EXO-BETA-D-GLUCOSAMINIDASE FROM *AMYCOLATOPSIS ORIENTALIS*: CATALYTIC RESIDUES, SUGAR RECOGNITION SPECIFICITY, KINETICS, AND SYNERGISM.

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Abstract

Structure and function of family GH-2 exo-β-D-glucosaminidase (GlcNase) from *Amycolatopsis orientalis* were investigated using the wild type and mutated enzymes. Mutations were introduced into the putative catalytic residues, and the four mutated enzymes (D469A, D469E, E541D, and E541Q) were successfully produced. The individual mutations were found to abolish the enzymatic activity, indicating that Asp469 and Glu541 are essential for catalysis as expected from the sequence alignment of enzymes belonging to family GH-2. Enzymatic products from mono-*N*-acetylated chitotetraose, [$(GlcN)_3$-GlcNAc], revealed that the enzyme split off the nonreducing end GlcN residue through a retaining mechanism. The rate of hydrolysis of the disaccharide, GlcN-GlcNAc, was slightly lower than that of $(GlcN)_2$, suggesting that *N*-acetyl group of the sugar residue located at (+1) site partly interferes with the catalytic reaction. The time-course of the enzymatic hydrolysis of the completely deacetylated chitotetraose [$(GlcN)_4$] was quantitatively determined by HPLC, and used for modeling *in silico* the enzymatic hydrolysis. The modeling study provided the values of binding free energy changes of the individual subsites, (-2)(-1)(+1)(+2)(+3)(+4), +7.0, -2.9, -1.8, -0.9, -1.0 and -0.5 kcal/mol, respectively. When chitosan polysaccharide was hydrolyzed by binary enzyme system consisting of *A. orientalis* GlcNase and *Streptomyces* sp. N174 endo-chitosanase, the highest synergy in the rate of product formation was observed at the molar ratio 2:1. Thus, GlcNase would be an efficient tool for industrial production of glucosamine monosaccharide.

Introduction

Chitin and chitosan are widely distributed in living organisms, including insects, crustaceans and fungi. In such organisms, chitinases and chitosanases are directly involved in biological phenomena important for their life; such as moulting, morphogenesis, and aggressive and defensive actions toward the targets. It is now widely accepted that two independent chemical processes catalyzed, respectively, by endochitinases and exo-β-*N*-acetyl-D-glucosaminidases are involved in chitin degradation. A similar mechanism has been considered for chitosan degradation: endochitosanases randomly hydrolyze chitosan polysaccharide, producing oligosaccharides, which are then hydrolyzed into the monosaccharide units by exo-β-glucosaminidase (GlcNase). Thus, GlcNase is an important enzyme for glucosamine monomer production. Nevertheless, very few studies have so far been dedicated to GlcNases. GlcNase was first purified from an actinomycete, *Nocardia orientalis* (present name: *Amycolatopsis orientalis*), and characterized by Nanjo et al. [1].

The enzyme was found to specifically hydrolyze the β-1,4-glucosaminide linkage of the non-reducing end GlcN residue, producing the monosaccharide unit. In our previous paper [2], we reported the deduced amino acid sequence of GlcNase from *Amycolatopsis orientalis* (GenBank/EBI/DDBJ accession number AY962188). The enzyme belongs to family GH-2 according to the CAZy database (http://afmb.cnrs-mrs.fr/CAZY/fam/acc_GH.html), and has a unique modular structure consisting of a sugar binding domain, an immunoglobulin-like β-sandwich domain, and a TIM barrel catalytic domain. In addition, these domains are followed by a putative carbohydrate binding module belonging to family CBM-6. Based on sequence alignments of *A. orientalis* GlcNase with other GH-2 members, we suggested that Asp469 and Glu541 are the putative catalytic residues [2]. In the current study, to further extend the knowledge of GlcNase from *A. orientalis*, we investigated the catalytic residues, sugar recognition specificity, and kinetic properties of the enzyme, using the wild type and its mutants. Moreover, using the binary enzyme system consisting of *A. orientalis* GlcNase and endochitosanase from *Streptomyces* sp. N174, the rate of product formation from chitosan hydrolysis was measured to examine the synergistic effect resulting from mixing the two enzymes.

Materials and methods

Materials

All glucosamine oligomers $(GlcN)_{2-6}$ were purchased from Seikagaku Kogyo Co. Chitosan 10B (99% de-*N*-acetylated) was from Funakoshi Chemical Co. The chitosan was partially degraded by nitrous acid, and the low molecular weight chitosan was used as the substrate for GlcNase. $(GlcN)_3$-GlcNAc and GlcN-GlcNAc were obtained by the method of Mitsutomi et al. [3]. Chitosanase from *Streptomyces* sp. N174 was produced by the expression system of *Streptomyces lividans* [4]. The other reagents were of analytical grade commercially available.

Site-directed mutagenesis and production of mutated enzymes

Site-directed mutagenesis was performed by the method of Ho *et al.*, [5] involving polymerase chain reaction using high fidelity *Pfu* DNA polymerase (Fermentas, Burlington ON). The procedure exploited the fact that the triplets encoding the residues to be mutated (D469; E541) were localized between unique restriction sites *Bsm*I and *Eco*RI. In a first series of amplifications, a common primer adjacent to the *Bsm*I site was used with the reverse primer specific for each mutation. In parallel series of amplifications, a common primer adjacent to the *Eco*RI site was used with the forward primer specific for each mutation. After purification of both amplicons on agarose gel, a second series of PCR was performed with the same external primers. The resulting mutated amplicons were cloned into the original vector using unique sites *Bsm*I and *Eco*RI. All mutants were sequenced in both orientations. Wild type and mutated GlcNases from *A. orientalis* were produced using the recombinant system established in the heterologous host *Streptomyces lividans* TK24 [2]. Structures of the mutated enzymes were assessed by CD spectra recorded in 50 mM sodium acetate buffer pH 5.3,using a Jasco J-720 spectropolarimeter at 20 ^{o}C.

Enzyme assay

GlcNase assay was performed combining 100 µl of 2 mM $(GlcN)_2$ in 50 mM sodium acetate buffer, pH 5.3, with 100 µl of diluted enzyme preparation. The reaction mixture was incubated for 10 min at 37 ^{o}C, and terminated by the addition of 25 µl of 0.2 M sodium tetraborate. The GlcN liberated from the dimer was *N*-acetylated by 25 µl of 1.5% acetic anhydride in acetone, and the resultant *N*-acetylglucosamine was determined by the method of Reissig et al. [6].

^{1}H-NMR spectroscopy

The oligosaccharide substrate was lyophilized three times from D_2O, and then dissolved in 0.5 ml of D_2O. The solution pH was adjusted to 5.0. The substrate solution was placed in a 5 mm NMR tube, and 60 µl of the enzyme solution (0.3 µM) was added to start the enzymatic reaction. The NMR tube was immediately set into a 5 mm probe on a JEOL EX-270 instrument, which was

thermostatically controlled at 25 °C. After an appropriate reaction time, the accumulation of a ^{1}H-NMR spectrum was started. One accumulation required 3 min. The substrate concentration was 8.3 mM

Time-course of the enzymatic reaction

Enzymatic reaction toward oligosaccharide substrate was conducted in 50 mM sodium acetate buffer pH 5.0 at 37 °C. Three microliters of the enzyme solution (0.48 μM) were added to 100 μl of the substrate solution (12.3 mM). After an appropriate reaction time, 10 μl of the reaction mixture were combined with the same volume of 0.1 M NaOH to terminate the enzymatic reaction. The oligosaccharide products obtained from enzyme digestion were determined by a gel-filtration HPLC on TSK-GEL G2000PW column (7.5 mm x 600 mm, Tosoh) using a Hitachi L-7100 intelligent pump. Elution was achieved with 0.1 M NaCl at room temperature at a flow rate of 0.3 ml/min. The products were detected by ultraviolet absorption at 220 nm or a refractive index detector.

In silico *modeling of the enzymatic hydrolysis of chitotetraose*

In silico modeling of the enzymatic hydrolysis of chitotetraose was carried out by the method of Fukamizo et al. [7]. Since the enzyme hydrolyzes the oligosaccharide substrate from the nonreducing end with an exo-splitting mode, the binding cleft of the enzyme was assumed to consist of the subsites (-2), (-1), (+1), (+2), (+3), and (+4), where the (-2) site should have an unfavorable positive binding free energy. Reaction parameters consist of three rate constants, k_{+1} (glycosidic bond cleavage), k_{-1} (transglycosylation), and k_{+2} (hydration), and six binding free energy changes of individual subsites, (-2)~(+4). In practical calculations, all of the possible binding modes were taken into consideration. By assuming rapid equilibrium, the concentrations of the ES-complexes were calculated from the binding constants, which were obtained from the binding free energy values of individual subsites occupied with the sugar residues assuming additivity. Details of the calculation method were described by Fukamizo et al. [7].

Chitosan hydrolysis by the binary enzyme system of GlcNase and endo-splitting chitosanase

To examine synergistic effect resulting from mixing *A. orientalis* GlcNase with endo-splitting chitosanase from *Streptomyces* sp. N174, we determined the rate of product formation from the low molecular weight chitosan by the binary enzyme system. At first, the two enzymes, dialyzed against 50 mM sodium acetate buffer pH 5.0 were mixed together at various exo/endo ratios, and the enzyme mixture solution (340 μl) was added to the chitosan solution (15 mg/ml, 160 μl) to start the enzymatic reaction. The reaction mixture was incubated for two hours at 37 °C. The enzymatic product was determined by the modified Elson-Morgan method with glucosamine as standards [8].

Results and Discussion

Catalytic residues

In the previous paper [2], we reported that two GlcNases define a new subfamily within family GH-2 glycosyl hydrolases. Sequence alignment of the GH-2 enzymes revealed that most GH-2 members possess a catalytically important NE diad, which is substituted with a SD diad in GlcNases from *A. orientalis*. The SD diad corresponds to Ser468 and Asp469 in the *Amycolatopsis* GlcNase. A strictly conserved glutamic acid was found at the 541st position of *A. orientalis* GlcNase, and is supposed to be the catalytic nucleophile. Thus, we mutated Asp469 and Glu541 residues of GlcNase to elucidate their role. We successfully produced and purified the wild type and four mutated enzymes (D469E, D469A, E541D, and E541Q). CD spectrum of D469E is different from that of the wild type, indicating that the mutation of Asp469 to glutamic acid would affect the secondary structure of the enzyme. In the spectra for the other mutated enzymes, however, the profiles are basically identical to that of the wild type. The global conformation of the enzyme is not affected in D469A, E541D, and E541Q. Enzymatic activities of these enzyme

preparations were determined by quantifying the liberated GlcN from the substrate $(GlcN)_2$. The mutation of Asp469 to alanine completely abolished the enzymatic activity as listed in Table 1, while D469E exhibited very low enzymatic activity. Both the E541-mutated enzymes were found to possess very low activity, but the activity of E541D is significantly larger than that of E541Q. These results clearly demonstrated that Asp469 and Glu541 are the catalytic acid and base, respectively.

Table 1. Enzymatic activities of the wild type and mutated exo-β-glucosaminidase from *Amycolatopsis orientalis*

Enzyme	Activity (Units/mg)	Relative activity (%)
Wild type	14.95	100.0
D469E	0.0011	0.007
D469A	not detected	——
E541D	0.016	0.10
E541Q	0.0019	0.012

Enzymatic hydrolysis of $(GlcN)_3$-GlcNAc, as determined by ^{1}H-NMR and HPLC

To examine the catalytic mechanism of the enzyme, we determined the anomeric form of the reaction products from the enzymatic hydrolysis of $(GlcN)_3$-GlcNAc. In ^{1}H-NMR spectra of the enzymatic reaction mixture, the doublet signal derived from the β-form of the GlcN monomer H1 appeared immediately after beginning the enzymatic reaction. However, the signal from the α-form did not appear until 30 min of the reaction time. The result suggests that the enzyme produces the β-form, which is then converted to the α-form by mutarotation. The enzyme was found to be a retaining enzyme.

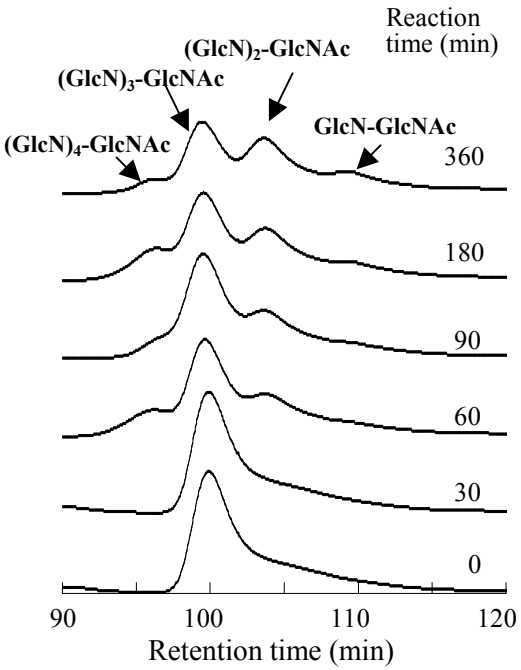

Fig. 1 Time-dependent HPLC profiles showing the enzymatic hydrolysis of $(GlcN)_3$-GlcNAc.

The enzymatic reaction was monitored by HPLC using detection by UV absorption (220 nm) originating from the *N*-acetyl groups of the substrate and the products. The resulting time-dependent HPLC profiles are shown in Figure 1. The initial substrate $(GlcN)_3$-GlcNAc was at first degraded into $(GlcN)_2$-GlcNAc, and then into GlcN-GlcNAc, but no *N*-acetylglucosamine monomer was produced at this stage. This indicates that the enzyme hydrolyzes the substrate from the nonreducing end in an exo-splitting manner. After a longer incubation period, the substrate was finally hydrolyzed into monosaccharides, GlcN and GlcNAc (data not shown). To evaluate the sugar recognition specificity at the (+1) site, we determined the rate of hydrolysis of GlcN-GlcNAc, and compared with that of $(GlcN)_2$. As shown in Figure 2, similar HPLC profiles were obtained for both substrates, suggesting that the (+1) site is specific to neither acetylated nor deacetylated residue. However, closer examination of the profiles indicated that the rate of the degradation of GlcN-GlcNAc is lower than that of $(GlcN)_2$, The *N*-acetyl group of the sugar bound to the (+1) site appears

to partly interfere with the catalytic reaction. Since this enzyme does not act toward $(GlcNAc)_2$, the (-1) site must have absolute specificity for GlcN.

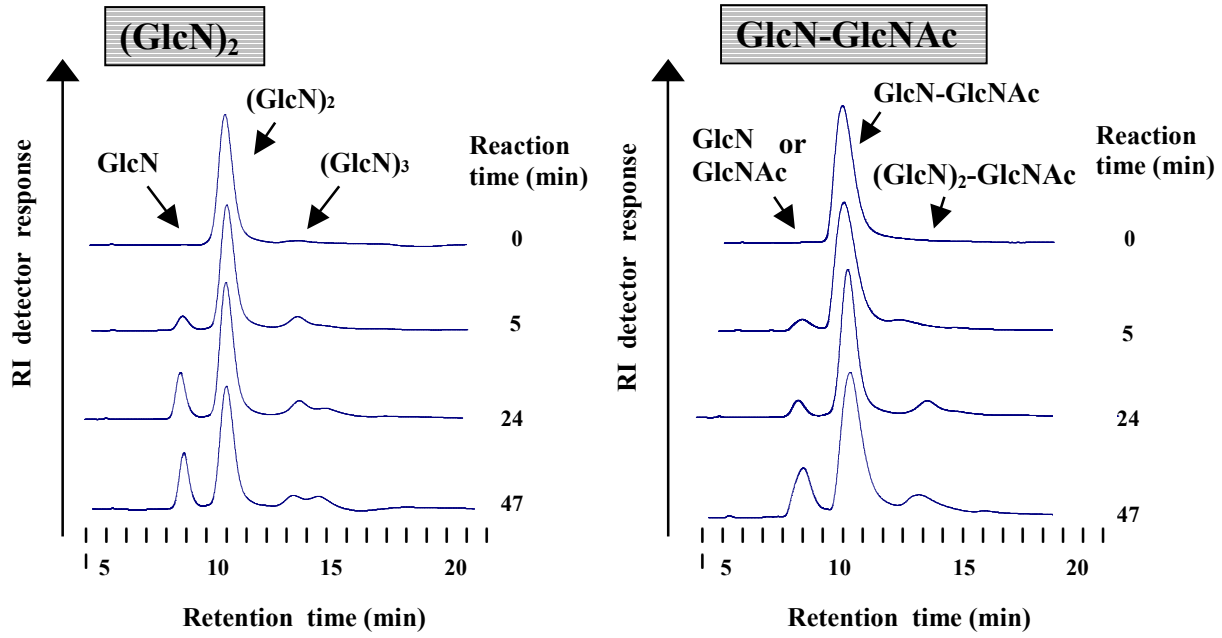

Fig. 2 Time-dependent HPLC profiles showing the enzymatic hydrolysis of $(GlcN)_2$ and GlcN-GlcNAc.

It should be noted that the transglycosylation products, whose molecular weights are larger than that of the initial substrate, were detected in significant amounts (Figure 1). The transglycosylation reaction catalyzed by the enzyme would be useful for producing GlcN-containing oligosaccharide derivatives with unique biological functions.

Experimental time-course of the enzymatic hydrolysis of $(GlcN)_4$

The experimental time-course of the reaction is shown in Figure 3A. At first, the enzyme predominantly produced GlcN and $(GlcN)_3$, which was further degraded into GlcN and $(GlcN)_2$. The course of the degradation exhibited a typical profile for an exo-splitting enzyme. The transglycosylation product, $(GlcN)_5$, was also produced, together with a lesser amount of $(GlcN)_6$. It appears that $(GlcN)_5$ is produced by the glycosyl transfer of the transition state GlcN to the initial substrate $(GlcN)_4$. Similarly, $(GlcN)_6$ appears to be produced by the transfer action to the product $(GlcN)_5$. The reaction time-course quantitatively determined by HPLC was used for the modeling study described below.

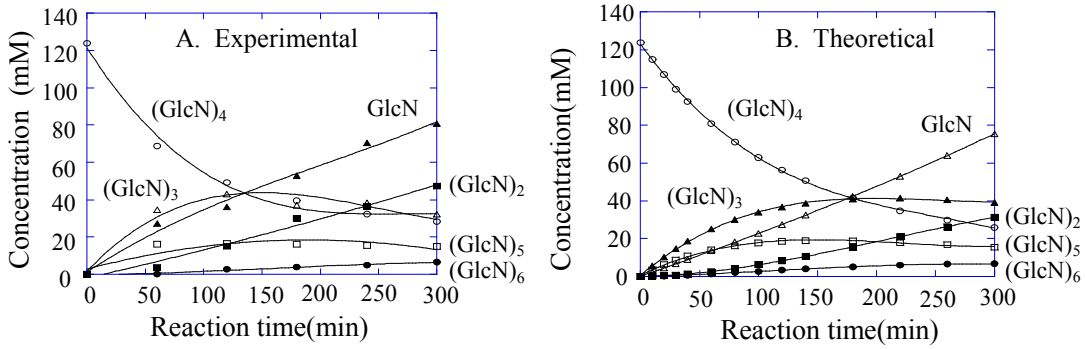

Fig. 3 Time-courses of $(GlcN)_4$ hydrolysis catalyzed by GlcNase from *A. orientalis*

In silico *modeling of the enzymatic hydrolysis of $(GlcN)_4$*

At first, we experimentally estimated the turnover numbers (k_{cat}) from the maximal velocity data of the individual oligosaccharide substrates at the saturated condition, and then the k_{cat} values were directly allocated to k_{+1}. The values are listed in Table 2. The individual k_{+1} values were fixed in the modeling calculation. The values of k_{-1} and k_{+2} could not be determined independently, but the ratio k_{-1}/k_{+2} could be determined based on the time-course data [7]. Thus, the k_{+2} value was tentatively fixed at 200 sec^{-1}, and the k_{-1} value was optimized based on the time-course. Since *A. orientalis* GlcNase split off the monomer unit from the non-reducing end of the substrate, (-2) site should be the most unfavorable for binding of the GlcN residue. Thus, a very high positive value (+7.0 kcal/mol) was tentatively allocated to the binding free energy value of (-2) site. In the modeling calculation, the values of k_{+1} and the binding free energy of (-2) site were kept constant, and the k_{-1} value (rate constant for transglycosylation) and the binding free energy values of individual subsites from (-1) to (+4) were optimized based on the experimental time-course (Figure 3A). The optimization successfully produced the k_{-1} value and the free energy values of the individual subsites as listed in Table 2. The time-course calculated with these values coincided satisfactorily with the experimental one as shown in Figure 3B. As to the free energy value of (-2) site, the product distribution of the calculated time-course was greatly affected upon descending the free energy value from +7.0 kcal/mol to +6.0 or +5.0 kcal/mol, resulting in the larger value of the cost function (Eq. (1)). However, the profile of the calculated time-course was hardly affected upon elevating the value from +7.0 kcal/mol. Thus, the free energy value of (-2) site should be larger than or almost equal to +7.0 kcal/mol, but could not be determined accurately. The affinities for sugar residues were found to be highest at subsites (-1) and (+1), while remote subsites were found to have lesser affinities. The free energy distribution in the substrate binding cleft is very similar to those of glucoamylases possessing similar cleavage specificity [9].

Table 2. Rate constants and binding free energy values used for the calculation of reaction time-course shown in Fig. 3B.

	Rate constants (s^{-1})			Binding free energy changes (kcal/mol)					
	k_{+1}	k_{-1}	k_{+2}	(-2)	(-1)	(+1)	(+2)	(+3)	(+4)
$(GlcN)_2$	120.0								
$(GlcN)_3$	120.0								
$(GlcN)_4$	60.0	220.0	200.0	+7.0	-2.9	-1.8	-0.9	-1.0	-0.5
$(GlcN)_5$	30.0								
$(GlcN)_6$	25.0								

Synergism between endochitosanase and exo-β-glucosaminidase

We measured the rate of product formation from chitosan by the binary enzyme system consisting of *A. orientalis* GlcNase and *Streptomyces* sp. N174 endochitosanase. The result is shown in Figure 4. In the presence of an excess amount of endochitosanase, the rate of product formation was enhanced by about two-fold of the sum of those obtained by individual enzymes; that is, the synergistic factor was about two. By increasing the ratio of GlcNase to the endo-splitting enzyme, the synergistic factor was gradually enhanced, and reached maximum (4.1) when the enzyme molar ratio was 2:1 (exo:endo). In the binary chitinase system (exo-□-*N*-acetylglucosaminidase and endochitinase) of tobacco hornworm, *Manduca sexta*, which is responsible for the destabilization of old cuticle, the greatest synergism of six takes place at a 1:6 (exo:endo) ratio of enzymes, typically found in the moulting fluid secreted from epidermal cells [10]. In the cellulase systems studied thus far, the synergistic factor was reported to be 2~10 [11].

Thus, we conclude that our binary chitosanase system exhibits a moderate synergism and is efficient for inductrial production of glucosamine monomer.

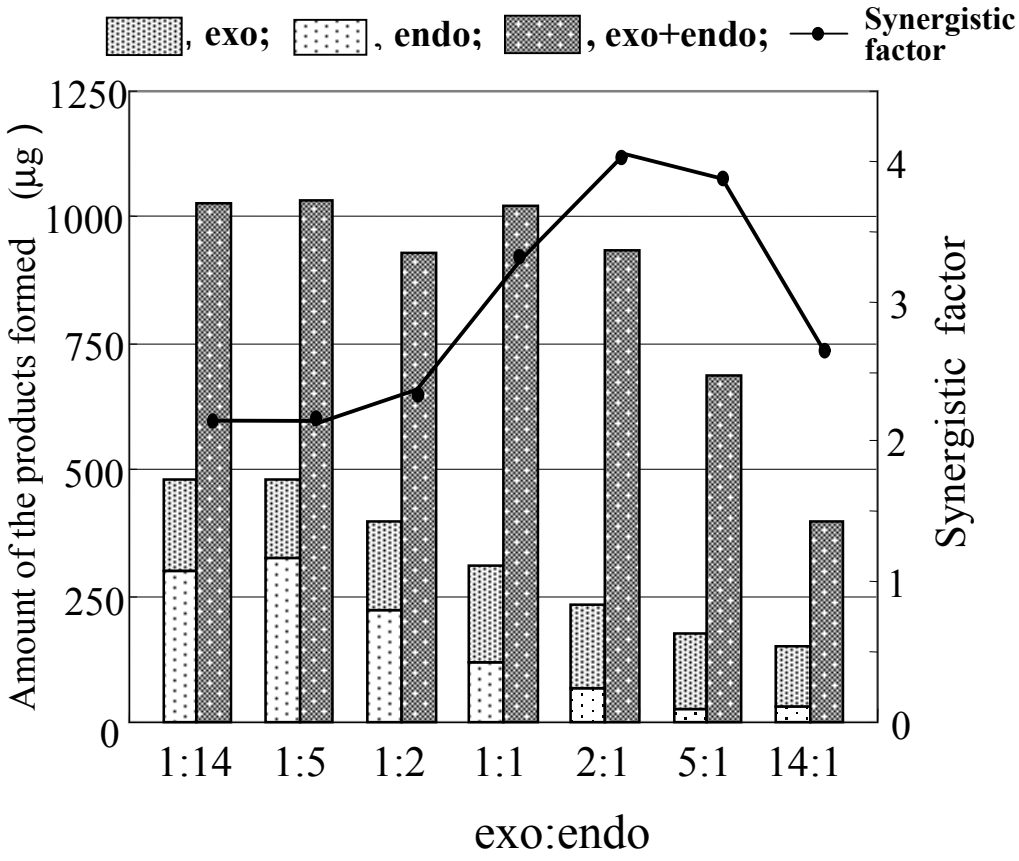

Fig. 4 Synergism resulting from mixing *A. orientalis* GlcNase (exo) with *Streptomyces* sp. N174 endochitosanase (endo).

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