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Notes

Activity Measurement of Chitosanase by an Amperometric Biosensor

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An amperometric biosensor for the detection of glucosamine (GlcN) and chitosan oligosaccharides ($(GlcN)_n$) was introduced for an activity measurement of chitosanase. By using the biosensor, an increase in the anodic current due to the production of GlcN and $(GlcN)_n$ by chitosanase was measured in a chitosan solution. The maximum value of the slope of the current increase was proportional to the enzyme concentration up to 1.4 μg mL^{-1}. The present method had the advantages of being simple and rapid over the conventional Elson–Morgan method.

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Introduction

Chitosanase (EC 3.2.1.132) is an *endo*-type glycosidase, which hydrolyzes β-(1→4)-linkages between D-glucosamine residues of chitosan. Recently, one of the authors (H. Kimoto) newly found a chitosanase that belongs to the family 8 glycosyl hydrolase group, in a *Paenibacillus fukuinensis* D2 culture supernatant.[1] This enzyme is expected to be a tool for manufacturing chitosan oligosaccharides with potential antibacterial, anticancer, or elicitor activity.[2] So far, the activity of the chitosanase has been assayed by the detection of amino sugars (here, glucosamine and chitosan oligosaccharides) released in the reaction mixture of chitosan and chitosanase after incubation for 10 – 120 min at 37°C. The amino sugars have been measured by the conventional Elson–Morgan method,[3-5] which consists of the following three steps: (i) amino sugars react with acetylacetone in an alkaline solution for 20 min at 100°C to form a chromogenic material, (ii) the chromogenic material gives chromophores upon a treatment in an acid solution with ethanolic *p*-dimethylaminobenzaldehyde for 30 min, and (iii) the absorbance of the chromophores at 530 nm, which is proportional to the concentration of the amino sugars in the original solution, is measured. However, boiling in an alkaline solution and a treatment in an acid solution are troublesome and time-consuming. Thus, a simpler and faster method for activity measurements of chitosanase has been desired.

In this work, we introduced an amperometric biosensor for the detection of amino sugars in an activity measurement of chitosanase. To measure the concentration of amino sugars in a chitosan solution with high sensitivity, we employed an electrochemical biosensor with pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH, EC 1.1.5.2), which had been previously constructed for cellobiose detection.[6] PQQ-GDH has a low substrate specificity,[7] and can use not only glucose, but also glucosamine and chitosan oligosaccharides as substrates. By using the PQQ-GDH-immobilized biosensor, the activity measurement of chitosanase was very much simplified. The experimental procedure and results are given in this paper.

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Experimental

Reagents and chemicals

Chitosanase was obtained by an overexpression of the recombinant chitosanase gene of *Paenibacillus fukuinensis* D2 in *Escherichia coli* XL1-Blue, and was purified as described previously.[1] In this work, the catalytic domain of the chitosanase, deficient in the discoidin domains (COOH-terminal 271-amino acid region),[2] was used. The specific activity of the enzyme was determined to be 20.1 units mg^{-1} by the conventional method described above with glycol chitosan as a substrate. Here, one unit of activity was defined as the amount of enzyme catalyzing the production of 1 μmol of the amino sugar per min, using glucosamine as the standard. Chitosan 10B (less than 10% acetylated chitosan) was obtained from Funakoshi Co. Ltd. (Tokyo, Japan). D-Glucosamine (GlcN) hydrochloride was purchased from Nacalai Tesque (Kyoto, Japan). Hydrochlorides of chitosan oligosaccharides ($(GlcN)_n$, n = 2 – 6) were purchased from Seikagaku Corporation (Tokyo, Japan). All other chemicals were of reagent grade, and used as received.

Electrochemical measurements

To measure the concentration of GlcN and $(GlcN)_n$ produced by the enzymatic hydrolysis of chitosan, we used a PQQ-GDH-immobilized benzoquinone-mixed carbon paste electrode (PQQ-GDH-BQ-CPE). The PQQ-GDH-BQ-CPE was prepared as described previously.[6] The electrode potential was fixed at +0.50 V *versus* a Ag | AgCl | 0.1 M KCl reference electrode, and the current (I) due to the enzymatic oxidation of GlcN and $(GlcN)_n$ was recorded as a function of time (t). The test solution was stirred by a magnetic stirrer at 500 rpm, at which the concentration polarization of GlcN and $(GlcN)_n$ could be neglected. The experiments were performed at 37 ± 1°C.

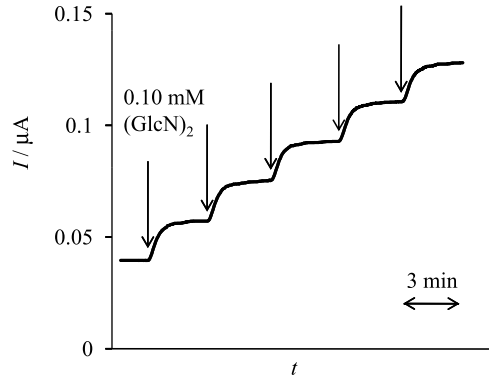

Fig. 1 Current response of the biosensor for the successive addition of 0.10 mM $(GlcN)_2$ in a 0.4% (w/v) chitosan solution (pH 5.9) at 37°C.

Table 1 Sensitivities, limits of detection, and response times of PQQ-GDH-BQ-CPE for GlcN and $(GlcN)_n$ (n = 2 – 6)

Analyte	Sensitivity/ μA mM^{-1} [a]	Limit of detection/ μM[b]	Response time/ min
GlcN	0.30 ± 0.02	8	1.5
$(GlcN)_2$	0.20 ± 0.03	6	1.5
$(GlcN)_3$	0.16 ± 0.01	6	2
$(GlcN)_4$	0.14 ± 0.02	8	3
$(GlcN)_5$	0.10 ± 0.03	8	4
$(GlcN)_6$	0.09 ± 0.01	6	6

a. Average with standard deviation from 3 separate experiments.
b. [S/N] = 3.

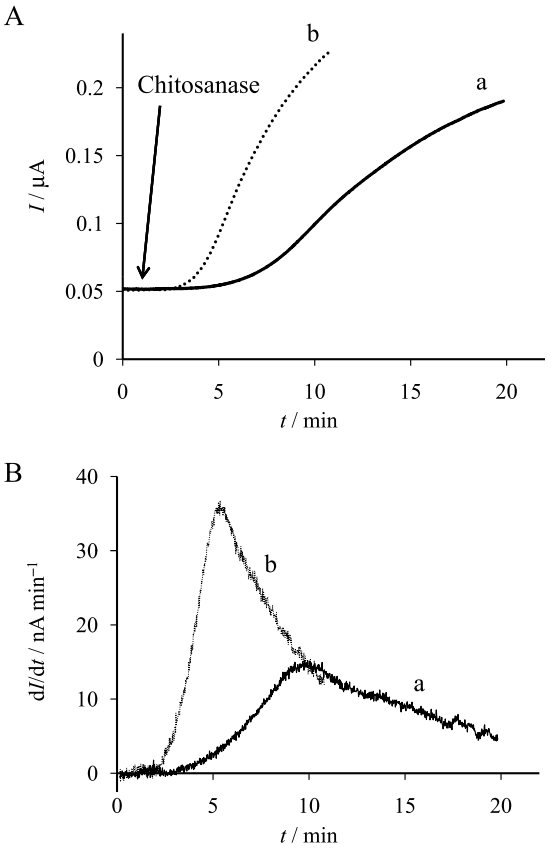

Fig. 2 (A) Current (I)–time (t) curve for the production of GlcN and $(GlcN)_n$ by the chitosanase-catalyzed hydrolysis of chitosan. Measurements were carried out in a 0.4% (w/v) chitosan solution (pH 5.9) at 37°C. Chitosanase ($[E]_0$ = (a) 0.74 μg mL^{-1}, (b) 2.0 μg mL^{-1}) was added at the point indicated by the arrow. (B) Differential curves of the corresponding I–t curves in (A).

Results and Discussion

Figure 1 shows the current response of PQQ-GDH-BQ-CPE for the successive addition of 0.10 mM $(GlcN)_2$ in a 0.4% (w/v) chitosan solution (pH 5.9) at 37°C. Upon each addition of $(GlcN)_2$, the current began to increase, and reached a steady-state value within 1.5 min. The catalytic steady-state current (I_s) was proportional to $[(GlcN)_2]$ up to 1.2 mM. From the slope of the regression line, the sensitivity for $(GlcN)_2$ was determined to be 0.20 μA mM^{-1}. The limit of detection (signal/noise, [S/N] = 3) was 6 μM. Similar current responses were also obtained with GlcN and $(GlcN)_n$ (n = 3 – 6). The I_s-values decreased and the response times increased as the degree of polymerization (n) of $(GlcN)_n$ became larger. The sensitivities, the limits of detection, and the response times for GlcN and $(GlcN)_n$ (n = 3 – 6) were determined in a similar manner as above, and are summarized in Table 1 together with the results for $(GlcN)_2$.

Figure 2A shows an I–t curve for a 0.4% (w/v) chitosan solution (pH 5.9) at 37°C. The small current observed before the addition of chitosanase to the solution indicated that the contamination of GlcN and $(GlcN)_n$ from chitosan was minor. In curve a of Fig. 2A, chitosanase (the total concentration of enzyme, $[E]_0$ = 0.74 μg mL^{-1}) was added at the point indicated by the arrow (t = 1 min). The current did not increase until t = 5 min. This is probably because at the initial stage of the reaction, higher chitosan oligosaccharides, for which the sensitivities of PQQ-GDH-BQ-CPE were very small, were produced predominantly. At t = 5 min, the anodic current began to increase due to the production of GlcN and $(GlcN)_n$ by the chitosanase-catalyzed hydrolysis of chitosan. The increase in the current may be attributed mainly to increases in the concentrations of $(GlcN)_2$ and $(GlcN)_3$, because the sensitivity of PQQ-GDH-BQ-CPE was large for small n, and the main products of the enzymatic hydrolysis (16 h) of soluble chitosan at 37°C and at pH 6.0, as determined by liquid chromatography, were $(GlcN)_2$ and $(GlcN)_3$ (44 and 37% of the total products, respectively). The slope of the I–t curve gradually decreased after t = 10 min, indicating that the hydrolysis rate decreased due to the formation of nonproductive complexes[8] as n of the substrate decreased. In curve b of Fig. 2A, chitosanase of $[E]_0$ = 2.0 μg mL^{-1} was added at t = 1 min. Although the shape of the I–t curve was similar to curve a, the anodic current began to increase earlier, and its slope was steeper than curve a. The slope decreased after t = 5 min. Figure 2B shows differential curves of the corresponding I–t curves in Fig. 2A. It was observed that the differential coefficients of I by t, that is dI/dt, of curves a and b had maximum values of 14.4 and 35.2 nA min^{-1}, respectively. Similar I–t curves and differential curves were also obtained with several other $[E]_0$'s. Figure 3 shows a plot of the maximum values of dI/dt, $(dI/dt)_{max}$, against $[E]_0$. The $(dI/dt)_{max}$-value was proportional to $[E]_0$ up to 1.4 μg mL^{-1}. The regression line in Fig. 3 is expressed by

$$(dI/dt)_{max}/\text{nA min}^{-1} = (18.8 \pm 1.6) \times [E]_0/\mu\text{g mL}^{-1} + (0.7 \pm 1.0). \quad (1)$$

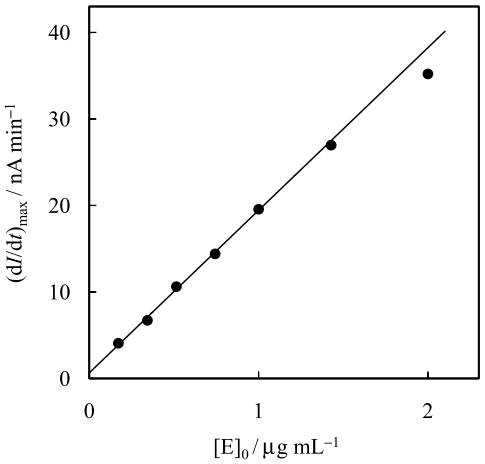

Fig. 3 Dependence of $(dI/dt)_{max}$ on $[E]_0$. The solid line is the regression line.

The limit of detection ([*S*/*N*] = 3) for chitosanase was 0.05 μg mL^{-1}. When $[E]_0$ = 2.0 μg mL^{-1}, the $(dI/dt)_{max}$ value obtained was smaller than that expected from the regression line. Perhaps the response time of PQQ-GDH-BQ-CPE (*e.g.*, 2 min for $(GlcN)_3$) was so slow that it could not follow a steep rise in current when $[E]_0$ was large (see curve b of Fig. 2A).

Thus, it is concluded that the present method using PQQ-GDH-BQ-CPE can be applied to an activity measurement of chitosanase. The method has the advantages of being simple and rapid over the conventional Elson–Morgan method.

Acknowledgements

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