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Short communication

Capillary electrophoresis of chitooligosaccharides in acidic solution: Simple determination using a quaternary-ammonium-modified column and indirect photometric detection with Crystal Violet

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ABSTRACT

Five chitosan oligosaccharides were separated in acidic aqueous solution by capillary electrophoresis (CE) with indirect photometric detection using a positively coated capillary. Electrophoretic mobility of the chitooligosaccharides (COSs) depended on the number of monomer units in acidic aqueous solution, similar to other polyelectrolyte oligomers. The separation was developed in nitric acid aqueous solution at pH 3.0 with 1 mM Crystal Violet, using a capillary positively coated with *N*-trimethoxypropyl-*N*,*N*,*N*-trimethylammonium chloride. The limit of the detection for chitooligosaccharides with two to six saccharide chains was less than 5 μM. CE determination of an enzymatically hydrolyzed COS agreed with results from HPLC.

1. Introduction

Chitosan is a cationic polysaccharide derived from chitin found mainly in the shells of crabs and shrimps. It has many applications in food, cosmetic, health-care and pharmaceutical products, agricultural chemicals, wastewater treatment, and biotechnology [1,2]. The chitooligosaccharide (COS) produced by its chemical or enzymatic hydrolysis has recently attracted interest in food and biotechnology fields due to its solubility in water [3,4]. With broadening potential application, a simple and rapid determination method for COS is of increasing importance for biotechnology researchers and those in the chitosan industry.

Many methods have been applied to separate COS: ion-exchange chromatography [5], amino acid analysis [6], immobilized metal affinity chromatography [7], gel electrophoresis [8], and normal phase HPLC with an amino column [9]. Although HPLC is commonly used, it requires a concentrated sample and large volumes of organic elution solvent. Capillary electrophoresis (CE) is useful for COS separation because of its high resolving power, and requirement for only a small amount of sample and solvent. Beaudoin et al. [10] successfully separated a submicromolar concentration chitin and COS mixture using CE with an aminopyrene–fluorophore conjugation procedure and laser-induced fluorescence detection. However, the method required relatively expensive materials and equipment, and derivatization of samples. Blanes et al. [11] developed a direct method using capillary zone electrophoresis with contactless conductivity detection for COS. No derivatization process was required. Successful separation occurred in a background electrolyte solution of 10% (v/v) acetonitrile–water containing 10 mM NaOH. Due to alkaline solution (pH > 12), negatively charged COS was separated as well as other saccharides [12]. Therefore the other oligosaccharides also will appear in its electropherogram.

In this paper we describe a simple and rapid CE determination for COS in an acidic solution. Although most of saccharides are neutral or negatively charged, only chitosan or COS can has positive charge in acid solution. It is easy to distinguish COS from other saccharides by CE in acidic solution. Successful, simple CE of COS was achieved using indirect photometric detection and a positively coated capillary. The background probe in this study was Crystal Violet, a triphenylmethyl derivative similar to malachite green used for indirect CE of metal cations [13]. The capillary column with chemical treatment of a quaternary ammonium silane coupling reagent can prevent COS adsorbing on itself. The method developed was applied to a mixture of COS produced by an enzymatic hydrolysis of chitosan. The determination of COS within this mixture by the simple CE method was compared with HPLC results.

Aider et al. had measured electromigration of COS monomers–hexamers at pH conditions from 2 to 9 [14,15]. The electromigrations of each oligomer and D-glucosamine were

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not significantly different in acidic solution. The similarity in mobility to D-glucosamine was unusual, in that electrophoretic mobility of a small DNA oligomer was strongly dependent on molecular weight [16]. Therefore, the electrophoretic mobility of COS in the aqueous solution needs to be verified.

2. Experimental

2.1. Reagent and materials

Five COS HCl salts, dimer (C2), trimer (C3), tetramer (C4), pentamer (C5), and hexamer (C6), were obtained from Dainichiseika Color & Chemicals Mfg. Co., Ltd. (Tokyo, Japan). COS from an enzymatic hydrolysis of fully deacetylated chitosan [17] was obtained from Katakura Chikkarin Co., Ltd. (Tokyo, Japan). Crystal Violet (chloride salt), D-glucosamine HCl (C1) and *N*,*N*-dimethylformamide (DMF) were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Crystal Violet was ion-exchanged to its nitrate on a long anion exchange resin column (Amber Lite IRA-400J), and the nitrate salt was obtained following evaporation and vacuum drying. *N*-Trimethoxypropyl-*N*,*N*,*N*-trimethylammonium chloride (TMPTMAC) 50% methanol solution was supplied by Gelest, Inc. (Morrisville, PA). Tetraphenyl phosphonium (TPP) chloride was purchased from Tokyo Chemical Ind., Ltd. (Tokyo, Japan).

As an electrophoretic background electrolyte solution an acidic solution was prepared by dissolving 0.03 g of Crystal Violet nitrate salt in 50 mL of water and adjusting the pH by adding a small amount of 1 M HNO_3. Crystal Violet is crystalloid salt and has high absorptivity of visible light. To obtain a large absorbance change on the indirect method, the concentration of Crystal Violet (1.4×10^{-3} M) was suitable for the COS determination in range of 10^{-3}–10^{-4} eq. mol L^{-1}.

2.2. Coating of the capillary column

A fused silica capillary (I.D. 50 μm, O.D. 375 μm, 1010-31842, GL Sciences Inc., Tokyo, Japan) was washed with solutions in the following order: 1 M NaOH solution for 1 h, 1 M HNO_3 solution for 1 h, and finally pure water for 30 min. A silane coupling solution was prepared by mixing 0.4 g of TMPTMAC 50% methanol solution, 0.5 mL of 0.1 M acetate buffer solution (pH 3.5), and 2.5 mL of ethanol. The silane coupling solution and ethanol were flowed in turn into the capillary for 20 and 15 min, respectively. After repeating this treatment, the capillary was dried at 100 °C for 18 h. Only first coating was insufficient and two coats indicated same electroosmotic force to third coats. The electroosmotic force of the coated capillary was determined by electromigration of DMF as a neutral maker at 214 nm. The electroosmotic force was $6.4 \pm 0.1 \times 10^{-4}$ cm^2 V^{-1} s^{-1} from pH 2.0 to pH 4.0.

2.3. Capillary electrophoresis

Capillary electrophoresis was performed using a high voltage supplier (HCZE-30P No. 25, Matsusada precision Inc., Osaka, Japan), a coated capillary column of 80 cm × 50 μm I.D. (55 cm effective length), a UV–vis detector (CV^4 capillary electrophoresis absorbance detector, ISCO Inc., CA), and a digital voltmeter (PC500 DIGITAL MULTIMETER, Sanwa Electric Instrument Co., Ltd., Tokyo, Japan). The digital signals per second were recorded with a personal computer, and an electropherogram was obtained.

The coated capillary was conditioned with the Crystal Violet acidic solution for 5 min at high pressure. Sample injection was performed at low pressure (4 kPa) for 3 s. After the Crystal Violet acidic solution was set, the separation voltage and detection wavelength were adjusted to −15 kV and 540 nm, respectively. The separation was performed at room temperature.

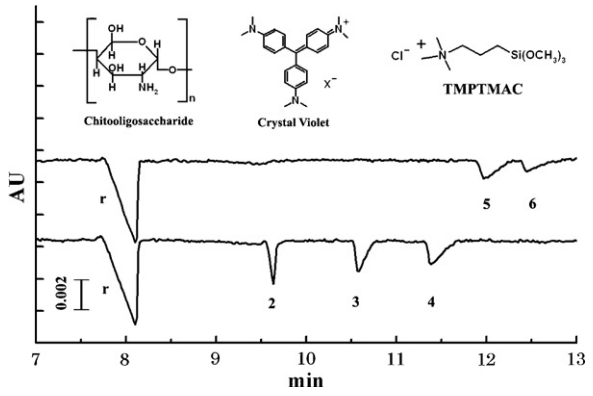

Fig. 1. Electropherograms of 2.5×10^{-4} eq. mol L^{-1} COS (C2–C6) with 1 mM Crystal Violet in an aqueous nitric acid solution at pH 3.0. Numbers and the letter r correspond to each chitooligosaccharide and the tetraphenyl phosphonium chloride used as an internal standard, respectively.

The electrophoretic mobility was measured by the electromigration of each COS with the neutral marker at 540 nm.

3. Results and discussion

3.1. Separation of COS

The positively coated capillary column decomposed in neutral and alkaline solutions. Therefore the COS migration conditions were examined only in acidic solution. Since COS bears positive charges in acidic aqueous solution, the electrophoretic separation must be due to an effective charge on each COS. Electropherograms were obtained of mixtures of C2, C3, and C4, and of C5, and C6 in pH 3.0 nitric acid solution, with each COS at 2.5×10^{-4} eq. mol L^{-1} (Fig. 1). COS migration time increased with the number of oligosaccharide chains, and the mixtures could be separated.

COS dissociation affects the charge carried at a given pH. The dissociation constant (Ka) for chitosan amines depends on its molecular weight. We reported apparent Ka for C1, COS, and large-molecular-weight chitosans [18], and also estimated the effect of salt concentration. The apparent pKa decreased with the degree of dissociation (α), particularly at low salt concentration. A calculation from the α dependence of the pKa for COS (apparent pKa > 6 at α = 0.8 in 1.5 mM NaCl solution) indicates that COS amine groups should be almost completely protonated at pH < 4 [18]. In this study, we observed equal electromigration at pH 3.0 and at pH 3.5, indicating that COS is completely dissociated at this low pH.

3.2. Electrophoretic mobility of COS

Aider et al. had reported the electrophoretic mobility of COS from pH 2–12 using a Zetasizer 2000 system equipped with a photon correlation spectroscopy [14,15]. Their results indicated that all of COS mobility were almost constant and strongly depended on the concentration of COS. Fig. 2 shows the results of present examination at pH 3.0. The negative mobility of five COSs and C1 increases with the number of oligosaccharide chains. As for the conflict of the results, we surmised that COS in an acidic solution adsorbed onto the measurement cell within their system. In fact, when we tried a bare fused silica capillary column, significant peaks of COS could not be obtained in an acidic solution.

Polyelectrolytes in aqueous solution are typically modeled as charged linear chains surrounded by counterions [19–23]. According to the counterion condensation theory [24], polyelectrolyte

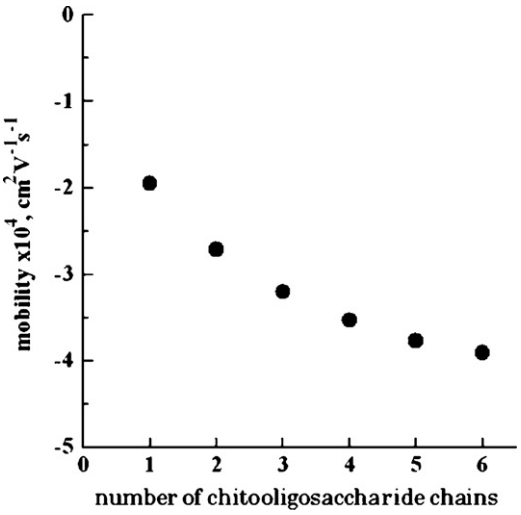

Fig. 2. Dependence of electrophoretic mobility on the number of positively charged chitooligosaccharide chains.

mobility follows the charge density when the polymer molecular weight is large and its charge density is low. Large molecular weight polyelectrolytes have similar electrophoretic mobility, with so-called free draining behavior occurring. However, the counterion condensation theory cannot explain the electrophoretic mobility of small oligomers [25,26]. As well as the COS in the present study, other small oligomers of natural and synthetic polyelectrolytes, such as DNA [16,25] and polystyrene sulfonate (PSS) [25], have displayed a strong molecular weight dependence of electrophoretic mobility. Although COS is positively charged, it is reasonable that its electrophoretic mobility is similar to DNA and PSS. The molecular weight dependence of single-strand DNA has been explained by hydrodynamic interaction between neighboring charged monomer units [25] or a decrease in solvent friction as the conformation of the oligomer gradually charges from a rigid rod to a worm-like coil [16]. The electrophoretic behavior of PSS oligomers has also has been explained by a semi-empirical approach involving physical parameters [26].

Peak shapes in electropherograms from indirect photometric detection can provide information on the relationship between analyte and background probe mobility [27]. Analytes which migrate faster than the probe will have a concentration distribution which is diffuse at the front and sharp at the back of the sample zone. This will result in fronted peaks as displayed by the TPP ion and C2 (Fig. 1). The negative electrophoretic mobility of the Crystal Violet probe is greater than that of both the mono-charged TPP ion and the di-charged C2 ion. Although, both Crystal Violet and TPP are bulky mono-charged ions at pH 3 and the di-charged C2 ion has a pKa = 0.8. The increased negative electrophoretic mobility of Crystal Violet is believed to occur due to formation of poly-charged aggregates under the experimental conditions. Crystal Violet has been reported to form aggregates greater than a dimer at concentrations above 10^{-3} M [28]. Optimum peak shape for indirect photometric detection can be obtained when the mobility of the probe is as close as possible to the mobility of the analytes [27]. Therefore, the electrophoretic mobility of Crystal Violet was suitable for detection poly-charged COS ions.

3.3. Analysis of COS

Analytical parameters for the five COSs (Table 1) were calculated from the peak area ratio between each COS and the internal standard (TPP) in Fig. 1. Parameters A and B are derived from the equation $y = Ac + B$, where y is the peak area ratio, and c is the normality (eq. mol L^{-1}) of COS. Good correlation coefficients (R) were observed for C2, C3, and C4. Low R-values were observed for C5 and C6 due to their small and tailing peak shapes. However, when the electropherogram was obtained with an increased molar concentration of COS, the peak areas and height for C2, C3, C4, C5, and C6 increased to two-, three-, four-, five-, and six-times those in Fig. 1, respectively. The limit of the detection for the five COSs was measured with the peak height at three-times the drift from base line (S/N = 3). The detection limits for C2, C3, C4, C5, and C6 were 2, 2, 2, 3, and 5 μM, respectively.

Table 1
Analytic parameters for each peak area with an internal standard of tetraphenyl phosphonium chloride.

	A	B	R[a]
C2	0.7784	−0.0263	0.9942
C3	0.9601	−0.0134	0.9950
C4	0.7769	0.0192	0.9913
C5	0.6278	0.0552	0.9788
C6	0.5126	−0.0044	0.9814

[a] R is the correlation coefficient for determination of more than 6 points. Refer to the text for descriptions of A and B.

Table 2
Determination of five chitooligosaccharides produced by an enzymatic hydrolysis of chitosan.

Present method			HPLC[a]
COS	Conc.[b] × 10^{-4} eq. mol L^{-1}	Cont. %	Cont. %
C2	1.46 ± 0.16	13.2	11.1
C3	3.02 ± 0.17	27.3	28.6
C4	3.38 ± 0.20	29.8	30.4
C5	2.38 ± 0.36	21.5	22.1
C6	0.91 ± 0.31	8.2	7.8

[a] HPLC condition was following in Ref. [17]; column, TSK gel NH_2-60 (TOSOH Co., Japan); eluent, acetonitrile/water = 55/45 (v/v); RI detector.
[b] Results of five runs.

Five peaks, one due to each oligomer, were detected in the electropherogram of a mixture of COS produced by an enzymatic hydrolysis of chitosan (Fig. 3). No peak was detected for the monomer C1 in our electropherogram, which is consistent with results showing little monomer present in the COS mixture from enzymatic hydrolysis of a chitosanase in contrast to acid hydrolysis [17]. The results from enzymatic hydrolysis (Table 2) almost agree with the HPLC results [17]. The maximum deviations from each mean value for five runs were 11% for C2, 6% for C3, 6% for C4, 15% for C5, and 34% for C6. The mobility of Crystal Violet was more than that of C2 and less than C3, and the determination method worked best for the oligomers closest in mobility to Crystal Violet (C2, C3, and C4). Improved determination for C5 and C6 requires a

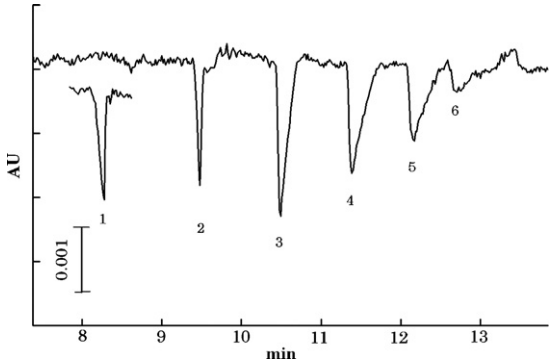

Fig. 3. Electropherogram of chitooligosaccharide produced by enzymatic hydrolysis of chitosan. The extra-peak indicates C1.

background probe with a negative electrophoretic mobility greater than that of Crystal Violet.

4. Conclusion

We have successfully developed a simple determination method for COS involving electrophoretic separation and studied its electrophoretic mobility in acidic solution. It was verified that electrophoretic mobility of positively charged small oligomers depends upon the number of saccharide chains in acidic aqueous solution, similar to DNA and PSS oligomers. Although the analytical parameters for C5 and C6 were not optimized, the results for enzymatically hydrolyzed COS agreed with previous HPLC results. The method developed is suitable for the monitoring of COS in chitosan biotechnology and in the chitosan industry. It requires no derivatization process, only a small amount of sample, no harmful solvents, and utilizes inexpensive equipment.

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