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NEW METHOD OF CHITIN DETERMINATION BASED ON DEACETYLATION AND GAS–LIQUID CHROMATOGRAPHIC ASSAY OF LIBERATED ACETIC ACID

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SUMMARY

An analytical method has been developed for chitin determination in biological materials; it is based on determination of the acetic acid liberated by acid or alkaline hydrolysis of chitin. The acetic acid is determined by gas–liquid chromatography on a Porapak Q column, with propionic acid as internal standard. The rates of hydrolysis of chitin and N-acetyl-D-glucosamine have been evaluated by power kinetics. Results obtained with crustacean chitin as a model agree with those for chitin in yeast-cell walls and for N-acetyl-D-glucosamine, even though the over-all deacetylation rate of the last-named substance is ten times greater. A simple nomogram is proposed for determination of the time required for deacetylation in respect of the acid concentration and the temperature.

INTRODUCTION

The determination of chitin in biological samples is routinely accomplished by colorimetry after acid hydrolysis. The original procedure, developed by Elson and Morgan[1], has been frequently modified[2–7]. Ion-exchange chromatography has been recommended because of its higher accuracy[8,9] and the possibility of automation[10]. Hexosamines can also be determined on an amino acid analyser[11–14] and by gas–liquid chromatography (GLC) in the form of their trimethylsilyl derivatives[15–17]. Other methods that may be used include, *e.g.*, the deamination of amino sugars and subsequent assay of free sugars[18] or distillation of the chromophores[19,20]. In a previous study[21], we deacetylated chitin by using dilute HCl, with highly satisfactory results, the resulting acetic acid being determined directly by GLC. The procedure was designed for determination of the N-acetyl groups of glycopeptidoglycans[22]; in our

modification, it can be used to determine chitin in the cell walls of, *e.g.*, yeasts and moulds.

The acetic acid was quantitatively determined by GLC on Porapak Q coated with 3% of H_3PO_4 (ref. 23). This technique represents a useful application of porous polymers in analysis for lower fatty acids. According to many studies on the properties of porous polymers, Porapaks Q and N or Chromosorbs 101 and 102 are the most suitable for this purpose. The internal-standard technique, with propionic acid as marker, was used for quantitative determination. Our method was far more accurate and rapid than, *e.g.*, the colorimetric or the distillation technique[24], including procedures based on deacetylation in alkaline medium[25]. Another advantage was the minute amount of sample needed for an analysis; hence, a single sample could be used for the determination of a wide spectrum of sugars and amino acids.

Further, the widely applied colorimetric method has several disadvantages, such as thermal degradation of the sugars during hydrolysis (the samples become dark and later form insoluble residues, which result in lower readings) and the attendant impossibility of attaining 99% hydrolysis.

In this paper, the term hydrolysis indicates hydrolysis of chitin to glucosamine (which is determined by a colorimetric method), whereas the term deacetylation means splitting off the acetyl groups from N-acetyl-D-glucosamine (in mono-, oligo- or polymeric form); these acetyl groups are then determined, as acetic acid, by GLC.

Our method was developed in an attempt to determine chitin (or N-acetyl-D-glucosamine and its oligomers) in yeast-cell walls as the next step after their qualitative determination by X-ray diffraction in various insoluble fractions of cell walls[21]. Some yeasts contain chitin located in the entire cell wall, whereas others have it solely in bud scars, and some contain no chitin. *Endomyces magnusii* was chosen as a representative of yeasts containing chitin (about 11%) in the entire cell wall, and *Saccharomyces cerevisiae* as one containing chitin (about 1%) only in bud scars.

EXPERIMENTAL

Apparatus

A Packard gas chromatograph, Model 7731 (Packard, Downers Grove, Ill., U.S.A.) equipped with a flame ionisation detector and a coiled glass column (200 cm × 4 mm I.D.) was used. The column was packed with Porapak Q (50–80 mesh) (Waters Assoc., Milford, Mass., U.S.A.) and coated with 3% of H_3PO_4 (ref. 23). Nitrogen was used as carrier gas at a flow-rate of 40 cm^3 min^{-1}, and the column was operated isothermally at 170°, with inlet and detector temperatures of 210° and 230°, respectively. Samples were injected with a 10-μl Hamilton micro-syringe. The columns were re-packed after 150 determinations.

Colorimetric determinations were carried out with a Prema K.56 spectrophotometer (Presná mechanika, Stará Turá, Czechoslovakia).

Reagents

N-Acetyl-D-glucosamine, *p*-nitrobenzaldehyde and mannose were purchased from Fluka (Buchs, Switzerland), D-glucosamine from Calbiochem (Los Angeles, Calif., U.S.A.), Dextrans 80 and 110 from Pharmacia (Uppsala, Sweden) yeast mannan from Koch-Light (Colnbrook, Great Britain), and glucose, galactose, xylose,

cellulose, acetic, propionic and hydrochloric acids, tetraethylammonium hydroxide, sodium and potassium hydroxides, ethanol and pyridine from Lachema (Brno, Czechoslovakia). Pyridine was re-distilled before use.

Sulphonated insulin was a gift from Dr. Zikán of this Institute. Chitin from Koch-Light and BDH (Poole, Great Britain) was dissolved in concentrated hydrochloric acid and precipitated in ice–water mixture; the precipitate was filtered off and washed in a sintered-glass funnel. After being washed, the white precipitate was freeze-dried, ground and sifted through a sieve (mesh size 0.05 mm).

Organism-cultivation techniques and preparation of cell walls

Yeasts from the collection of the Institute of Microbiology of the Czechoslovak Academy of Sciences were used throughout. After batch cultivation and disintegration[27], the cell walls were washed as described by Mitchell and Taylor[28], and the resulting material was freeze-dried.

Glucan protein (GP) from the cell walls of *S. cerevisiae* was the insoluble residue after extraction of the walls with performic acid (12 h), 2 *M* hydroxylamine (12 h)[29] and 1 *M* KOH (1 h)[30].

Glucomannan protein (GMP) was a fraction obtained from the solution in 1 *M* KOH by dialysis and freeze-drying.

Soluble fractions were obtained from the solutions in performic acid and hydroxylamine by dialysis and freeze-drying.

Calibration curves

Portions (0.05, 0.1, 0.15, 0.25, 0.35, 0.5, 0.75 and 1 cm^3) of acetic acid were added to samples of propionic acid (0.75 cm^3 in 500 cm^3 of 2 *M* HCl), and 3-μl aliquots of the resulting mixtures were injected into the column. The peak area for each component was determined by multiplying the peak height by its width at half height, and the calibration graph was constructed by plotting the ratio of peak area for acetic acid to that of propionic acid against the known concentration of acetic acid injected. The concentration of acetic acid liberated by hydrolysis of chitin could be read immediately from the calibration graph. A linear detector response for acetic acid was obtained by establishing an absolute calibration curve over the sample volume range *ca.* 0.1–250 μl.

Procedure

For the acid hydrolysis (de-acetylation) of chitin, 25 mg of chitin (or N-acetyl-D-glucosamine) was allowed to react with 4 μl of propionic acid in 5 cm^3 of HCl (usually 2–4 *M*) for 15 min–24 h (96 h in one experiment) on a rotary shaker at 96–100° in a nitrogen atmosphere in a sealed tube. After hydrolysis, the tube was cooled in ice–KCl mixture and opened, and 3-μl samples were injected into the GLC column. The minimum concentration of acetic acid detectable was 0.05 $\mu g\ \mu l^{-1}$ for yeast-cell-wall chitin; the sensitivity was $3 \cdot 10^{-11}$ mA.

Alkaline deacetylation

A 25-mg portion of chitin (or N-acetyl-D-glucosamine) with 4 μl of propionic acid in 2.5 cm^3 0.75 *M* NaOH was heated in a sealed tube in a nitrogen atmosphere in a water bath at 60° and at 100° for 15 min to 2 h. The sample was then frozen in

ethanol–solid CO_2, the tube was opened, 2.5 cm^3 of 1 *M* HCl were added, and the mixture was cooled in the ice–KCl mixture; 3-μl samples were injected into the GLC column.

Acid hydrolysis of yeast-cell walls.

Cell walls (25 mg) of *E. magnusii* (or *S. cerevisiae* and its fractions) were allowed to react with 4 μl of propionic acid in 5 cm^3 4 *M* HCl for 90 min to 24 h on a rotary shaker at 98° in a nitrogen atmosphere in sealed tubes. The subsequent procedure was the same as with samples containing chitin in HCl.

Colorimetric method

After determination of acetic acid by GLC, the samples were evaporated, and the total glucosamine was estimated according to Gatt and Berman's modification[7] of the Elson–Morgan method[1] or by the simpler method of Nakamura *et al.*[31], which is based on the reaction of unacetylated hexosamine with *p*-nitrobenzaldehyde.

A 0.5-cm^3 volume of 1% (w/v) *p*-nitrobenzaldehyde solution in pyridine (freshly prepared) was added to 1 cm^3 of sample, and the mixture was shaken, set aside for 20 min at 27–28°, then cooled in ice for 5 min and diluted with 10 cm^3 of ice-cold 0.3% (v/v) tetraethylammonium hydroxide in ethanol; after 10–25 min, the absorbance was measured at 504 nm in 1-cm cuvettes.

Data processing

The data were processed by means of our library program[32] for non-linear regression. Minimisation was carried out by an optimum-seeking method in the direction of a random gradient. The method has the character of a global optimization[33]. Computation was done on an IBM 370/135 computer; the central processor unit time for a typical run did not exceed 10 sec.

RESULTS AND DISCUSSION

The data for the calibration curve are presented in Table I. The results for several conditions (2, 3 and 4 *M* HCl at 96° or 98°) of deacetylation and hydrolysis of

TABLE I

ACCURACY OF GLC DETERMINATION OF DIFFERENT CONCENTRATIONS OF ACETIC ACID WITH PROPIONIC ACID AS INTERNAL STANDARD

Weight ratio of acetic to propionic acid	*Peak-area ratio of acetic acid to propionic acid*		
	$\bar{x}$ ($n = 7$)	$\sigma_{abs.}$	$\sigma_{rel.}$ (%)
0.065	0.06	0.0014	2.33
0.14	0.1	0.0014	1.4
0.213	0.16	0.0031	1.9
0.355	0.24	0.00141	0.58
0.497	0.35	0.0057	1.62
0.723	0.505	0.0076	1.50
1.063	0.700	0.0061	0.87
1.41	0.975	0.007	0.78

chitin and N-acetyl-D-glucosamine are shown in Fig. 1. It is evident that chitin can be more easily deacetylated than hydrolysed, and, for this reason, the distinction between the two terms should be precise. Further, the low yields obtained by hydrolysis in 2 *M* HCl could be increased only by increasing the HCl concentration and the temperature. On the other hand, a higher molarity of HCl as well as a longer hydrolysis results in more complete destruction of carbohydrate. The destruction of released D-glucosamine was measured by a colorimetric method and was observable after 10 h in 4 *M* HCl both at 96° and 98°.

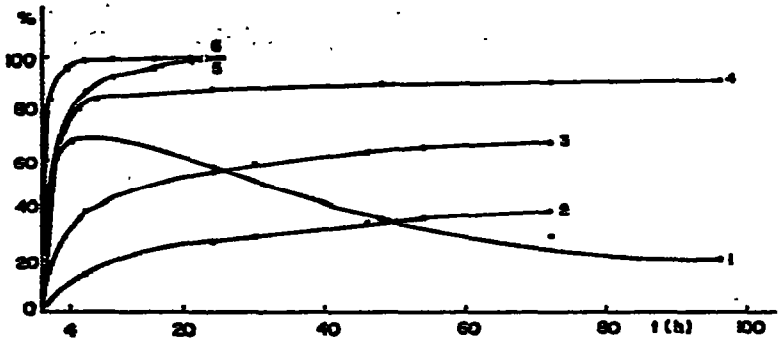

Fig. 1. Deacetylation of crustacean chitin and N-acetyl-D-glucosamine in HCl. Curve 1, hydrolysis of chitin in 4 *M* HCl at 96°, with spectroscopic determination of glucosamine[32]. Curve 2, as for curve 1, but in 2 *M* HCl. Curve 3, deacetylation of chitin in 2 *M* HCl at 96°; determination of acetic acid as in this paper. Curve 4, deacetylation of chitin in 4 *M* HCl at 96°. Curve 5, as for curve 4, but at 98°. Curve 6, deacetylation of N-acetyl-D-glucosamine in 4 *M* HCl at 98°.

The 4 *M* HCl did not damage the syringe, the Porapak Q or the detector. We tried to reduce the concentration of HCl by neutralisation with NaOH. The results were comparable with those without this procedure, which, in addition, had several disadvantages, *viz.*, additional operations and pollution of the column by sodium salts.

Kinetics of acid deacetylation of chitin

Chitin hydrolysis should follow first-order kinetics (with respect to chitin) if all glycosidic bonds are equally accesible to the acid. Fourth- to fifth-order kinetics should be followed with regard to the acid. These results can be explained in two ways. The first hypothesis is based on the assumption that all interior bonds have the same reactivity (which, however, differs from that of terminal bonds with different susceptibility to hydrolysis). The second hypothesis suggests that the high order of reaction with respect to acid is due to the physical character of the chitin chains. Chitin, as a semicrystalline structure, forms aggregates less accessible to hydrolysis, whereas outer superficial aggregates composed of several hydrogen-bond-interacting chains may exist in solution. Considering the long half-period of the reaction, the significant decrease in the over-all rate of hydrolysis by diffusion can be ignored.

Power kinetics are suitable for expressing the reaction rate. The liberated acetyl groups can serve as a better indicator of chitin hydrolysis than does the D-glucosamine (Fig. 1). The reaction rate can be expressed in the form:

$$r = k[H^+]^{n_1} \cdot (1 - y)^{n_2} \qquad (1)$$

where k is a rate constant dependent on temperature according to the Arrhenius relation:

$$k = A \cdot \exp\left(-\frac{E}{RT}\right) \tag{2}$$

and y is the fraction of liberated acetyl groups related to the total content of acetyl groups in the sample, H^+ is the molarity of hydrogen ions, n_1 is the order of reaction with respect to the concentration of hydrogen ions and n_2 is the order of reaction with respect to the fraction of non-deacetylated chitin. The criterion of the least squares of residuals was used in fitting the experimental data to the integrated rate equation (eqn. 3) to provide an explicit evaluation of the degree of liberation of acetyl groups:

$$y = 1 - [(k \cdot [H^+]^{n_1} \cdot t)(n_2 - 1) + 1]^{1/(1-n_2)} \tag{3}$$

where t is time of hydrolysis and n_1 and n_2 have the same meaning as in eqn. 1.

The resulting values were: $A = 8.45 \cdot 10^8$; $E/R = 10^4$; $n_1 = 4.32$; and $n_2 = 1.97$.

The activation energy, E, and the order of reaction with regard to the acid are in keeping with the data of Rupley[34]. The high orders of reaction lead to the conclusion that the limiting step of the reaction is the splitting of the outer bonds between the N-acetyl groups and the chitin chains, which is slower than the over-all rate of physical transfer between the fluid and the surface of the chitin particles.

Effect of temperature

As the activation energy was found to be fairly high, an estimation of the temperature effect on the essential time of chitin hydrolysis could easily be performed by inserting the above data into eqn. 3.

Effect of acid concentration

The effect of acid concentration is considerable, as is seen from the high order of reaction (4.32). The time needed for a fixed degree of hydrolysis of chitin can be calculated from eqn. 3.

Effect of agitation

Absence of agitation causes a fall of *ca.* 40% in the reaction rate. Hydrolysis proceeds on partially released aggregates that pass into solution during agitation; in the absence of agitation, the same aggregates block the non-hydrolysed surface of chitin. The effect of agitation must therefore be taken into account.

Effect of quantity

The effect of the amount of chitin is insignificant in the range of 5–50 mg per 5 cm^3 of 4 *M* HCl at 100°.

Alkaline hydrolysis of chitin and N-acetyl-D-glucosamine

Partial hydrolysis in acids and then in alkalis was used for the isolation of insoluble chitin residues in, *e.g.*, bud scars[26,35]. Therefore, it is useful to know the extent of deacetylation of chitin or N-acetyl-D-glucosamine for the purpose of quantitative determination.

Alkaline hydrolysis of chitin and N-acetyl-D-glucosamine was carried out at 60° and 100° in 0.75 *M* NaOH. The course of deacetylation is shown in Table II. Thorough study was performed by Sannan *et al.*[36], according to whom $E/R = -1.05 \cdot 10^4$, $n_1 = 2$ and $n_2 = 1$; from these data, $A = 3.28 \cdot 10^{12}$. The kinetics of deacetylation of N-acetyl-D-glucosamine were pseudo-first order, and the authors showed that the reaction could be second order with regard to OH^-.

TABLE II

DEACETYLATION OF CRUSTACEAN CHITIN AND N-ACETYL-D-GLUCOSAMINE IN 0.75 *M* NaOH

Values are percentages of deacetylation.

Compound	*Temperature (°C)*	*Reaction time*				
		0.25	*0.5*	*1*	*2*	*3*
N-Acetyl-D-glucosamine	100	28.5	31.2	34.5	44.7	
	60	10.3	13.5	19.6	29.8	
Chitin	100	3.4	4.7	6.8	9.8	
	60	0.7	1.1	2.0	2.1	3.4

Our results differ from those of Sannan *et al.* in that the conversion rates are much lower. This may be due to the fact that Sannan *et al.* used solubilised chitin which yields a three-fold higher conversion in comparison with our particulate chitin; therefore, their data for chitin conversion almost correspond to ours for N-acetyl-D-glucosamine.

Although the rate of chitin deacetylation in alkaline medium is higher than that in acid, use of alkali has two main disadvantages: the need for subsequent acidification, and greater contamination of the GLC column by sodium salts.

Acid hydrolysis of N-acetyl-D-glucosamine

Acid hydrolysis was performed at 98° and 96° in 3 *M* and 4 *M* HCl; power kinetics were used for evaluation. The order of reaction with regard to the acid ($n_1 = 4.32$), and the activation energy ($E/R = 10^4$), were assumed to be the same as with chitin, yielding $A = 9.20 \cdot 10^9$ and $n_2 = 1.91$. The mechanism of deacetylation was essentially the same as with chitin, since the order of reaction, n_2, was almost identical; however, the rate of reaction was 10 times higher than with chitin, which might be attributed to easier accessibility of the bonds to the deacetylating agent in solution.

The orders of deacetylation of chitin and N-acetyl-D-glucosamine were almost identical, but the Arrhenius factor was higher with N-acetyl-D-glucosamine. Although the dependence of the rate of chitin deacetylation on the size of the chitin particles was not studied, we can assume that the reaction rate increases as the size of the chitin particles decreases, until the value for the monomer unit, *i.e.*, N-acetyl-D-glucosamine is reached.

Acid hydrolysis of yeast cell-wall chitin

The acid hydrolysis was performed at 98° in 4 *M* HCl. The order of reaction and the activation energy were assumed to be the same as with chitin in order that

the over-all rates of reaction could be compared; $A = 8.47 \cdot 10^8$ was found for chitin in *E. magnusii*. The rate of reaction was almost identical with that of the deacetylation of crustacean chitin.

The cell wall of *S. cerevisiae* consists of an alkali-insoluble part (GP), which contains chitin, a water-insoluble part (GMP) and a water-soluble part; the last two fractions contain oligomers of N-acetyl-D-glucosamine. Fractionation by partial hydrolysis is performed to yield a fraction with a high content of chitin.

The over-all kinetics of hydrolysis of entire cell walls therefore reflect the kinetics of hydrolysing the individual cell-wall components. The value of $A = 6.72 \cdot 10^8$ for GP is slightly lower than that for both crustacean chitin and the chitin of *E. magnusii*. The chitin-containing structures (bud scars) have thus first to be unmasked by hydrolysis of the surrounding material.

The value of $A = 1.36 \cdot 10^9$ was found for GMP. The Arrhenius factor was 1.62 times higher than that for chitin, but 87 times lower than that for N-acetyl-D-glucosamine. This may be due to the fact that, in acids, GMP forms flakes that might lead to a spatial conformation less accessible to deacetylation or to a protective effect exerted by the protein moiety on the sugar moiety, so that the extent of deacetylation of GMP will be lower than that of the monomer unit (N-acetyl-D-glucosamine). The rate of destruction of such ternary structures then nearly approaches the rate of hydrolysis of chitin.

A value of $A = 6.45 \cdot 10^8$ was found for the entire cell wall. It was lower than that for crustacean chitin, for the chitin of *E. magnusii* and for GP.

The determination of chitin in yeast-cell walls and N-acetyl-D-glucosamine in their fractions requires reaction conditions almost identical to those used with chitin. For partial hydrolysis of yeast-cell walls[26,35], we recommend treatment with 1 *M* KOH for 1 h at room temperature[30]; neither chitin nor N-acetyl-D-glucosamine is deacetylated under these conditions. Mild hydrolysis with acetate buffer[35] was unsuitable because of the presence of acetic acid, which could distort the results.

It appears that the distribution of N-acetyl-D-glucosamine units in individual fractions of the cell wall of *S. cerevisiae* is proportional to the composition of the cell walls (Table III).

TABLE III

CONTENT OF ACETYL GROUPS IN CELL-WALL FRACTIONS OF BAKERS' YEAST

Component	*Content (%)*	*Acetyl-group content (%)*
Cell wall	100	0.730
GP	23.33	0.512
GMP	24.76	0.096
Soluble fractions	47.16	0.133
Loss	4.75	0.009

The nomogram for estimation of the time required for 95% deacetylation of chitin in relation to temperature and acid concentration is presented in Fig. 2; the curves were calculated on the basis of the kinetics of chitin deacetylation. It is more convenient to use concentrations of acid higher than 4 *M* as recommended by Rupley[34]; a rise of 5° in the reaction temperature roughly corresponds to a lowering of the acid concentration by *ca.* 0.5 *M*, and *vice versa*.

Fig. 2. Nomogram for estimation of time for 95% deacetylation of chitin in relation to temperature and acid contration.

After a longer time, the samples darkened. To ascertain whether or not additional reactions leading to acetic acid were involved, various monosaccharides (glucose, mannose, galactose, xylose and arabinose), polysaccharides (cellulose, dextran, mannan and starch), amino acids (glycine and aspartic acid) and insulin (as a model protein), were investigated as substrates for possible thermal hydrolytic reactions. Glycine was not deaminated to acetic acid under our conditions of hydrolysis (4 *M* HCl; 98°; 15–24 h), nor was acetic acid liberated from insulin or the cited sugars or polysaccharides or from apyrogenic glucose. Thus, formation of acetic acid during thermal degradation of sugars seems to occur most readily in a dry state at higher temperatures[37–39].

Our method can be successfully used not only for the determination of chitin and N-acetyl-D-glucosamine in biological samples, but also in studying the deacetylation of amino acids[40], and for determination of the chitin–chitosan ratio in moulds and yeasts[41].

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