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Detection of Chitinase Activity Using Fluorescence-Labeled Substrate on Polyacrylamide Gel

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Abstract: Gradient polyacrylamide gel electrophoresis was used to analyze the products formed by chitinases acting on N-acetylchitohexaose-fluorescent conjugate. N-Acetylchitooligosaccharides 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. N-Acetylchitohexaose-fluorescent conjugate and chitohexaose were exhaustively treated with three kinds of chitinases from *Serratia marcescens*, *Streptomyces griseus*, and green onin (*Allium fistulosum* L.). The bands were visualized under long wavelength of UV light. Analysis of reaction products provided the information on the action of chitinase action from different sources.

Chitin, the second most plentiful natural polymer, is mainly composed of β1,4-linked N-acetylglucosamine (Austin *et al.*, 1981). Chitinase is an enzyme that typically hydrolyzes chitin. Either chitobiose or chitotriose is formed, depending on the mode of action of the chitinase (Deshpande, 1986; Cabib, 1987). Recent biomedical applications of chitooligosaccharides has increased interest in finding new kinds of chitinase (Takiguchi *et al.*, 1989; Usi *et al.*, 1990; Hara *et al.*, 1989). Chitinase activity has been assayed by a variety of methods. These include: 1. the estimation of substrate degradation by viscosity measurements using soluble derivatives of chitin (Ohtakara, 1988) and 2. the determination of chitooligosaccharides of N-acetylglucosamine liberated in the reaction using colloidal chitin (Boller *et al.*, 1988). In the second case, the reducing power of free N-acetylhexosamines has been measured with p-dimethylaminobenzaldehyde. Here, it is necessary to include β-N-acetylhexosaminidase in the reaction mixture to convert oligosaccharides formed into the monosaccharides. In addition, high sensitivity, radiochemical methods have been developed using tritiated chitin (acetyl-^{3}H) (Molano *et al.*, 1977).

Recently, fluorometric assays using sugar-fluorophore conjugate have been used as the sensitive methods for the detection of oligosaccharides (Yamamoto *et al.*, 1989; Lee *et al.*, 1991a). We demonstrated that N-acetylchitooligosaccharides ranging from monosaccharides to hexasaccharides could be efficiently labeled with a fluorescent tag containing two fixed negative charge (Lee *et al.*, 1991b). These labeled oligosaccharides could then be analyzed by capillary zone electrophoresis with very high sensitivity (Lee *et al.*, 1991b).

This paper focuses on the use of a homogeneous, structurally defined, charged, fluorescent conjugate as a substrate for chitinase assay. Gradient polyacrylamide gel electrophoresis (PAGE) was used for the detection of reaction mixture.

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Materials and Methods

Materials

N-Acetylglucosamine, N,N′-diacetylchitobiose and N,N′,N″-triacetylchitotriose were from Sigma, St Louis, MO, USA. N-Acetylchitooligosaccharides were prepared from crab chitin (Sigma), by the partial hydrolysis and separated on a Bio-Gel P-4 column (Eikeren *et al.*, 1977) or purchased from Seikagaku Co, Japan. Acryamide, N,N′-methylenebisacrylamide (BIS), N,N,N′N′-tetramethylenediamine (TEMED), and ammonium persulfate were obtained from Sigma. 7-amino-1,3-naphthalenedisulfonic acid, monopotassium salt (Amido-G-Acid, AGA, Fig. 1) was purchased from Aldrich, Milwaukee, WI, USA. The monopotassium salt of AGA was used after recrystallization from deionized water. All other chemicals were of reagent grade.

Nylon 66 plus membrane, 32×16 cm vertical slab gel unit, and TE70 semi-dry transfer unit were from Hoefer Scientific Instruments, San Francisco, CA, USA. The electrophoresis power supply (KMC 101) was purchased from Vision Scientific Co, Korea.

N-Acetylglucosaminidase (EC 3.2.1.30 jack bean), chitinase (EC 3.2.1.30) from *Streptomyces griseus* and chitinase from *Serratia marcescens* were products of Sigma. N-Acetylglucosaminidase and chitinase from green onion (*Allium fistulolsum*) were purified in our laboratory (Kim *et al.*, 1991). N-Acetylglucosaminidase activity was negiligible in chitinase fraction from green onion.

Preparation of fluorescently labeled sugar by reductive amination

The derivatization and recovery of sugars were essentially followed by the procedure of Lee *et al.* (1991 a). N-Acetylchitooligosaccharides (3.5 μmol) were dissoived in 750 μl of AGA solution (50% w/v in water adjused with 10 N sodium hydroxide to pH 6.2). After heating at 18℃ 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℃. After the reaction was complete the products were desalted on a 2.5×45 cm Bio-Gel P-2 column eluted with distilled water.

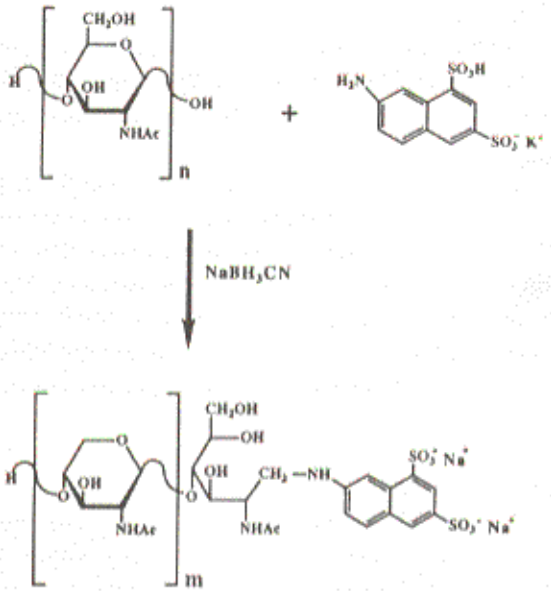

Fig. 1. Conjugated of AGA to N-acetylchitooligosaccharides, where n=1~6, m=n−1.

Preparation of polyacrylamide gels

The preparation of gels were essentially followed by the procedure of Al-Hakim *et al.* (1990). The resolving gel buffer and lowere chamber buffer contained 0.1 M boric acid, 0.1 M Tris and 0.01 M disodium EDTA at pH 8.3. Gradient polyacrylamide resolving gel was prepared from two resolving gel buffer solutions, one containing 11.52% acryamide, the othe other containing 20.02% w/v acrylamide with 0.48 and 2% w/v N,N′-methylenebisacrylamide and 1 and 15% w/v sucrose, respectively. Gels were poured vertically between glass plated (16 cm×32 cm) seperated by 0. 75 and 1.5 cm. For the 1.5 mm gel, gradients were poured by adding 35 ml of 12% solution to the resorvoir and 35 ml of 22% solution to the mixing chamber of the linear gradient maker. Ammonium persulfate, 100 μl of 10% in water and 15 μl TEMED were added to both resorvoir and mixing chamber. After pouring the polyacrylamide solution, the top was overlayed with water. After polymerization was completed, the water layer was removed. A 10 ml solution of stacking gel made of 4.75% w/v acrylamide and 0.25 w/v BIS in stacking gel buffer (which was identical to the resolving gel buffer but was adjusted to pH 6.3 with

hydrochloric acid, containing 10 μl of TEMED and 150 μl of 10% w/v of ammonium persulfate) was added to the top of the resolving gel. The upper chamber buffer were composed of 0.2 M Tris and 1.25 M glycine at pH 8.3.

Samples were combined with an equal volume or 50% w/v sucrose in water containing trace quantities of phenol red (10 μg/ml) and bromophenol blue (10 μg/ml) and loaded into the bottom of each well. Electrophoresis was performed for 16 h at 250 V while cooling with circulating tap water. The gel was visualized under long wavelength of UV light and the picture was taken.

Semi-dry electrotransfer

The desired fluorescent band was removed from the gel by carefully cutting the gel and soaked in transfer buffer consisting of Tris base (5.82 g), glycine (4.35 g) and methanol (200 ml) made up in 1 l with water. Several layers of blotting paper and positively charged transfer nylone membranes were cut to the same size as the gel and soaked in transfer buffer. Two pieces of transfer buffer-saturated blotting papers (3 mm) were placed on top of the Mylar Mask. Several layers of nylon membrane (depending on the concentration of sample in the gel) were placed on top of the blotting papers. The soaked gel was placed directly on the nylon membranes followed by 3 layers of blotting paper, thus constructing a transfer sandwich. The cover of the semi-dry transfer was performed at 7～10 V for 30 min. Completion of the transfer process was ensured by examining the gel under ultraviolet light and making sure that no material was left behind.

Elution and recovery of membrane-bound sugar-fluorescent conjugates

Nylon membrane containing conjugate were cut into small pieces and immersed in test tubes containing 3 ml of 2.0 M sodium chloride and placed on a shaker for several hour at room temperature. The salt solution was freeze-dried and desalting ws performed on a 2.5 cm×45 cm Bio-P-2 gel low pressure column. The salt free solution was concentrated by freeze-drying.

High performance liquid chromatographic (HPLC) analysis

HPLC was performed to analyze the N-acetylchitohexaose and chitinase reaction mixture. The system was equipped with a SP 8860 ternary HPLC pump (Spectra Physics), fixed-volume loop Rheodyne (Cocati, CA) #7125 injector and Spectra 100 variable wavelength detector. The data was processed using SP4270 integrator (Spectra Physics). The reaction mixture (10 μl, 20 μg) was injected on a Spheri-5 amino column of dimension 4.6 mm×22 cm from Spectra Physics and eluted with acetonitrile (75%) and water (25%) at 1.0 ml/min.

Chitinase reaction

N-Acetylchitohexaose 0.16 μmol was dissolved in 10 μl of citrate buffer (pH 5.0) and treated with 0.2 U of each chitinase. N-Acetylchitohexaose-AGA conjugate 45 nmol was dissolved in 80 μl of citrate buffer and incubated with 0.2 mU of microbial chitinases and 0.1 U of chitinase from green onion. The reaction was complete after incubating for 12 h at 37℃.

Results and Discussion

Reductive amination of N-acetylchitooligosaccharides

Reductive amination is a useful method of labeling sugars with probes to permit their detection and faciliate their separation (Fig. 1). AGA was dissolved in 10 N NaOH and made to 50%. The molar ratio of sugar to AGA and sodium cyanoborohydride was based on the results of the labeling experiments using D-glucose and D-N-acetylglucosmine (Lee *et al.*, 1991 a). It was necessary to use excess AGA to drive the reaction to completion. Thus a purification step to remove AGA was necessary to obtain sugar-AGA conjugate of sufficient purity for analysis and sequencing. 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 temperature) required to ensure complete reaction. The yield was decreased with higher oligosaccharides. Further studies are required to optimize the yield for labeling each N-acet-

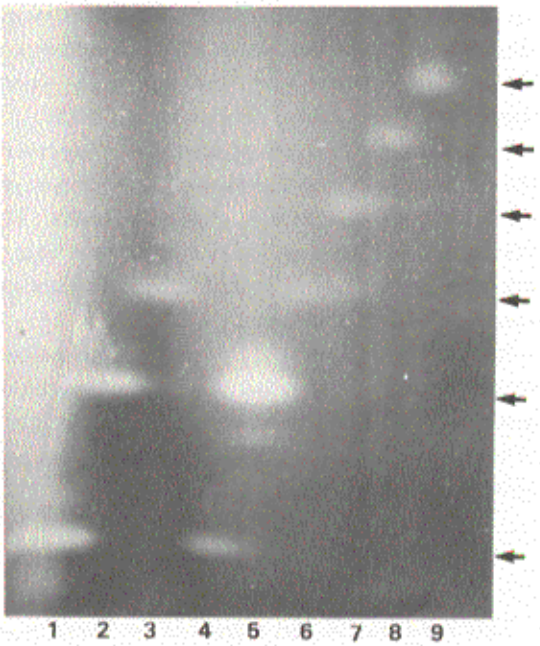

Fig. 2. Analytical gradient polyacrylamide gel photographed under ultrabiolet light of N-acetylchitooligosaccharides-AGA and the reaction products of chitinases acting on N-acetylchitohexaose-AGA. Lane 1, 2, and 3 represent the reaction mixture of chitinases from *Serratia marcescens*, *Streptomyces griseus*, and green onion, respectively. Lane 4 shows the reaction mixture of N-acetylchitohexaose-AGA treated first with chitinase and then with N-acetylglucosaminidase. Lanes 5~9 and 10 are the standards: N-acetylchitobiose-AGA, -triose-AGA, -tetraose-AGA, -pentaose-AGA, and-hexaose-AGA, respectively. Each lane contained approximately 10~15 μg of sample.

ylchitooligosaccharide. Recently, Suzuki *et al.* (1991), reported the simple procedure for the reductive amination of sugars using 2-aminopyridine and dimethylamine-borane complexes.

Isolation and purification of N-acetylchitooligosaccharide-AGA conjugates

The crude reaction product was loaded directly on the preparative gradient gel. Electrophoresis gave a major band corresponding to the desired oligosaccharide-conjugate, bands corresponding to unreacted AGA and multiple minor bands. These fluorescent bands which were easily visualized under UV light were cut from the gel and recovered by semi-dry electrotransfer from the gel slice to a positively-charged nylone membrane.

Semi-dry electrotransfer has been used for the recovery of glycosaminoglycan-derived acidic oligosaccharides from PAGE gel (Ali-hakim *et al.*, 1991). Electrotransfer by the semi-dry procedure (15 min at 8 V) also resulted in the quantitative transfer of N-acetylchitooligosaccharides-fluorescent conjugates. Optimum detection sensitivity by fluorescence was obtainesat $\lambda_{ex}=250$ nm and $\lambda_{em}=420$ nm (Lee *et al.*, 1991a).

Chitinase reaction

The chitinases used for the reactions were from *Streptomyces griseus*, *Serratia marcescens* and green onion (*Allium fistulosum* L.). The cleavage pattern for chitohexaose-AGA is in Fig. 2 and 3. Chitinase from *Serratia marcescens* cleaved N-acetylchitohexaose-AGA to N-acetylglucosamine-AGA (Lane 1 of Fig. 2). The major reaction product is N-acetylglucosamine upon incubations with chitohexaose (a of Fig. 3). In contrast, chitinase from *Streptomyces griseus* hydrolyzed N-acetylchitohexaose-AGA to N-acetylchitobiose-AGA (Lane 2 of Fig. 2) and converted chitohexaose to chitobiose and N-acetylglucosamine in about 4 to 1 ratio (b of Fig. 3). Plant chitinase from green onion hydrolyzed N-acetylchitohexaose-AGA to chitotriose-AGA (Lane 3 of Fig. 2). When this chitotriose-AGA product was treated with N-acetylglucosaminidase (0.5 U) from green onion, N-acetylglucosamine-AGA was obtained (Lane 4 of Fig. 2). Neither N-acetylchitobiose-AGA nor N-acetylchitotetraose-AGA could be seen on PAGE. In contrast, chitinase from green onion cleaved chitohexaose to chitotriose, chitobiose and chitotetraose (c of Fig. 3). When this mixture was treated with N-acetylglucosaminidase from green onion, the amount of N-acetylglucosamine formed increased gradually (data not shown).

Chitinase from *Serratia marcescens* appears to be an exo type enzyme or it has contaminating β-N-acetylglucosaminidase activity (Roberts *et al.*, 1982). When N-acetylchitohexaose (200 μg) was treated with chitinase (0.6 U) from *Serratia marcescens* for 20 min, the products were mixture of N-acetylchitooligosaccharides ($n=1\sim6$) (date not shown). The chitinase from *Streptomyces griseus* acted as an exo type enzyme. This is consistent with previous results using colloidal chitin as a substrate (Jeuniaux, 1966). Since the major

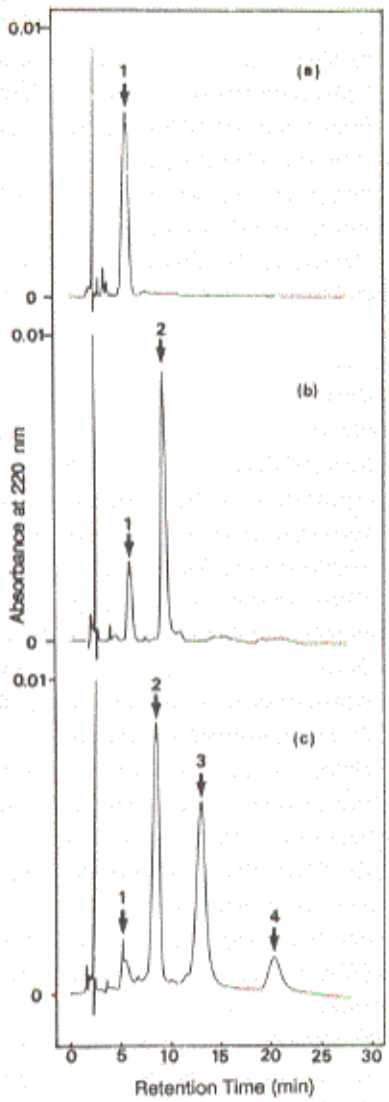

Fig. 3. High performance liquid chromatography of N-acetylchitohexaose treated with various chitinases using N-acetylchitohexaose a) chitinase from *Serratia marcescens*, b) *Streptomyces griseus* c) chitinase from green onion: The peakes were identified by co-injection with standardds and are: 1. N-acetylglucosamine, 2. N-acetylchitobiose, 3. N-acetylchitotriose, 4. N-acetylchitotetraose.

band of reaction products of enzyme from green onion was N-acetylchitotriose-AGA, the action mode was endo type. It was not clear why this chitinase did not yield N-acetylchitobiose-AGA or N-acetylchitotetraose-AGA. An endochitinase induced by ethylene has also been reported in bean leaves (*Phaseolus vulgaris*) (Boller *et al.*, 1983; Park *et al.*, 1991). Reaction products of chitinase from bean leaves were the same as those from green onion (Boller *et al.*, 1983).

In fact, some differences have been observed in the cleavage pattern of chitinases from different sources acting on N-acetylchitooligossaccharides. These results agree with this study and suggest that two microbial enzyme act as exochitinases and plant enzymes acts as endochitinase.

In addition to studies of the enzymatic depolymerization of linear oligosaccharides and polysaccharides, gradient PAGE can be also applied to the analysis of other sugars including oligosaccharides from glycoprotein and glycolipids.

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초록 : 형광기-기질 결합체를 이용해 폴리아크릴아미드겔 위에서 Chitinase 활성의 검정

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농도기울기 폴리아크릴아미드 전기영동을 이용하여 N-acetylchitohexaose-형광기 결합체에 작용하여 얻어지는 chitinase 생성물을 분석하였다. N-Acetylchitooligosaccharides는 7-amino-1,3-naphthalene disulfonic산과 환원성 아민화반응에 의해서 결합되어졌다. 각각의 소당-형광기 결합체는 농도기울기 폴리아크릴아미드 전기영동과 양이온 나일론 막으로 전기적 전이에 의해서 성제되어졌다. 나일론 막에 결합된 당은 염에 의해서 막을 씻어 회수하였다. N-Acetylchitohexaose-형광기 결합체와 형광기가 결합되어 있지 않은 N-acetylchitohexaose를 *Serratia marcescense, Streptomyces griseus,* 식용의 파(*Allium fistulosum* L.)로부터 얻어진 세 종류의 chitinase와 반응시켰다. 형광기 결합체와의 반응물을 분석용 전기영동에 걸었을 때 자외선 장파장에서 띠가 인식될 수 있었다. 이와 같은 전기영동 방법에 의한 반응생성물의 분석을 통해서 서로 다른 종류의 chitinase의 작용에 관한 특징을 알 수 있었다.