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A new spectrophotometric method of assay for chitosanase based on Calcofluor white dye binding

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A new spectrophotometric method for the assay of chitosanase based on complex formation of the substrate chitosan with Calcofluor white dye is described. The absorption maximum for the chitosan–Calcofluor complex is determined to be 406 nm. The apparent minimum size of chitosan for complex formation is 5–7 kDa. Therefore, those enzymes that do not generate glucosamine or reducing groups as products of hydrolysis at levels not measurable by the available methods of assay can be assayed by the present method. In the standardized procedure 200 μg of chitosan in acetate buffer pH 4.5 with the enzyme in a reaction volume of 1.5 ml is incubated at 45°C for 1 h, after which 1.5 ml of Calcofluor white (0.05%) is added, kept for 1 h and absorbance at 406 nm measured by a spectrophotometer. The chitosanase unit is arbitrarily defined as the reduction in absorbance by 0.01/min.

INTRODUCTION

Chitosanases (EC 3.2.1.99) that catalyse hydrolytic depolymerization of chitosan are found widely distributed among microorganisms and also in some plants (Monaghan *et al.*, 1973; Somashekar and Joseph, 1996). The assay of chitosanase is based on the measurement of the glucosamine (Rondle and Morgan, 1955) or reducing groups (Nelson, 1944; Dygert *et al.*, 1965; Imoto and Yagishita, 1971) generated by the hydrolysis of chitosan. In the present work, we have encountered a relatively rare chitosan hydrolysing enzyme produced by a strain of *Rhodotorula gracilis* (Somashekar and Joseph, 1992) which does not generate measurable amounts of either glucosamine or reducing groups which can be quantified by the existing methods. We therefore tested the possibility of using the Calcoflour dye binding technique which has been adopted in the qualitative identification of the presence of chitosanases in gel electrophoresis by Trudel and Asselin (1989). In this approach we have been able to standardize a new spectrophotometric technique using the ability of the dye to complex with unhydrolysed chitosan leading to a characteristic absorption spectrum. The usefulness of this method for assay of the *Rhodotorula gracilis* chitosanase is demonstrated.

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MATERIALS AND METHODS

Shrimp chitosan was procured from CFTRI Regional Centre, Mangalore, India (its preparation and properties have been described by Moorjani *et al.* (1975) and Rao *et al.* (1987)). As per the specification the chitosan had 25% acetylated amino groups. Calcofluor white M2R was from Sigma Co., USA.

Chitosanase was obtained as extracellular enzyme by growing the locally isolated *Rhodotorula gracilis* CFR-1 (ATCC No:90950) in a minimal salts medium containing KH_2PO_4 6.0, Na_2HPO_4 3.0, $CaCl_2$ $2H_2O$ 0.04, $MgSO_4$ $7H_2O$ 0.02, peptone 10, glucose 20 g/litre and calcium pantothenate 50; thiamine 20 μg/litre, pH 6.0. The culture was grown for 48 h in shake flasks at 30°C, after which the cells were pelleted by centrifugation and discarded. The supernatant formed the crude enzyme preparation.

For assay of chitosanase the following procedure was standardized. Chitosan was solubilized in 1% acetic acid and the pH was adjusted to 4.5 with sodium acetate buffer 0.5 M, pH 5.0. The final concentration of chitosan was adjusted to 0.5 mg/ml. The reaction mixture (1.5 ml) containing 0.4 ml of chitosan solution, 0.6 ml of sodium acetate buffer 0.05 M, pH 4.5 and 0.5 ml of enzyme was incubated for 1 h at 45°C. To the control tubes, either heat-killed enzyme was added prior to incubation or enzyme was added after

incubation of the reaction mixture. The reaction was stopped by inserting the test tubes containing the reaction mixture in a boiling water bath for 10 min. After cooling the tubes in cold water, 1.5 ml of Calcofluor white (0.01% in distilled water) was added and allowed to stand at ambient temperature for 1 h. The absorbance was measured at 406 nm. One unit of enzyme is arbitrarily defined as the reduction in optical density (OD) by 0.01/min.

To determine the minimum size of chitosan for complex formation with Calcofluor, 100 mg of chitosan was hydrolysed in 10 ml of 1 to 6 N HCl for 0.5 to 6 h and the samples were spotted on Whatman No. 1 chromatography paper. The paper electrophoresis was carried out according to the method of Tharanathan *et al.* (1993). The paper was sprayed with 0.25% ninhydrin in acetone. Based on this data, it was found that the chitosan that was hydrolysed with 1 N HCl for 1 h gave an appropriate profile of very large to very small oligosaccharides (data not shown). This was subjected to Biogel P-100 (100–200) fractionation. The sample was eluted with sodium acetate buffer pH 4.5, 0.05 M, containing 0.02 M NaCl. The fractions were analysed for total sugars based on the method of Updegraff (1969) and were also subjected to Calcofluor white binding as described above. The relative molecular mass of the chitosan that does not form a complex with Calcofluor white was determined in the above-mentioned gel with dextrans of molecular mass 10, 40 and 70 kDa (Pharmacia) as standards (Fig. 4).

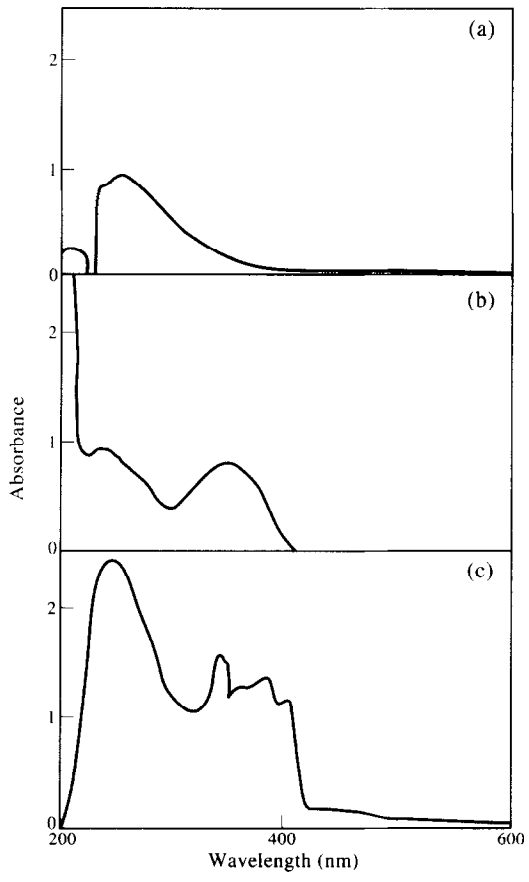

Fig. 1. Absorption spectrum of chitosan (A), Calcofluor white (B) and the chitosan–Calcofluor white complex (C).

RESULTS AND DISCUSSION

The absorption spectra of chitosan in sodium acetate buffer 0.05 M, pH 4.5 (Fig. 1(A)) and aqueous Calcofluor white (Fig. 1(B)) spanning wavelengths 200–600 nm were obtained. It was found that both the compounds do not show any absorbance in the visible range (400–600 nm). On the other hand the chitosan–Calcofluor white complex was found to have an entirely new absorption spectrum quite different from that of either Calcofluor white or chitosan. The spectrum of the complex spanned both the visible and the ultraviolet regions of the electromagnetic range. There was distinctly a significant level of absorption at 406 nm (Fig. 1(C)). This wavelength was thus chosen for quantification of unhydrolysed chitosan. The reduction in absorbance at 406 nm of the chitosan–Calcofluor white complex after predigestion of chitosan with enzyme was taken as a measure of enzyme activity. It was convenient to arbitrarily define an enzyme unit in terms of reduction in absorbance by 0.01 OD. A minimum of 60 min duration was found to be necessary for completion of the formation of the chitosan–Calcofluor white complex under the conditions of the experiment (Fig. 2).

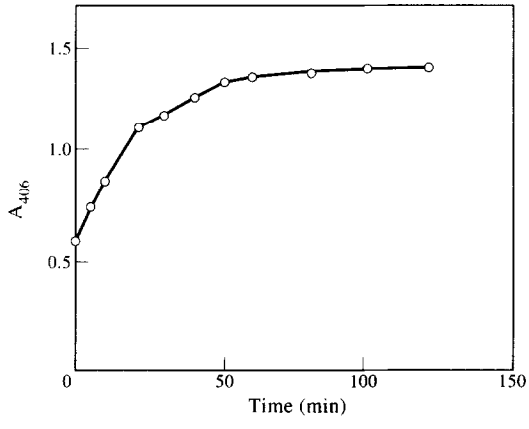

Fig. 2. Influence of incubation period on Calcofluor white and chitosan interaction (at ambient temperature 26±2°C).

In the experiment performed to determine the minimum size of chitosan for complex formation we found that this has to be larger than 5–7 kDa (Figs 3 and 4). In the sample of chitosan subjected to partial acid hydrolysis and fractionated on Biogel P-100, the 5 kDa low molecular weight chitosan containing fraction failed to form a complex with Calcofluor

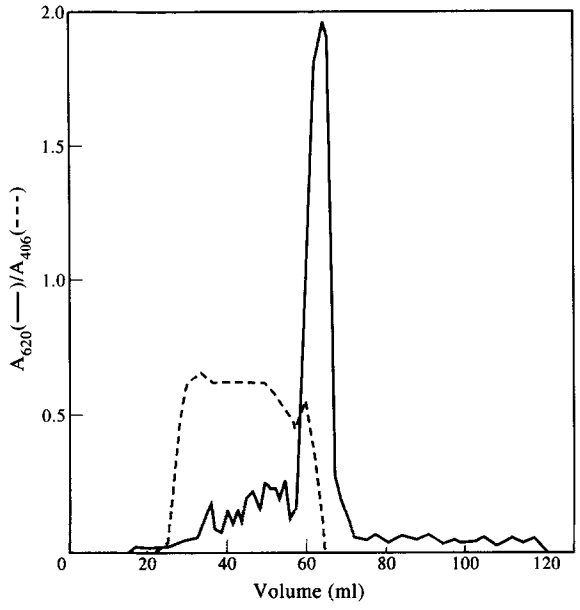

Fig. 3. Elution profile of partially acid-hydrolysed chitosan in Biogel P-100 showing total sugar content (———) as anthrone reaction at 620 nm and Calcofluor white complexing fractions (— — —) at 406 nm.

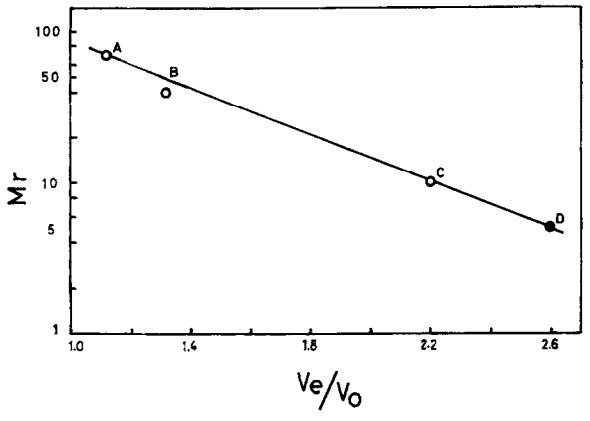

Fig. 4. Calibration curve for the molecular mass determination on the Biogel P-100 column. A, B, and C dextrans of molecular weight 70, 40 and 10 kDa, respectively. The D chitosan fraction does not form a complex with Calcofluor white.

white. The fraction that had chitosan in the range of 5–7 kDa formed complexes partially and not in proportion to the total carbohydrate content. This was possibly due to the presence of a few large oligomers in the total oligomer population of these fractions. Fractions that had oligomers larger than 7 kDa formed complexes, as indicated by spectrophotometric reading, proportional to the total carbohydrate content. The precise size of the partially acid-hydrolysed chitosan was, however, difficult to ascertain as we have employed standard dextrans for calibration and it is known that dextrans are globular molecules while chitosan is linear. Nevertheless, the procedure provides an idea of the relative behaviour of unhydrolysed and hydrolysed chitosan for formation of complexes with the dye. The procedure is somewhat similar to the assay of α-amylase by the iodine reaction (Smith and Roe, 1949).

The concentration of chitosan as substrate for enzyme was standardized to be 200 μg in the 3 ml reaction mixture. The time course of the enzyme reaction is given in Fig. 5. As the crude enzyme preparation used in the assay of chitosanase contains traces of media components, their interference with the chitosan–Calcofluor white complex formation was tested. Of the different media components only peptone and yeast extract were found to reduce the reading at 406 nm by about 30% over the control (Table 1). However, the concentrations of these media components are bound to be much less after the culture has grown. This problem can also be significantly reduced in dialysed or partially purified chitosanase preparations (data not shown). Even otherwise the enzyme control samples will have the same interference that would be compensated in the test.

Maeda and Ishida (1967) found the affinity of Calcofluor white M2R for chitin, on the basis of which some authors have employed this dye for detection of chitosanolytic enzymes in polyacrylamide gel electrophoresis incorporating the substrate to the gel matrix (Trudel and Asselin, 1989; Grenier *et al.*, 1991). But this method has not so far been used for quantification of unhydrolysed chitosan which in turn can be used for assay of chitosanase. The present method will be useful where the levels of glucosamine or its small oligomers released are too low for measurement by conventional methods.

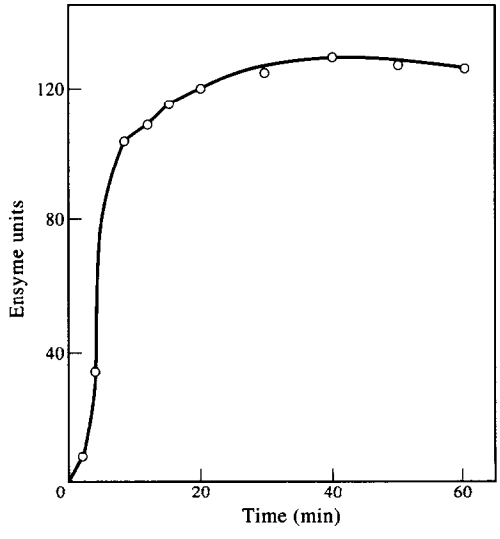

Fig. 5. The time course of enzyme reaction.

Table 1. Influence of the presence of media components on the chitosan–Calcoluor white interaction

Addition[a]	Concentration (mg)	Absorbance at 406 nm	Relative absorbance
None (control)	—	0.820	100.0
Peptone	10.0	0.641	78.0
Yeast extract	5.0	0.614	74.8
Glucose	10.0	0.817	99.6
Starch	0.5	0.848	103.4
KH_2PO_4	3.0	0.794	96.8
Na_2HPO_4	1.5	0.860	104.9
$CaCl_2$	0.5	0.819	99.9
$MgSO_4$	0.5	0.881	107.4

[a] The reaction mixture contains chitosan (200 μg) and other compounds added separately in a total volume of 1.5 ml sodium acetate buffer, 0.05 M, pH 4.5.

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