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A dye-labelled soluble substrate for the assay of endo-chitosanase activity

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ABSTRACT

Coupling Remazol Brilliant Blue R (RBB) to chitosan that has been subjected to partial enzymatic hydrolysis gave rise to a new dyed derivative of chitosan which is highly soluble in slightly acidic buffers or microbiological media. The RBB:chitosan ratio has been carefully optimized, as well as the reaction conditions, to obtain a quasi-linear, sensitive assay for chitosanase. The substrate (sRBB-C) is useful for microtitre plate-based screening and for assay of chitosanase activity in the presence of high backgrounds of reducing sugars. Due to its solubility, sRBB-C is particularly suitable for the detection of chitosanase-positive organisms on agar media.

1. Introduction

Dye-coupled polysaccharides are universally used for the detection or assay of glycoside hydrolases of endo-type such as 1,4-β-xylanases, 1,4-α-amylases or 1,4-β-glucanases (Bailey, Biely, & Poutanen, 1992; Biely, Mislovicová, & Toman, 1985; Farkaš, Lišková, & Biely, 1985; Kluepfel, 1988). Most of these substrates consist of native polysaccharides or their derivatives (such as hydroxyethyl cellulose) coupled to pigments used in textile technology such as Remazol Brilliant Blue R (RBB) or Ostazin Brilliant Red H-3B (OBR). The Remazol series of dyes was shown to be coupled to cellulose through a covalent bond (Bohnert & Weingarten, 1959). For enzymes participating in the hydrolysis of polymeric aminosaccharides, such as lysozymes or chitinases, similar substrates were proposed (Gómez Ramírez, Rojas Avelizapa, Rojas Avelizapa, & Cruz Camarillo, 2004; Wirth & Wolf, 1990). The protocols for enzyme assay using such substrates differ according to the solubility of the dye-coupled polysaccharide. For insoluble or colloidal substrates used as a suspension, the enzyme activity is determined by measuring the soluble chains liberated from the substrate after their separation by centrifugation or filtration. For soluble substrates, the assay involves a precipitation step with a concentrated salt or organic solvent aiming to separate the unhydrolyzed chains from the shorter fragments produced as the result of enzyme activity.

These assays are generally more sensitive when used with soluble substrates. However, some polysaccharides, such as cellulose or chitin, must be chemically modified to increase their solubility by the addition of substituents such as hydroxyethyl or carboxymethyl groups (Biely et al., 1985; Wirth & Wolf, 1990). Such modifications could result in a decreased sensitivity against specific enzymes, modifying or restricting the spectrum of detected enzymes (Burzyński, Piślewska, & Wojtaszek, 2000; Martins, Kolling, Camassola, Dillon, & Ramos, 2008; Zhang, Barr, & Wilson, 2000).

Chitosan, a partly or totally *N*-deacetylated derivative of chitin has numerous potential applications in biotechnology (Harish Prashanth & Tharanathan, 2007; Rinaudo, 2006). For many applications, moderate or low molecular weight chitosans are preferred over the native polymer (Blanchard, Park, Boucher, & Brzezinski, 2003; Harish Prashanth & Tharanathan, 2007). While chitosan hydrolysis can be easily achieved using inorganic acids (Allan & Peyron, 1995; Roberts, 1992), enzymatic methods are preferable as being environmentally friendly yet allowing a subtle control over the extent of hydrolysis. Enzymatic hydrolysis is also the method of choice for the preparation of chitosan oligosaccharides. Thus, there is a growing interest in chitosanases as tools for the study and the preparation of chitosans of varying chain length. The known endo-chitosanases belong to six families of glycoside hydrolases, GH5, GH7, GH8, GH46, GH75 and GH80 and more than a 100 chitosanase gene sequences are reported in databases (Cantarel et al., 2009). However, the use of chitosanases at an industrial scale remains limited so far, indicating the need for the identification of new, more efficient and/or stable enzymes.

In this context, the dye-labelled Remazol Brilliant Blue substrates are particularly convenient for screening purposes, as the assay procedures involving such substrates can be easily scaled-down to microtitre plates (Wirth & Wolf, 1990). Recently,

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a Remazol Brilliant Blue–chitosan substrate has been described (Fen, Illias, Kamaruddin, Maskat, & Hassan, 2006). However, this substrate is used as a suspension (both in its powdered or colloidal forms) which precludes its use in microtitre-style screening. Here, we describe a soluble-dyed chitosan (sRBB-C) which has not been chemically modified in anyway except coupling to RBB. The soluble substrate, very simple to use, displays an increased sensitivity and allows chitosanase assays in the presence of high backgrounds of reducing sugars.

2. Materials and methods

2.1. Reagents

Chitosan (8% *N*-acetylated, food grade) was kindly provided by Diversified Natural Products Canada (Granby, Québec, Canada). Endo-chitosanase from *Streptomyces* sp. N174 has been purified from recombinant *Streptomyces lividans* TK24 (pRL270) by ion-exchange and hydroxylapatite chromatography as described previously (Boucher, Dupuy, Vidal, Neugebauer, & Brzezinski, 1992; Dennhart, Fukamizo, Brzezinski, Lacombe-Harvey, & Letzel, 2008). All the other chemicals were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Preparation of soluble RBB-chitosan (sRBB-C)

2.2.1. Partial hydrolysis of chitosan

Chitosan was dissolved in 0.4 M acetic acid at a final concentration of 5 g/L. The viscous solution was heated to 50 °C and hydrolysis was initiated by the addition of 0.6 U/L of N174 chitosanase (Boucher, Dupuy, Vidal, Neugebauer, & Brzezinski, 1992) while stirring. Incubation was conducted until the weight average molecular weight (M_w) of the polymer (estimated by the triple detector array method, Viscotek, Houston, TX) reached an approximate value of 30 kDa. Hydrolysis was terminated and the polymer recovered by salting out with trisodium citrate (3:1 salt:solute ratio) at 50 °C for 45 min (Dupuis & LeHoux, 2007) followed by centrifugation. The pellet, which contains an approximate proportion of 25% of salt (Dupuis & LeHoux, 2007) was dried, and then dissolved in 0.2 N HCl at a final chitosan concentration of 25 g/L. Chitosan was re-precipitated by the addition of 5 N NaOH to a final pH of 9.5. After centrifugation, the pellet was washed with distilled water until neutral pH obtaining the partly hydrolyzed chitosan paste.

2.2.2. Coupling reaction

Remazol Brilliant Blue R (Sigma–Aldrich; approximate dye content 60%) was used as a 4% (w/v) aqueous solution. The coupling reaction followed essentially previously published procedures (Fen et al., 2006; Wirth & Wolf, 1990). For 50 mL of coupling reaction mixture, 2.5 g of partly hydrolyzed chitosan (dry weight equivalent) were combined with varying proportions of 4% RBB solution and distilled water, depending on the desired RBB:chitosan ratio. The best result was achieved using 17.8 mL of 4% RBB (corresponding to a w/w ratio RBB:chitosan 0.28:1). The suspension was incubated for 30 min at 60 °C with occasional vortexing. The suspension was then centrifuged for 10 min at 4000 rpm and the supernatant was discarded. The pellet was combined with distilled water to a final volume of 75 mL. This suspension was supplemented with 18.5 mL of 1.5% sodium potassium tartrate and 18.5 mL of 1.5% sodium dichromate and incubated for 15 min at 90 °C with occasional stirring. The dyed pellet was recovered by centrifugation and washed with distilled water until obtaining a clear supernatant. However, a too extensive washing resulted in some loss of dyed chitosan. Finally, the pellet was dried at 50 °C and ground into a fine powder.

2.2.3. Biochemical assays

Endo-chitosanase activity on sRBB-C was determined at 1 mg/mL substrate concentration in 100 mM Na-acetate buffer pH 4.5. Routinely, 950 μL of substrate solution were mixed with 50 μL of appropriately diluted enzyme sample and incubated for 60 min at 37 °C. The reaction was terminated by the addition of 500 μL of 0.6 N NaOH. After 20 min of incubation in ice, the unhydrolyzed substrate was pelleted by centrifugation (10 min) in a microcentrifuge and the optical density (OD) of the supernatant was measured at 595 nm against an appropriate blank in 1 cm polystyrene cuvettes. The OD_{595} measured in a reference tube (where the NaOH solution was added before the enzyme sample solution) was subtracted from the OD_{595} of the reaction sample and the resulting value was used to estimate the enzyme activity from a standard curve (see Section 3).

Endo-chitosanase activity was also measured by the release of reducing sugars from chitosan (Sigma–Aldrich; degree of *N*-acetylation of 18%) as described previously (Boucher et al., 1995) except that the *p*-hydroxybenzoic acid reagent (Lever, 1972) was supplemented as proposed by Schep and coworkers (Schep, Shepherd, & Sullivan, 1984) to achieve increased sensitivity.

2.3. Agar medium for detection of chitosanase-positive microorganisms

A basal medium was prepared containing $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.02 g/L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), $FeCl_3$ (0.05 g/L), NaCl (2.5 g/L), glucose (1 g/L), $(NH_4)_2SO_4$ (1 g/L), malt extract (Difco) (1 g/L), NaOH (5 mM) and agar (15 g/L) pH 6.5. About 900 mL of water were added and the medium was autoclaved. Separately, the sRBB-C powder (a batch synthesized at a 2:1 RBB:chitosan ratio) was dissolved at 5 g/L in 100 mL of 50 mM HCl and autoclaved. After sterilization, the hot agar medium (60 °C) was combined with the sRBB-C solution and poured on Petri plates.

3. Results and discussion

3.1. Optimization of substrate synthesis and assay method

Sixteen chitosan derivatives were synthesized using various RBB:chitosan ratios. We showed in (Fig. 1) their intrinsic optical densities (OD_{595}) when solubilized at 1 mg/mL as well as the OD_{595} of non-precipitable reaction product liberated after 60 min of incubation with 10 mU of endo-chitosanase from *Streptomyces* sp. N174. Both values were increased up to a RBB:chitosan ratio

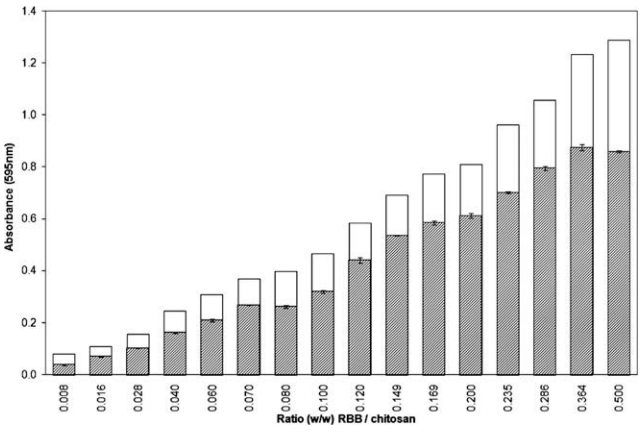

Fig. 1. Release of dyed alkali-soluble fragments from sRBB-C preparations synthesized at various RBB:chitosan ratios. Each column corresponds to the absorbance of a 1 mg/mL solution of intact sRBB synthesized at the indicated RBB:chitosan ratio. The hatchet portion represents the maximal absorbance of alkali-soluble fragments released from the substrate after treatment with excess amounts of enzyme.

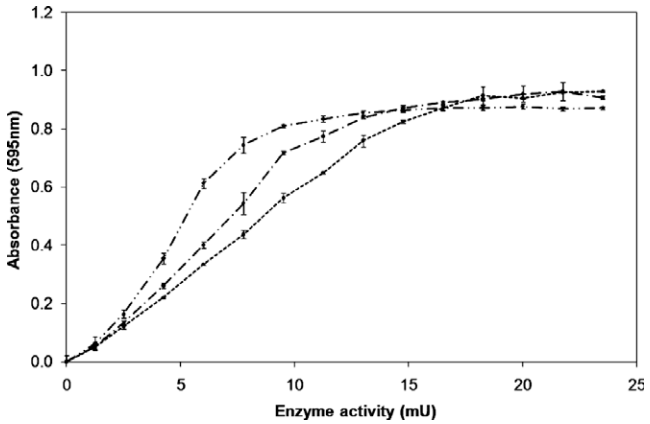

Fig. 2. Release of dyed alkali-soluble fragments from sRBB-C synthesized at 0.365:1 (w/w) RBB:chitosan ratio as a function of enzyme activity and NaOH concentration. During the precipitation step, NaOH concentration was: 0.2 N (——); 0.6 N (– • –); 1 N (–••–).

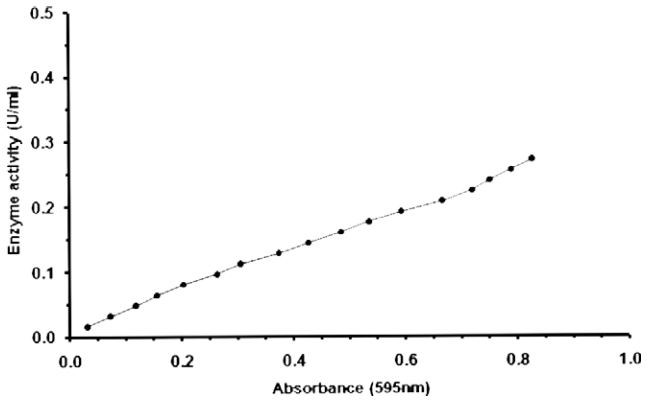

Fig. 3. Relationship between absorbance and chitosanase enzyme activity obtained for sRBB-C synthesized at 0.365:1 (w/w) RBB:chitosan ratio. Standard protocol was applied for all the samples in triplicate. Only the linear part of the curve is shown.

of 0.364:1 (w/w). At higher ratios, the intrinsic optical density was further increased, as expected, but the product liberated by enzyme activity began to decrease – probably because of steric hindrance between the active site of the enzyme and the RBB residues coupled to chitosan.

As in the case of Remazol Brilliant Blue–colloidal chitosan (Fen et al., 2006), enzyme assay with sRBB-C involves treatment with an alkaline solution to stop the reaction and initiate the precipitation of unhydrolyzed polymer fragments. We compared the amount of dyed fragments released by various chitosanase amounts with the amount that remained in solution after treatment with three different concentrations of NaOH (Fig. 2). The shape of the optical density curve of unprecipitated product as a function of enzyme activity was markedly influenced by the NaOH concentration during the precipitation step. At higher concentrations, the plateau was reached at lower enzyme quantities. At 0.2 N NaOH, the quasi-linear portion of the curve was much more extended, allowing assaying a wider range of enzyme concentrations before reaching the maximum plateau. This was also the minimal concentration at which unhydrolyzed sRBB-C was entirely precipitated (data not shown). We then adopted this concentration for our routine procedure.

In a final step of optimization, we examined the length of the cooling and centrifugation steps. The optimal times found for these two steps were 20 and 10 min, respectively. Shorter cooling time gave less stable results at low enzyme quantities, while shorter centrifugation times did not allow for complete pelleting of unhydrolyzed chitosan (data not shown).

3.2. Quantitative assay of endo-chitosanase activity

Using the optimized protocol, a complete standard curve allowing the calculation of enzyme activity from the optical density of alkali-soluble fragments was determined (Fig. 3). As shown, within the effective range of OD_{595} (0.03–0.9) the relationship OD vs. activity was essentially linear, corresponding to the empirical equation of:

$$Activity(U/mL) = 0.304OD_{595} + 0.013$$

with a R^2 value of 0.9968.

The sRBB-C substrate allowed the assay of chitosanase solutions having activities as low as 15 mU/mL. The chitosanase assay was also performed in the presence of high concentrations of reducing sugars (glucose; xylose or glucosamine; up to 5 g/L) and no interference was observed (data not shown). The sRBB-C assay is then particularly useful in situations where low enzyme concentrations have to be determined in complex liquid media, for instance at early stages of microbial cultures where most of the carbon sources are still present in the culture supernatant. As an example of this use, we show the study of chitosanase induction in *Kitasatospora* sp. N106, a **known chitosanase producer (Dubeau, Broussau, Gervais, Masson, & Brzezinski, 2005). Chitosanase production by this strain was induced by chitosan oligosaccharides and somewhat less efficiently by D-glucosamine, while no production was observed in medium with mannitol (Fig. 4). The high concentrations of sugars present in the samples at short induction times would make it very difficult to measure precisely the enzyme activities (in the milliunit/mL range) with the reducing sugars assay.

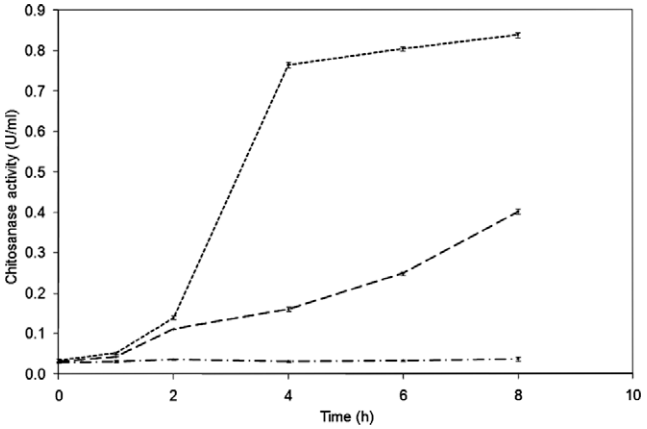

Fig. 4. Induction of chitosanase production in *Kitasatospora* sp. N106. Cultures grown in Tryptic Soy Broth were divided into equal aliquots, the mycelia in each aliquot were collected by centrifugation and resuspended in minimal medium with 5 g/L of chitosan oligosaccharides (– – –),D-glucosamine (—) or mannitol (– • –). Data are averaged from triplicate experiment.

3.3. Use of sRBB-C for plate screening

As discussed above, the previous attempt to synthesize a RBB-derivative of chitosan resulted in a relatively poorly soluble polymer (Fen et al., 2006) which rendered difficult its application to direct screening of microbial colonies on agar media. The lower molecular weight derivative presented in this work can be easily included in agar media for detection of chitosanolytic microorganisms by plate clearing, giving the same advantages as the well known substrates for cellulose or xylanase producers (Farkaš et al., 1985). Well defined clearing zones could be observed after

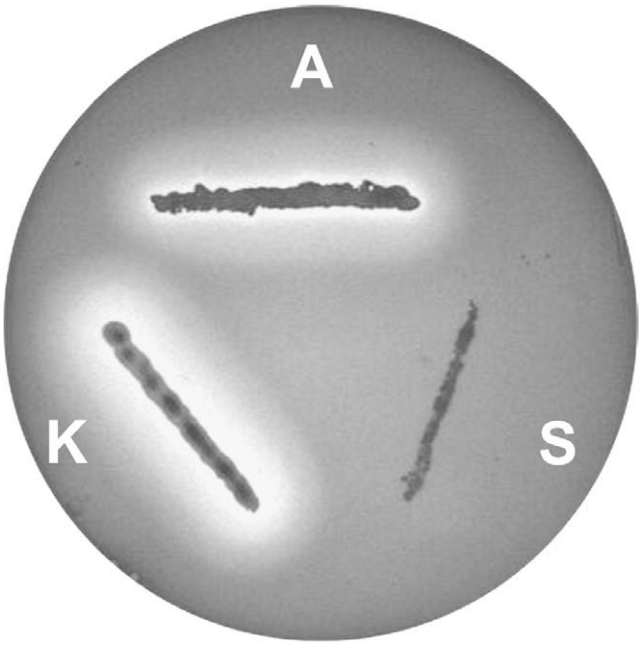

Fig. 5. Detection of chitosanolytic bacteria on agar medium with sRBB-C. Incubation was for 24 h at 30 °C. A: *Amycolatopsis orientalis* subsp. *orientalis;* S: *Saccharopolyspora erythtraea* NRRL2338, K: *Kitasatospora* sp. N106.

24 h of incubation at 30 °C for two known chitosanolytic bacteria chosen as an example (Fig. 5). Thus the sRBB-C chitosan derivative was recognized as an inducer of chitosanase by the tested microorganisms, probably due to the incomplete substitution of D-glucosamine residues in the chitosan chain.

This new soluble RBB-derivative of chitosan, simple in use and responding almost linearly to chitosanase activity is useful for screening procedures. It offers an interesting alternative for precise determination of chitosanase activity in situations where the interference from the media components does not allow to measure enzyme activity with the assays based on reducing sugar determination. The use of the substrate for studies of the regulation of expression of chitosanase genes is in progress in our laboratory.

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