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Note

A convenient assay for chitinase that uses partially *N*-acetylated chitosans as substrates

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Chitinase activity has been assayed variously by viscosimetry, turbidimetry, and colorimetry, using colloidal chitin and glycol chitin as substrates[1,2]. The modified Schales colorimetric method[2] is convenient, but it is necessary to remove residual colloidal chitin by centrifugation before the absorbance is measured. Glycol chitin is useful for the colorimetric and viscosimetric assays because it does not precipitate at any pH. Solutions of partially *N*-acetylated chitosans (PNAC), prepared by *N*-acetylation of highly deacetylated chitosans (HDC) under homogeneous conditions, in aqueous acetic acid gave neither precipitates nor gels on the addition of excess of alkali, in contrast to chitosan[3,4]. The use of PNAC as substrates for the assay of chitinase is now reported.

PNACs were prepared as described by Hirano et al.[5] and their characteristics are listed in Table I. Chitinase activity was assayed easily by the modified Schales method[2] during which the PNAC did not precipitate. In contrast, reaction mixtures containing moderately deacetylated chitosans (MDC: K-13, K-21, and K-30 in Table II), obtained by *N*-deacetylation of chitin under heterogeneous conditions, precipitated during the period of color development, and centrifugation was necessary. Table II summarises the amounts of reducing sugars produced by the chitinase, as expressed by the decrease of absorbance (D_{415}). The absorbances of the unincubated solutions were in the range 0.886–0.939. D_{415} was proportional to the amount (M in mg) of 2-acetamido-2-deoxy-D-glucose as the standard ($M = 0.146D_{415}$). In the pH range 5.4–8.2, PNACs were qualitatively more digestible than glycol chitin, and the D_{415} values were 4–6 times greater. It was possible to measure the amounts of reducing sugars at low concentrations of PNAC with small amounts of the chitinase. With 0.01% PNAC (4C-5-56) and 1 μg of chitinase, D_{415} was 0.168 and 0.299 at pH 5.4 for reaction times of 0.5 and 1.0 h, respectively.

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TABLE I

Characterisation of chitosans

Sample	M_v [a] ($\times 10^6$)	M_w [b] ($\times 10^6$)	Degree of *N*-acetylation [c] (%)
Moderately deacetylated chitosans (MDC)			
K-13	1.5	1.2	13
K-21	2.1	2.1	21
K-30	1.8	1.8	30
Highly deacetylated chitosans (HDC)			
2C-5	1.2	0.83	5
4C-5	0.98	0.63	5
N-*Acetylated chitosans (PNAC) prepared from HDC*			
2C-5-49		0.84	49
4C-5-56		0.81	56

[a] Viscosity-average molecular weight[6]. [b] Weight-average molecular weight determined by gel-permeation chromatography. [c] Averaged values[3] of data obtained by IR spectroscopy, colloid titration, gel-permeation chromatography, and elemental analysis.

The data in Table II indicate that the use of PNAC as a substrate provides a highly sensitive assay of chitinase. However, the digestibility of PNAC and glycol chitin cannot be compared directly because the enzyme reactions were not linear with time. The initial rates of the enzyme reactions, calculated by the method of Algranati[7] and summarised in Table III, are expressed as μmol of 2-acetamido-2-deoxy-D-glucose released/min/mg of enzyme preparation. PNAC was ~5 times more digestible than glycol chitin. The digestibility of MDC was proportional to the degree of *N*-acetylation. The chitinase from *Streptomyces griseus* specifically

TABLE II

Effect of pH on the amounts of reducing sugars produced in the reaction mixture [a]

Sample [b]	Reaction time (h)	pH			
		5.4	6.3	7.4	8.2
Glycol chitin	0.5	0.055	0.074	0.060	0.063
	1.0		0.091	0.122	0.088
2C-5-49	0.5	0.315	0.277	0.393	0.382
	1.0	0.540	0.464	0.651	0.624
4C-5-56	0.5	0.331	0.310	0.381	0.375
	1.0	0.521	0.486	0.629	0.630
K-13	0.5	0.105			
K-21	0.5	0.154			
K-30	0.5	0.220			

[a] Expressed by the decrease of absorbance at 415 nm. The absorbance of unincubated solutions ranged from 0.886 to 0.939. The standard deviation was < 0.02. [b] See Table I.

TABLE III

Effect of pH on chitinase activity [a]

Sample [b]	pH			
	5.4	6.3	7.4	8.2
Glycol chitin		2.7	1.5	2.1
2C-5-49	9.5	8.0	11.4	11.6
4C-5-56	10.8	10.2	12.2	11.8
K-13	3.9			
K-21	4.7			
K-30	6.5			

[a] Expressed by μmol of 2-acetamido-2-deoxy-D-glucose released/min/mg of enzyme preparation. [b] See Table I.

cleaves[8] the 2-acetamido-2-deoxy-D-glucosidic linkages in MDC. The chitinase activity toward PNAC was relatively low at pH 6.3, possibly due to the low ionic strength of the 0.05 M citrate buffer used. The amino groups of PNAC are half protonated even at pH 6.3.

Ohtakara et al.[9] also showed that MDC (20–45% *N*-acetylation) were hydrolysed by microbial chitinases and suggested their use for the assay of chitinase activity. Although the MDC were soluble in aqueous acid, they were not soluble in alkaline media, in contrast to PNAC. The optimum pH of some chitinases is in the alkaline region[10,11].

Hirano et al.[12] showed that PNACs with $> 40\%$ acetyl content were more digestible with chitinase than chitosan and chitin, but the reaction mixtures were heterogeneous. The low activity of the chitinase from *Streptomyces griseus* (0.75 U/mg) was measured at 37° and the highest value was ~ 0.015 mg of 2-acetamido-2-deoxy-D-glucose released/min/mg of enzyme preparation, in contrast to the highest value of 2.7 in our experiments.

The data in Tables II and III indicate that PNACs are suitable for the assay of chitinase activity over a wide range of pH values. The solubility and sensitivity of PNACs are higher than those of colloidal chitin, glycol chitin, and MDC.

EXPERIMENTAL

Materials. — Chitinase [EC 3.2.1.14] from *Streptomyces griseus* (1.12 U/mg, Sigma) and glycol chitin (Seikagaku Kogyo) were commercial products and were used without further purification. Chitosan samples, obtained from the Katakura Chikkarin Co., were used to prepare highly deacetylated chitosans (HDC) and moderately deacetylated chitosans (MDC). The chitosan samples were heated at 110° in aq 47% alkali for 1 h under a N_2 atmosphere to afford HDC. In the case of MDC, chitosan solutions in aq 2% acetic acid were centrifuged, the supernatant solutions were poured dropwise into aq 4% alkali, and the precipitates were thoroughly washed with water and dried. Partially *N*-acetylated chitosans (PNAC)

were prepared from HDC by the method of Hirano et al.[5]. The molecular weights and the degrees of *N*-acetylation are listed in Table I.

Assay of chitinase activity. — Each chitosan derivative was dissolved in 0.24 M acetic acid, 0.066 M citric acid, 0.057 M HCl, or 0.033 M HCl, and M NaOH or 0.2 M Tris was added to adjust the pH to the desired value. A mixture of solutions of each substrate (0.05%, 1 mL) and chitinase (1 μg in 0.5 mL buffer) was shaken at 37° for 0.5 or 1.0 h. The reducing sugar produced was determined by the modified Schales method[2]. The absorbance at 415 nm was measured with a Corona Electric MTP-22 microplate photometer. The buffer solutions were as follows: pH 5.4, 0.2 M acetate; pH 6.3, 0.05 M citrate; pH 7.4 and 8.2, 0.05 M Tris.

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