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Note

Determination of the degree of *N*-acetylation of chitin–chitosan with picric acid

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Chitin, namely, poly[(1→4)-*N*-acetyl-β-D-glucosamine], the natural polymer second in abundance to cellulose, has become a subject of considerable interest[1,2]. The deacetylated form of chitin is called chitosan, a term that covers a wide range of chitin-related polymers that differ in their content of free amino groups, including a totally *N*-deacetylated form. Many physical and chemical properties of chitin–chitosan are closely related to its molecular weight and the extent of *N*-acetylation. Any study of these polymers requires an accurate and rapid technique for determination of the degree of acetylation. The *N*-acetyl content can be determined by i.r. spectroscopy[3–6], titration[7–9], elemental analysis, pyrolysis–gas chromatography[10], u.v. spectrophotometry[11], first-derivative u.v. spectrophotometry[12], c.d. measurements[13], n.m.r. spectroscopy[14], and thermal analysis[15]. None of the methods mentioned seems to be simultaneously adequate for highly deacetylated chitosan and highly acetylated chitin. Furthermore, many of them are laborious, or need sophisticated laboratory equipment, or both.

We now describe a method for determining the degree of *N*-acetylation of a wide range of chitin–chitosans. The method is simple and relatively fast, and requires only milligram quantities of these polymers.

EXPERIMENTAL

General. — All spectrophotometric measurements were performed with a Beckman DU-8B spectrophotometer equipped with 1-cm quartz cuvettes. Chitin from crab shells, and chitosan (practical grade) were supplied by Sigma. Colloidal

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chitin was prepared[16] by treatment with concentrated HCl. A set of samples of partly deacetylated chitin was prepared by treating chitin with 40% NaOH[17]. After various periods of time (30 min, sample A; 45 min, sample B; 70 min, sample C; and 30 h, sample D), portions of polymer were removed, thoroughly washed with water, and lyophilized. A set of reacetylated chitin–chitosan samples (E, F, G, and H) was prepared from commercial chitosan by acetylation with various proportions of acetic anhydride–methanol[18,19].

Determination of the degree of N*-acetylation of chitin–chitosan.* — Carefully dried, finely powdered polymer (5–30 mg) was placed in a small column for liquid chromatography, or in a Pasteur pipette plugged with glass wool and provided with a stopper to keep liquid in the column for a desired period of time. The weight of the polymer was determined accurately by weighing the column before and after introduction of the sample. In order to remove residual salts from the amino groups, a preliminary washing step was performed: the polymer in the column was exposed for 15 min to 0.1M diisopropylethylamine (DIPEA)–methanol, and then washed with methanol (10 mL).

Binding of picric acid to the amino groups was performed by introducing 0.1M picric acid–methanol (0.5–1.0 mL), and allowing the reaction to proceed for 6 h. The column was then washed with methanol, usually 30 mL, at the rate of 0.5 mL/min, in order to remove the unbound picric acid completely. Usually, this was achieved by washing with methanol (30 mL) at the rate of 0.5 mL/min. The bound picric acid was then quantitatively removed from the chitin–chitosan amino groups by proceeding as follows. 0.1M Methanolic DIPEA (0.5–1.0 mL) was introduced into the column, allowed to stay for 30 min, and then eluted with methanol (~8 mL). The total eluate was then diluted to exactly 10 mL with methanol.

The concentration of DIPEA picrate in the eluate was measured at 358 nm after suitable dilution. The absorbance should not exceed 1.8. In this range (corresponding to DIPEA-picrate concentrations of 0 to 115 μM), the calibration curve for this salt in methanolic solution is linear and gives a molar absorptivity value of 15,650.

The fraction of amino groups acetylated (degree of *N*-acetylation, d.a.,) is given by:

$$\text{d.a.} = \frac{m - 161n}{m + 42n}$$

where m = weight of sample (mg), n = mmoles of picric acid eluted from sample, 161 = mol. wt. of D-glucosamine unit, and 42 = mol. wt. of *N*-acetyl-D-glucosamine − mol. wt. of D-glucosamine.

Reference methods. — For the determination of amino groups by reaction with salicylaldehyde, a modified version[4] of the original protocol[20] was used. The degree of acetylation in chitin–chitosan was also determined by titration of the polymer (suspended in dilute HCl) with[8] 0.1M NaOH.

TABLE I

MEASURED DEGREE OF *N*-ACETYLATION (d.a.) AS A FUNCTION OF TIME OF REACTION BETWEEN PICRIC ACID AND AMINO GROUPS OF CHITOSAN

Time of reaction (h)	*Measured degree of* N-*acetylation*[a]	
	Chitosan, lot H[b]	*Chitosan (Sigma)*
0.083	0.900	0.892
4	0.714	0.614
6	0.637	0.255
20	0.637	0.255
24	0.637	0.255

[a]Values are the average of at least three measurements. For identical samples, the measured d.a. varied no more than ±0.020. [b]Chitosan, lot H, was prepared as described in the Experimental section.

RESULTS

Picric acid is currently used for monitoring the amino group content of insoluble polymers, for instance during solid-phase peptide synthesis[21]. The same principle can be used for chitin–chitosan polymers. In methanol, chitin and chitosan remain insoluble, but can still react with picric acid through their free amino groups. In the presence of a strong base, the picric acid forms a methanol-soluble salt which is released from the polymer.

In order to establish the kinetics of the reaction, portions (~5 mg) of two different lots of chitosan were analyzed by the procedure described in the Experimental section, using various times of exposure to picric acid. As shown in Table I, uptake of picric acid was complete at the end of 6 h of treatment.

Our method (for which 5-mg samples were used) was compared with other methods of similar complexity: the titrimetric method[8], in which samples (~500 mg) of chitin–chitosan in dilute HCl are titrated with 0.1M NaOH, and the method based on determination of residual salicylaldehyde after reaction of the sample for 48 h with the reagent (*N*-salicylidene formation[4]) which requires 100-mg samples of polymer. The degree of acetylation of chitin–chitosan samples as determined by the picric acid method, the titrimetric method, and the detemination of residual salicylaldehyde is shown in Table II.

The method with picric acid gave reproducible results for a full range of chitin–chitosan polymers having different degrees of *N*-acetylation. The *N*-acetyl content calculated from determination of picrate was comparable with that obtained from the two other reference methods (when applicable). For instance, our method allowed monitoring of the kinetics of the deacetylation process (see samples A–D, Tables II) for even relatively short periods of treatment with alkali.

TABLE II

COMPARISON OF THE PICRATE PROCEDURE WITH STANDARD PROCEDURES FOR DETERMINATION OF THE DEGREE OF *N*-ACETYLATION

Sample	*Degree of acetylation*		
	Picrate determination[a]	*Titrimetric method*[b]	*Residual salicylaldehyde*[c]
Chitin (Sigma, practical grade)	0.988 (0.001)	N/A[d]	0.988
Colloidal chitin (from practical grade, Sigma)	0.988 (0.002)	N/A	free amino groups not detected
Chitosan (Sigma)	0.255 (0.051)	0.274	
Partly deacetylated chitin			
Sample A	0.965 (0.002)	N/A	0.961
Sample B	0.945 (0.003)	N/A	0.927
Sample C	0.912 (0.006)	N/A	0.914
Sample D	0.281 (0.020)	0.276	
Partly reacetylated chitosans			
Sample E	0.626 (0.020)	0.613	0.703
Sample F	0.836 (0.020)	N/A	0.854
Sample G	0.914 (0.006)	N/A	0.923

[a]Values are means of six to seven independent determinations; the standard deviation is given in the parentheses. [b]Two determinations per sample. [c]Three to four determinations per sample. [d]N/A, not applicable: the method is not suitable for samples in this range of degree of acetylation.

DISCUSSION

In this work, a new method for the determination of the degree of *N*-acetylation of chitin–chitosan is described. The method has been shown to be reliable for a large spectrum of substrates, and relatively fast, and simpler and less expensive than other methods available. It seems to be particularly useful for highly acetylated chitin–chitosans, for which some methods[11–14] are not applicable at all, and others[3,5–9] give poor accuracy. Thus, the method described here can be used for (*a*) monitoring *N*-acetyl loss during preparation of colloidal chitin from practical-grade chitin[16], (*b*) the measurement of deacetylation during chemical or enzymic treatment of chitin, as the very high molar absorptivity of the picric salt permits the detection of even small changes in *N*-acetyl content, and (*c*) characterization of the regenerated chitin prepared from chitosan by acetylation with acetic anhydride[18,19].

In all of these cases, the small amounts of sample required, and the maintenance of the sample in the undissolved state make our procedure the method of choice.

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