *o*-phthalaldehyde. *Russian Journal of Bioorganic Chemistry, 30*(5), 403–408.

Karrer, P., & Hofmann, A. (1929). Polysaccharides XXXIX. Enzymic degradation of chitin and chitosan. *Helvetica Chimica Acta, 12*, 616–637.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265–275.

Muzzarelli, R. A. A. (1998). Colorimetric determination of chitosan. *Analytical Biochemisty, 260*, 255–257.

Muzzarelli, R. A. A., & Muzzarelli, C. (2002). Natural and artificial chitosan-inorganic composites, a mini-review. In R. A. A. Muzzarelli & C. Muzzarelli (Eds.), *Chitosan in pharmacy and chemistry* (pp. 233–243). Grottammare, Italy: Atec Edizioni.

Olmos, J. M., Lasuncion, M. A., & Herrera, E. (1992). Dextran sulfate complexes with potassium phosphate to interfere in determinations of high-density lipoprotein cholesterol. *Clinical Chemistry, 38*(2), 233–237.

Pan, Y., Zheng, J.-M., Zhao, H.-Y., Li, Y.-J., & Xu, H. (2002). Relationship between drug effects and particle size of insulin-loaded bioadhesive microspheres. *Acta Pharmacologica Sinica, 23*(11), 1051–1056.

Paul, W., & Sharma, C. P. (2000). Chitosan, a drug carrier for the 21st century. *S.T.P. Pharma Science, 10*(1), 5–22.

Petrov, A. I., Antipov, A. A., & Sukhorukov, G. B. (2003). Base-acid equilibria in polyelectrolyte systems: from weak polyelectrolytes to interpolyelectrolyte complexes and multilayered polyelectrolyte shells. *Macromolecules, 36*(26), 10079–10086.

Sekmokienė, D., Speičienė, V., Šalaševičienė, A., & Garmienė, G. (2005). Determination of chitosan in food products. *Veterinarija Ir Zootechnika, 31*(53), 87–92.

Švedas, V.-J. K., Galaev, I. J., Borisov, I. L., & Berezin, I. V. (1980). The interaction of amino acids with *o*-phthaldialdehyde: A kinetic study and spectrophotometric assay of the reaction product. *Analytical Biochemistry, 101*(1), 188–195.

Wischke, C., & Borchert, H.-H. (2006). Increased sensitivity of chitosan determination by a dye binding method. *Carbohydrate Research, 341*(18), 2978–2979.