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Capillary zone electrophoresis for the quantitation of oligosaccharides formed through the action of chitinase

Capillary zone electrophoresis with fluorescence detection was used to analyze the products formed by chitinase acting on N-acetylchitooligosaccharide-fluorescent conjugates. Six oligosaccharides of the structure [N-acetylglucosamine(1→4)]_n (where n = 1–6) were conjugated to 7-amino-1,3-naphthalene disulfonic acid by reductive amination. Each oligosaccharide-fluorescent conjugate was purified by preparative gradient polyacrylamide gel electrophoresis, semi-dry electrotransfer to a positively-charged nylon membrane and recovered by washing the membrane with salt solution. The products formed by treating each oligosaccharide-fluorescent conjugate with chitinase were analyzed by capillary zone electrophoresis. The chitinase treatment hexasaccharide-fluorescent conjugate was also examined kinetically to study the action pattern of this enzyme.

1 Introduction

Recently, we demonstrated the utility of gradient polyacrylamide gel electrophoresis (PAGE) for analyzing fluorescent conjugates of neutral, linear oligosaccharides before and after treatment with exoglycosidases and endoglycosidases [1]. Chitinase is a glycosidase, obtained from animal [2,3], plant [4, 5] and microbial sources [5, 6, 7], which acts on chitin through a unique and complex mechanism. Chitinase hydrolyzes chitin, a water insoluble polymer of 1→4 linked 2-acetamido-2-deoxy-D-glucose (GlcNAc) [8]. It can exhibit endo-type, exo-type, and random-type action pat-

terns [2, 4, 5]. Chitinase can also catalyze transglycosylation reactions [9]. Previous workers relied on conventional methods of analysis to study chitinases. These include the use of radiolabeled chitin [10] or chromogenic substrates such as *p*-nitrophenyl-β-D-N,N'-diacetylchitobiose [5]. Analysis by paper chromatography [10], thin layer chromatography (TLC) [5] and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection [2, 4, 6] have been used to study the mechanism and substrate specificity of this enzyme. In our earlier study [1] we demonstrated the high sensitivity of fluorescence detection of sugar conjugates but also the limitations of gel electrophoresis. These limitations are primarily difficulties in sample quantitation and in the automation of gel-based analyses. High voltage capillary electrophoresis is rapidly becoming an indispensable tool for the separation and microanalysis of biopolymers including peptides, proteins, nucleotides, and nucleic acids [11]. The utility of capillary zone electrophoresis (CZE) to quantitatively analyze sulfated carbohydrates such as chondroitin sulfate and dermatan sulfate derived disaccharides has recently been de-

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Abbreviations: AGA, amido-G-acid or 7-amino-1,3-naphthalenedisulfonic acid; CZE, capillary zone electrophoresis; GlcNAc, 2-acetamido-2-deoxy-D-glucose; [GlcNAc(1→4)]_n-AGA, N-acetylchitooligosaccharide-AGA; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis

monstrated [12]. However, to date there have been few reports of capillary electrophoresis being applied to the analysis of neutral oligosaccharides [1, 13]. This may be due to the absence of fixed charges, which facilitate separation by electrophoresis, and the absence of chromophores in most carbohydrates, used in their detection by absorbance or fluorescence spectrometry [14]. This paper describes CZE as a method to examine the action pattern of chitinase, obtained from *Streptomyces griseus*, using fluorescent conjugates of chitin-derived oligosaccharides.

2 Materials and methods

2.1 Materials

N-acetylchitooligosaccharides ($[\text{GlcNAc}(1\rightarrow4)]_n$, $n = 1-6$) were obtained from Seikagaku America (Rockville, MD, USA). Chitinase (EC 3.2.1.14, *Streptomyces griseus*) and β -*N*-acetylglucosaminidase (EC 3.2.1.30, jack beans) were from Sigma (St. Louis, MO, USA). Glycine, sucrose, ethylenediaminetetraacetic acid, boric acid, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine were from Fisher Chemical Company (Fair Lawn, NJ, USA). Biotrace RP nylon (positively charged nylon membrane) was from Gelman Science (Ann Arbor, MI, USA) and 3 mm paper was from Whatman (Hillsboro, OR, USA). The 32×16 cm vertical slab gel unit (SE 620), 250 mL SG500 linear gradient maker apparatus and the TE70 semi dry electro-transfer unit were from Hoefer Scientific Instruments (San Francisco, CA, USA). The electrophoresis power unit model 1420B and Bio-Gel P-2 were purchased from Bio-Rad (Richmond, CA, USA).

2.2 Preparation of fluorescently labeled sugar by reductive amination

The monopotassium salt of 7-amino-1,3-naphthalenedisulfonic acid (Amido-G-Acid, AGA, Fig. 1) was used after recrystallization from deionized water. *N*-Acetylchitooligosaccharide (3.5 μmol) was dissolved in 750 μL of AGA solution (50% w/v in water adjusted with sodium hydroxide to pH 6.2). After heating at 80°C for 60 min, sodium cyanoborohydride (16 μmol) was added (the pH changed <0.1 unit). The mixture was then heated for 24 h at 70°C, in an incubator-shaker. After the reaction was complete the products were desalted on a 2.5×50 cm Bio-Gel P-2 column eluted with distilled water.

2.3 Purification of *N*-acetylchitooligosaccharide-AGA conjugates

N-Acetylchitooligosaccharide-AGA conjugates were purified by preparative PAGE using a linear gradient from 12% to 22% w/v acrylamide that containing 0.5%–2% w/v *N,N'*-bisacrylamide [15]. Electrophoresis was performed on a $32 \times 16 \times 0.75$ cm gel for 18 h at 400 V (constant voltage) with cooling using circulating tap water. Following semidry-electrotransfer onto a positively charged nylon membrane nylon, *N*-acetylchitooligosaccharide-AGA conjugates were recovered by washing the membrane with salt [15]. The salt was removed using a Bio-Gel P-2 column eluted with distilled water and the sample was freeze-dried.

2.4 Proton nuclear magnetic resonance (NMR) spectroscopy

Proton NMR spectroscopy as performed on a Bruker WM-360 spectrometer operating under ASPECT 2000 control. Purified *N*-acetylchitooligosaccharide-AGA conjugate (2 mg) was dissolved in deuterium oxide (>99.996%) and residual water was removed by freeze drying the sample three times from deuterium oxide. Samples were prepared in $^2\text{H}_2\text{O}$ containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard and spectra were obtained at room temperature.

2.5 Chitinase digestion of *N*-acetylchitooligosaccharide-AGA ($[\text{GlcNAc}(1\rightarrow4)]_n$ -AGA)

Substrate 45 nmole was dissolved in 80 μL of 50 mM citrate buffer (pH 5.0) containing 0.2 mU of chitinase. Substrate was completely converted to product by incubating for 12 h at 37°C. The $[\text{GlcNAc}(1\rightarrow4)]_n$ -AGA was also partially converted to product in a kinetic study. Six vials containing enzyme and this substrate were incubated at 37°C. Reaction vials were removed from water baths after 0, 0.1, 2, 15, 30, 240, and 500 min and heated at 100°C for 3 min. Each sample was freeze-dried and reconstituted in 40 μL deionized, distilled water before analysis by CZE.

2.6 Kinetic analysis of chitinase by CZE

The kinetic profile of chitinase acting on $[\text{GlcNAc}(1\rightarrow4)]_n$ -AGA was analyzed by CZE. The experiments were performed with Dionex Capillary Electrophoresis System (Sunnyvale, CA) equipped with a fluorescence detector. Separation and analysis were carried out using a fused silica capillary tube (measuring 75 μm ID, 375 μm OD, 68 cm long, externally coated except where the tube passed through the detector) from Dionex. The capillary tube was washed extensively with 0.1 M phosphoric acid, 0.5 M sodium hydroxide, deionized, distilled water, filled with the operating buffer (10 mM sodium borate and 50 mM boric acid, having a pH of 8.8), and inserted into the machine. Electrophoresis was performed by gravity injection (1 nL) at 20 kV using the above operating buffer.

2.7 Optimization of emission and excitation wavelengths

Fluorescence detection requires installation of a long-pass optical emission filter in the cell. Optical filters having cut-off wavelengths of 305, 375, 400, 420, 455, 515 nm were investigated. Using an emission filter of 420 nm, the excitation wavelength was varied at 50 nm intervals from 200 nm to 350 nm to obtain the optimum excitation wavelength. Using the optimum excitation wavelength, each emission filter was tested to determine which gave the greatest sensitivity.

2.8 Calibration curves

Using chitotriose-AGA ($[\text{GlcNAc}(1\rightarrow4)]_3$ -AGA) conjugate, a series of dilutions were prepared at concentrations of 1 pmol/nL, 0.62 pmol/nL, 0.21 pmol/nL and 42 fmol/nL. Calibration curves were obtained by injecting 1 nL of each concentration of chitotriose-AGA conjugate solution. The

integrated peak area was obtained using Dionex AI-450 chromatography software.

3 Results and discussion

3.1 Reductive amination of *N*-acetylchitooligosaccharides

Reductive amination is a useful method of labeling sugars with probes to permit their detection and facilitate their separation. Hase *et al.* [16] have optimized the labeling of sugars with 2-aminopyridine by reductive amination and have used this chemistry in an effort to develop an HPLC-based method for sequencing oligosaccharides. *N*-Acetylchitooligosaccharides ($[\text{GlcNAc}(1\rightarrow4)]_n$, $n = 1-6$) were obtained commercially and successfully labeled with an alternative fluorescent reagent, 7-amino-1,3-naphthalenedisulfonic acid to form an oligosaccharide-AGA conjugate (Fig. 1). This fluorescent probe contains two fixed negative charges used to drive a separation by electrophoresis [1]. In an earlier study, we found that the greater the number of sugar residues, in particular oligosaccharide, the more vigorous were the conditions (*i.e.*, longer reaction times, higher temperatures) required to ensure complete reaction [1]. For the *N*-acetylchitooligosaccharides studied here, recovered yields of 60-80% were obtained using a single reaction time and temperature. Further studies would be required to optimize the yield for labeling *N*-acetylchitooligosaccharide of each size.

3.2 Isolation and purification of *N*-acetylchitooligosaccharide-fluorescent conjugates

Each *N*-acetylchitooligosaccharide-fluorescent conjugate prepared was contaminated with excess fluorescent tag,

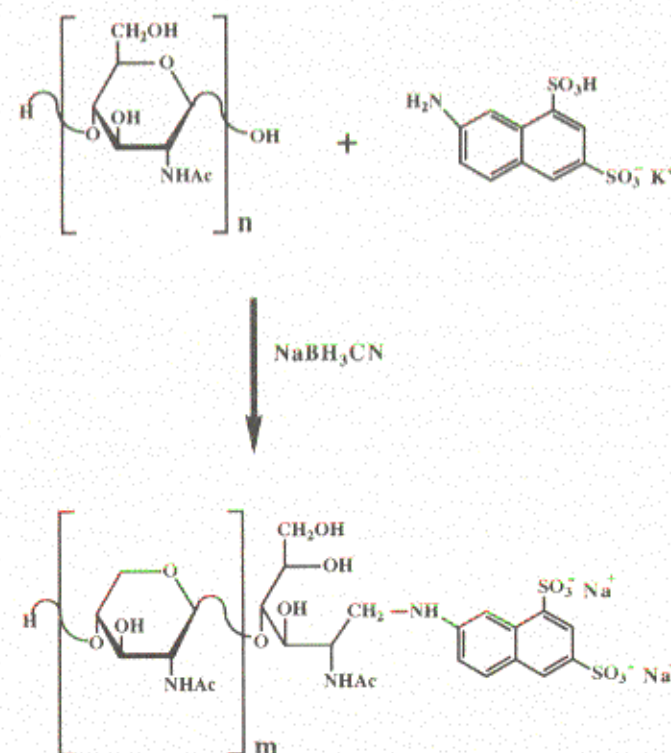


Figure 1. Conjugation of AGA to *N*-acetylchitooligosaccharides, where m ($n-1$) = 0-5.

(AGA) required to drive the reaction to completion, and also with minor reaction side products [1]. The *N*-acetylchitooligosaccharide starting material was not 100% pure, also leading to the formation of additional minor products during reductive amination. Preparative gradient PAGE was used due to its effectiveness and simplicity in resolving, identifying and separating the desired products in the crude reaction mixture. The crude reaction product was loaded directly on the preparative gradient gel. Electrophoresis gives a major band corresponding to the desired oligosaccharide-conjugate, a band corresponding to unreacted AGA and multiple minor bands. These fluorescent bands were easily visualized under UV light. Previous workers have used multiple separation steps including extraction, HPLC and thin-layer chromatography to obtain the purified oligosaccharide conjugates. Iwase *et al.* [17] has recently developed a simpler extraction method for preparing pyridylamino sugar derivatives. The method described here uses a single preparative electrophoresis step [15] to replace these time-consuming, labor-intensive procedures. Crude products are loaded directly on gradient gel and fractionated by electrophoresis. After electrophoresis of the crude reaction product for 18 h at 200 V, and gel is visualized under UV light. Shorter separation times were possible using either higher voltages or smaller gels. The sugar-fluorescent conjugates appearing as well-resolved band are then cut from the gel and the desired product is recovered by semidry electrotransfer from the gel slice to a positively charged nylon membrane [15].

Semidry electrotransfer has been used for the quantitative recovery of glycosaminoglycan-derived acidic oligosaccharides from a PAGE gel [15]. Electrotransfer by the semidry procedure (15 min at 8 V) also resulted in the quantitative transfer of *N*-acetylchitooligosaccharide-fluorescent conjugates. For transferring large quantities of sugar-fluorescent conjugates, multiple layers of positively charged nylon membranes were required to avoid membrane saturation. The *N*-acetylchitooligosaccharide-fluorescent conjugates were eluted from the membrane with 2 M sodium chloride until no additional fluorescent material was detected on membranes. After removing the salt using a Bio-Gel P-2 column, the purity of *N*-acetylchitooligosaccharide-fluorescent conjugates were determined by CZE and strong anion exchange HPLC [1] to be >90%. Optimum detection sensitivity by fluorescence was obtained at $\lambda_{\text{ex}} = 250$ nm and $\lambda_{\text{em}} = 420$ nm. CZE analysis of 1 nL of distilled water containing 1 ng of each *N*-acetylchitooligosaccharide-AGA conjugate using fluorescence detection showed only a single peak. Migration times (in min) for $[\text{GlcNAc}(1\rightarrow4)]_n$ -AGA were: $n=6$, 6.63 ± 0.06 ; $n=5$, 7.20 ± 0.12 ; $n=4$, 7.58 ± 0.09 ; $n=3$, 8.17 ± 0.10 ; $n=2$, 9.53 ± 0.15 ; and $n=1$, 10.12 ± 0.09 . A calibration curve was constructed for $[\text{GlcNAc}(1\rightarrow4)]_n$ -AGA for sample amounts ranging from 42 fmol to 1 pmol (Fig. 2).

3.3 Characterization of *N*-acetylchitooligosaccharide-AGA conjugates

Two of the oligosaccharide conjugates were prepared in multimilligram quantities required for detailed structural characterization. GlcNAc and chitotriose ($[\text{GlcNAc}]_3$) were conjugated with AGA and purified to >95%. High resolution $^1\text{H-NMR}$ of these fluorescent conjugates at 360 MHz

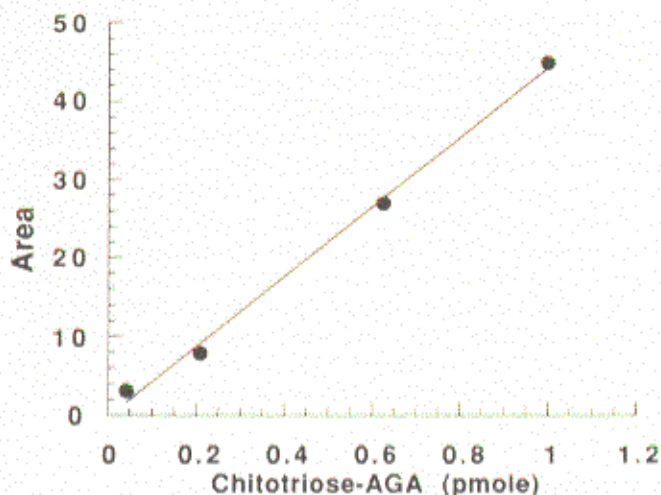


Figure 2. Standard curve of peak area as a function of amount of sample analyzed by CZE using fluorescence detection ($\lambda_{ex} = 250$ nm; $\lambda_{em} = 420$ nm).

gave spectra consistent with each structure [1, 18]. Full assignment of GlcNAc-AGA was possible using two-dimensional homonuclear correlation spectroscopy [1, 18].

3.4 Products obtained from chitinase treatment of *N*-acetylchitooligosaccharide-AGA conjugates

The AGA conjugates of *N*-acetylchitooligosaccharides ($[\text{GlcNAc}(1 \rightarrow 4)]_n$, $n = 1-6$) were each treated for 24 h at 37°C with chitinase and analyzed by CZE. The products obtained from each *N*-acetylchitooligosaccharide-AGA conjugate is shown in Table 1. The monosaccharide and disaccharide-AGA conjugates were stable towards chitinase treatment. Trisaccharide-AGA conjugate was very slowly hydrolyzed to monosaccharide-AGA while tetrasaccharide-AGA conjugate was slowly converted to a disaccharide-AGA product. Pentasaccharide-AGA gave rise to disaccharide-AGA product. Exhaustive treatment of hexasaccharide-AGA resulted in the formation of a mixture containing 8% trisaccharide-AGA, 80% disaccharide-AGA and 12% monosaccharide-AGA products. Disaccharide-AGA conjugate and trisaccharide-AGA conjugate were both substrates for the exolytic enzyme, β -*N*-acetylglucosaminidase (data not shown). The absence of significant amounts of monosaccharide-AGA product from disaccharide-AGA suggests that this chitinase preparation is essentially free of β -*N*-acetylglucosaminidase activity. The specificity of this enzyme for certain linkages within specific *N*-acetylchitooligosaccharide-AGA conjugates is similar to that previously reported for this enzyme. This chitinase reportedly is an exo-type chitinase that removes two sugars at a time from the substrate's non-reducing terminus [5, 7, 10].

This study confirms that *Streptomyces griseus* chitinase is different from the endo-type chitinases isolated from prawns [2], yams [4] and insects [3].

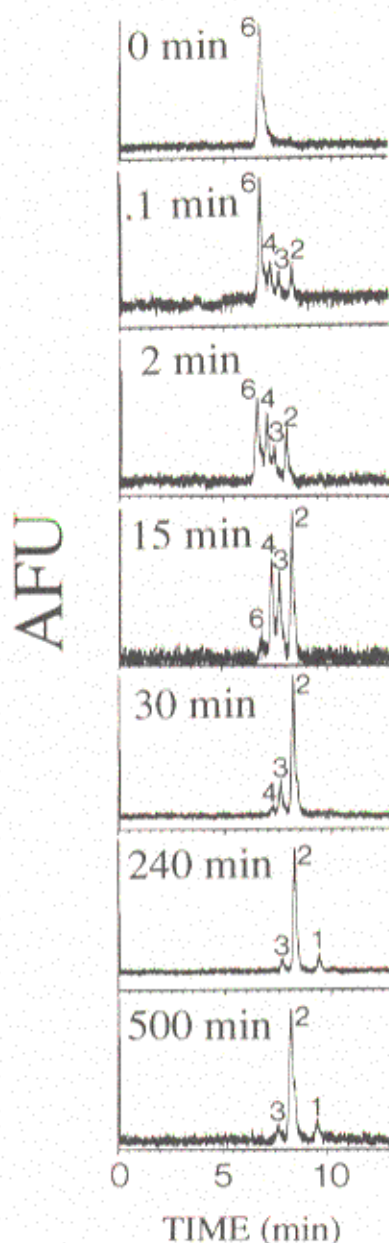


Figure 3. Seven electropherograms (fluorescence detection at $\lambda_{ex} = 250$ nm, $\lambda_{em} = 420$ nm) are shown at various time during the treatment of *N*-acetylchitohexasaccharide-AGA ($[\text{GlcNAc}(1 \rightarrow 4)]_6$ -AGA) with chitinase. The reaction time (in min) is indicated. The peaks are identified as $n = 1-6$, where the products formed are of the structure $[\text{GlcNAc}(1 \rightarrow 4)]_n$ -AGA.

Table 1. Products formed from AGA conjugates of *N*-acetylchitooligosaccharides on exhaustive treatment with chitinase

Substrate, $[\text{GlcNAc}(1 \rightarrow 4)]_n$ -AGA	Products, $[\text{GlcNAc}(1 \rightarrow 4)]_n$ -AGA (mole %) ^{a)}
Monosaccharide, $n = 1$	$n = 1, 100$;
Disaccharide, $n = 2$	$n = 1, 0$; $n = 2, 100$
Trisaccharide, $n = 3$	$n = 1, 5$; $n = 2, 0$; $n = 3, 95$
Tetrasaccharide, $n = 4$	$n = 1, 0$; $n = 2, 10$; $n = 3, 0$; $n = 4, 90$
Pentasaccharide, $n = 5$	$n = 1, 0$; $n = 2, 100$; $n = 3, 0$; $n = 4, 0$; $n = 5, 0$
Hexasaccharide, $n = 6$	$n = 1, 12$; $n = 2, 80$; $n = 3, 8$; $n = 4, 0$; $n = 5, 0$; $n = 6, 0$

a) The mole % of each product was obtained from relative peak areas.

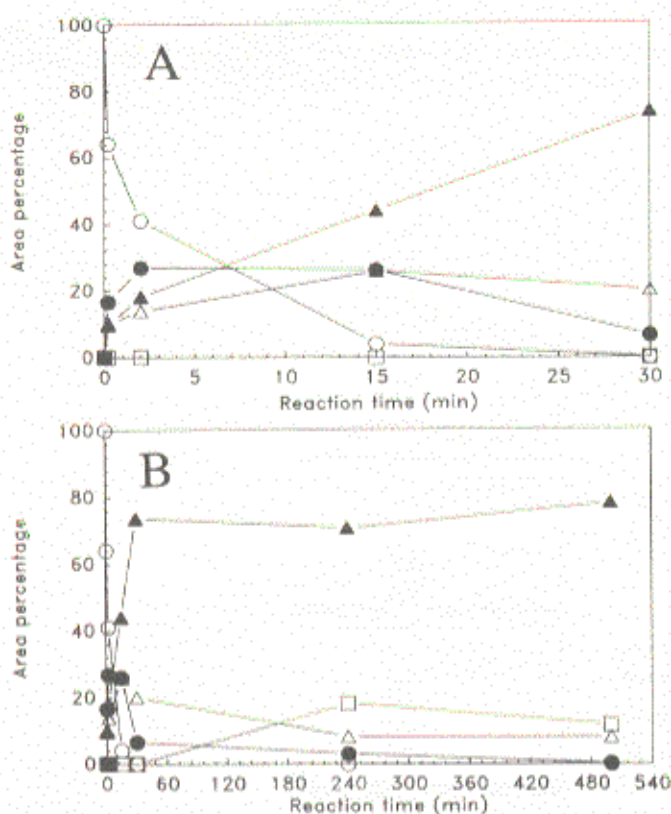


Figure 4. (A) Disappearance of *N*-acetylchitohexaoligosaccharide and the appearance of *N*-acetylchitooligosaccharide during the first 30 min of the reaction monitored in Fig. 3. (B) Reaction over its entire time course. The symbols correspond to $[\text{GlcNAc}(1\rightarrow4)]_n\text{-AGA}$ where $n=1$ (□), $n=2$ (▲), $n=3$ (△), $n=4$ (●), and $n=6$ (○).

3.5 Kinetics of chitinase action on *N*-acetylchitohexaoligosaccharide

A detailed kinetic study was performed on the hexasaccharide-AGA conjugate. The substrate concentration selected for this study was 4.5 mM. The K_m reported for this enzyme on insoluble chitin is 4 mM while the K_m of similar chitinases on soluble *N*-acetylchitooligosaccharides range from 10–25 μM [4, 6]. To a solution of this substrate 0.2 mU of chitinase was added, time points taken, the reaction terminated by heating and the products analyzed by CZE. The electropherograms (Fig. 3) show the disappearance of hexasaccharide-AGA over time and the appearance of smaller *N*-acetylchitooligosaccharide-AGA products. Only the product found at the reducing end of the site where chitinase acts (containing the fluorescent tag) was detected. The hexasaccharide-AGA substrate gave rise nearly immediately to tetrasaccharide-AGA, trisaccharide-AGA and disaccharide-AGA products in comparable amounts (Fig. 4A). Each product is clearly formed directly from hexasaccharide-AGA. Within 15 min nearly all the hexasaccharide-AGA had disappeared and the tetrasaccharide-AGA, trisaccharide-AGA and disaccharide-AGA accounted for 23, 24 and 42%, respectively, for a total of 89% of the area observed in the electropherogram (Fig. 3 and 4A). Tetrasaccharide-AGA, exhaustively treated with chitinase, afforded disaccharide-AGA product (Table 1). Trisaccharide-AGA although relatively stable towards exhaustive treatment with chitinase, slowly gives rise to monosaccharide-AGA product. Thus, the concentration of the stable disaccharide-AGA product continued to increase throughout the reac-

tion (Fig. 3 and 4B) while the concentration of tetrasaccharide-AGA and trisaccharide-AGA both decreased after reaching their maximum concentration at 15 min. No pentasaccharide-AGA product was observed although it migrates very close to the hexasaccharide-AGA product and thus could be hidden under this larger peak. In a separate study using gradient PAGE, pentasaccharide-AGA was observed to first give a tetrasaccharide-AGA product before finally forming disaccharide-AGA. In the kinetic studies using CZE (Fig. 3 and 4), the concentration of hexasaccharide-AGA and the concentration of each product is measured as a function of time. These data when taken together with the end-point determinations (Table 2) give a better understanding of the exo-type action pattern of chitinase on soluble oligosaccharide substrates.

4 Concluding remarks

This paper demonstrates that *N*-acetylchitooligosaccharides ranging from monosaccharide to hexasaccharide can be efficiently labeled with a fluorescent tag containing two fixed negative charges. Preparative gradient PAGE combined with semidry electrotransfer can be used to purify these *N*-acetylchitooligosaccharide-fluorescent conjugates. Some of these conjugates serve as substrates for chitinase as demonstrated by their CZE analysis following exhaustive enzymatic treatment. Chitinase action in the hexasaccharide-fluorescent conjugate was also examined kinetically using CZE. This analysis resulted in a better understanding of the action pattern of this important enzyme and demonstrated the utility of CZE in such studies.

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