Enhancement of Transglycosylation Activity of Lysozyme by Chemical Modification

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An attempt to enhance the transglycosylation activity of lysozyme was made by chemical modification. Computer simulation of the lysozyme-catalyzed reaction indicated that inhibition of the sugar residue binding to the binding subsite A caused a significant increase in transglycosylation activity. Therefore, the binary modification of Asp 101 and Trp 62 in hen egg white lysozyme was made in order to inhibit the sugar residue binding to subsite A. The modified lysozyme, in which the affinity of the sugar residue to subsite A was decreased by about 2 kcal/mol of binding free energy change, increased the amounts of transglycosylation products in comparison with the native lysozyme. In particular, the octamer of N-acetylglicosamine was abundantly produced from the initial substrate, pentamer. The modified lysozyme should be useful for synthesis of oligosaccharides with a high degree of polymerization.

Chitin and chitosan are noteworthy for their immunological activities, controllabilities in fungal-plant interactions, and some other biological functions.1 In this situation, the lower molecular weight analogues of chitin and chitosan, chitooligosaccharides, are considered to be more advantageous for their use as biological regulators because of their water solubilities.2 Chitooligosaccharides can be obtained by partial hydrolysis of chitin with hydrochloric acid followed by charcoal chromatography and gel filtration.3,4 In acid hydrolysis, oligosaccharides with high degrees of polymerization can be obtained only in low yield. However, using the enzymatic transglycosylation reaction, it is possible to obtain high molecular weight oligosaccharides from the lower ones. In fact, several types of oligosaccharides with chromophores, useful substrates for carboydrolases, were synthesized by the enzymatic transglycosylation reaction which is often observed in the reaction catalyzed by some carboydrolases.5 In this case, however, it is important to find optimum conditions for the transglycosylation.

Hen egg white lysozyme [EC 3.2.1.17], whose structure and reaction mechanism are well understood, is known to catalyze the transglycosylation more efficiently than other carboydrolases.6 Various kinds of chemical modification methods were developed in this enzyme, and the functional variations in company with the modifications were investigated.7,8 Therefore, the chemical modification of lysozyme that is site-directed to a specific binding subsite may enhance the transglycosylation activity.

In this paper, the binary modification of Asp 101 and Trp 62 in the lysozyme molecule was done with D-glucosamine and 2-nitrophenyl-

Abbreviations: GlcNAc, 2-acetamido-2-deoxy-β-D-glucopyranose; (GlcNAc)_n, β-1,4 linked n-mer of GlcNAc; ΔG‡, free energy change of the sugar residue binding to subsite X; DABITC, 4-dimethylaminoazobenzene-4-isothiocyanate; DABTH, 4-dimethylaminoazobenzene-4-thiohydantoin; PITC, phenylisothiocyanate; HPLC, high performance liquid chromatography.
Materials and Methods

Materials. Six times recrystallized hen egg white lysozyme was purchased from Seikagaku Kogyo Co., Ltd. The substrates, chitooligosaccharides, were prepared by acid hydrolysis of chitin followed by charcoal chromatography. Each of the oligosaccharides showed a single peak on high performance gel filtration. Bio-Rex 70 and Sephadex G-25 were the products of Bio-Rad Laboratories and Pharmacia, respectively. 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride and d-glucosamine hydrochloride were from Wako Pure Chemical Industries, and 2-nitrophenylsulfonyl chloride from Tokyo Chemical Industries. Other reagents were of analytical grade commercially available.

Chemical modification of lysozyme. The selective modification of Asp 101 with d-glucosamine was done by the method of Yamada et al., followed by the purification with ion exchange chromatography on Bio-Rex 70. Nitrophenylsulfonylation of the Asp 101-modified lysozyme was done by the method of Schechter et al., and also followed by purification with Bio-Rex 70 column chromatography. A linear gradient elution system of 1 l of 0.1 M sodium phosphate, pH 7.0, and 1 l of 0.4 M sodium phosphate, pH 7.0, was used for the purification of the Asp 101-modified lysozyme, and that of 700 ml of 25 mM sodium borate buffer, pH 10.0, and 700 ml of the same buffer containing 0.2 M sodium chloride was used for the purification of the nitrophenylsulfonyl chloride derivative.

HPLC peptide mapping. Carboxymethylations of the native and the modified lysozyme were done by the method of Crestfield et al., and followed by tryptic hydrolysis at 37°C, pH 8.0, for 12 hr. The tryptic digest was applied on a reverse-phase HPLC column (ODS T120A, 4.6 x 250 mm, Tosoh) using a JASCO 800 HPLC. Peptides were eluted with a linear gradient solvent system of 0.1% trifluoroacetic acid (solvent A) and 60% acetonitrile in solvent A (solvent B).

Composition and sequence of amino acids. Each of the tryptic peptides was hydrolyzed in an evacuated sealed tube at 110°C for 20 hr using constant boiling HCl containing 0.05% β-mercaptoethanol. The hydrolysate was analyzed with a Hitachi Amino Acid Analyzer, Model 835. The amino acid sequence was analyzed by the method of DABITC/PITC double-coupling micro sequencing.

Enzymatic reaction. The enzymatic reaction was done with the substrate (GlcNAc),. The reaction mixture containing 0.03 or 0.04 mM modified lysozyme and 1 mM substrate dissolved in 10 mM sodium acetate, pH 5.0, was incubated at 50°C. At a given reaction time, 500 μl of the reaction mixture was withdrawn and rapidly chilled in solid CO2/acetone. Seventy mg of Bio-Rex 70 was added to it in order to remove the lysozyme. After centrifugation in the cold, the supernatant was lyophilized. The dried sample was dissolved in 50 μl of ice-cold water, and 25 μl of the solution was applied on a gel filtration column (TSK-GEL G2000PW, 7.5 x 600 mm, Tosoh) using a Shimadzu LC-3A. Elution was done with distilled water at room temperature and at a flow rate of 0.3 ml/min. Each chitooligosaccharide concentration was calculated from the peak area monitored by ultraviolet absorption at 220 nm, using the standard curve obtained from authentic saccharide solutions.

Computer simulation. The rate equation of the lysozyme-catalyzed reaction on the initial substrate (GlcNAc), (see Appendix) was numerically solved to obtain the calculated time-courses by the method previously reported. In the calculation, the rate equation was repeatedly solved by changing the value of the binding free energy change at each of the six binding subsites (A, B, C, D, E, and F). Using the concentrations of the products, chitooligosaccharides, obtained by the numerical analysis of the rate equation, the gel filtration profile was simulated by standard Gaussian functions:

\[ A_i(t) = h_i \exp \left[ -2.773 \left( \frac{t - t_i}{w_i} \right)^2 \right] \]  

where \( t \) represents the retention time and \( t_i \) the retention time corresponding to the maximum of peak \( i \), \( w_i \) is the width of peak \( i \) at half-maximum height, and \( h_i \) the maximum height of peak \( i \). \( t_i \) and \( w_i \) for each of the oligosaccharides were obtained from the experimental profile of the gel filtration, and \( h_i \) was calculated from each of the concentrations of the chitooligosaccharides obtained by the numerical analysis of the rate equation assuming the existence of proportionality between the peak height and the concentration. The peak profile for each of the oligosaccharides, \( A_i(t) \) in Eq. (1), was then superimposed on the neighboring peaks, and formed the theoretical profile for gel filtration at a given reaction time.

Data-fitting. Calculation of the time-courses was repeated by varying the value of the binding free energy change for each of the subsites to obtain a minimum of the cost function:

\[ F = \sum \sum |(\text{GlcNAc})_{x,i} - (\text{GlcNAc})_{x,i}^e| \]  

Here, \( e \) and \( c \) are the experimental and the calculated
values, \( n \) is the size of the chito-oligosaccharide, and \( i \) the reaction time. A set of values of the reaction parameters giving the minimum value of \( F \) in Eq. (2) was regarded as the most reliable values of the reaction parameters. In the definition of the cost function, the data for \((\text{GlcNAc})_{3}\) in the early stage of the reaction were not used, because the chromatographic separation of \((\text{GlcNAc})_{3}\) and \((\text{GlcNAc})_{5}\) was not satisfactory in the early stage of the reaction and error could not be avoided.

**Results**

**Computer simulation**

It can be supposed that the selective modification of a specific amino acid participating in the substrate binding weakens the sugar residue binding at the corresponding subsite. Thus, the computer simulation of the lysozyme-catalyzed reaction with the substrate, \((\text{GlcNAc})_{5}\), was done by increasing the value of the free energy change of the sugar residue binding to each of the six binding subsites.

In these calculations, the values of the binding free energy changes which were already estimated for hen egg white lysozyme\(^{13}\) were used as standards. First, the time-courses were calculated by changing the free energy value at subsite \( A \) (\( \Delta G_{A} \)), while fixing the values of the other reaction parameters. Figures 1(a) and 1(b) show the time–courses calculated with 0.0 kcal/mol of \( \Delta G_{A} \), which is higher than the standard value by 2 kcal/mol, and the calculated time–courses as standard, respectively. Due to the inhibition of sugar residue binding to subsite \( A \), the rate of \((\text{GlcNAc})_{5}\) degradation was accelerated, and the amount of each transglycosylation product, whose polymerization degree is higher than that of the initial substrate, clearly increased. The inhibition of the sugar residue binding to subsite \( A \) may increase the amount of the productive ES-complex and may facilitate

![Fig. 1. Calculated Time-Courses of the Lysozyme-catalyzed Reaction with (GlcNAc)\(_5\).](image)

Initial concentrations of the lysozyme and \((\text{GlcNAc})_{5}\) were 0.1 mM and 1.0 mM, respectively. The standard values of the reaction parameters are \( \Delta G_{A} = -2.0 \), \( \Delta G_{B} = -3.0 \), \( \Delta G_{C} = -5.0 \), \( \Delta G_{D} = +4.5 \), \( \Delta G_{E} = -2.5 \), \( \Delta G_{F} = -1.5 \) kcal/mol, \( k_{-1} \) (rate constant for cleavage of \( \beta \)-1,4 glycosidic linkage)=0.93, \( k_{+1} \) (rate constant for regeneration of glycosidic linkage)=40.0, and \( k_{+2} \) (rate constant for hydration)=0.3 sec\(^{-1}\). Numerals in the figures are the polymerization degree of the reaction product species. (a) Calculated time–courses with \( \Delta G_{A} = 0.0 \) kcal/mol. The other values were fixed at the standard ones. (b) Calculated time–courses with the standard values. (c) Calculated time–courses with \( \Delta G_{F} = +0.5 \) kcal/mol. The other values were fixed at the standard ones.
the transglycosylation acceptor binding to sub-
sites E and F. These could enhance the initial
substrate degradation and the transglycosyla-
tion. When the sugar residue affinities at sub-
sites B, C, and D were respectively lowered
with increasing the free energy values by
2 kcal/mol, the rates of the product forma-
tions equally decreased and no significant varia-
tions in the product distribution were found (data
not shown). In the reaction model scheme used
for these simulations, the oligosaccharide ac-
ceptor for the transglycosylation should bind
to subsites E and F of the lysozyme-carbonium
intermediate complex before the regeneration
of the glycosidic linkage.13,14 As expected
from the reaction model, lowering the sugar
residue affinities at subsites E and F is to
decrease the amount of each transglycosyla-
tion product. In fact, a 2 kcal/mol increase
in the free energy value at subsite F markedly
suppressed the amount of each transglycosyla-
tion product (Fig. 1(c)). From these results,
it is concluded that the chemical modification
which can induce a suppression of sugar resi-
due binding to subsite A results in the en-
hancement of the transglycosylation activity of
lysozyme.

Chemical modifications of lysozyme

In our previous papers,7, 8 we reported that
the chemical modification of Asp 101 or Trp
62 inhibited the sugar residue binding to sub-
site A, increasing the $\Delta G_A$-value by 1.1, or
1.7 kcal/mol, respectively. Therefore, in this
study, the binary modification of Asp 101 and
Trp 62 was attempted for obtaining the strong
suppression of the sugar residue binding at
subsite A. First, Asp 101 modification of hen
egg white lysozyme was done with D-glucos-
amine using water soluble carbodiimide.9
After purification of the Asp 101-modified ly-
sozyme, Trp 62 in the modified lysozyme was
2-nitrophenylsulfenylated by the method of
Schecter et al.10 Figure 2 shows the ion-
exchange chromatogram of the lysozyme
treated with the binary modification. The
peak fraction which can be detected with ab-
sorbances both at 280 nm and at 365 nm was
rechromatographed and its amino acid se-
quence was analyzed.

Identification of modified Trp residue

For identification of the modified Trp resi-
due, tryptic digests of carboxymethylated
lysozymes were applied on a reverse-phase
HPLC column. Figures 3(a) and 3(b) show the
HPLC profiles of the tryptic peptides from the
native lysozyme and the lysozyme treated with
the binary modification, respectively. Each
peak was identified by amino acid analysis as
indicated in the figures, referring to the Can-
field’s nomenclature of tryptic peptides.15 In
Fig. 3(b), the peak corresponding to the pep-

Fig. 2. Ion-exchange Chromatography of the Nitro-
phenylsulfenylated Derivative from the Asp 101-modified
Lysozyme on Bio-Rex 70 (100 ~ 200 mesh, 1 x 70 cm).
Elution was done with the linear gradient of 700 ml of 25
mM sodium borate buffer (pH 10.0) and 700 ml of the
same buffer containing 0.2 M sodium chloride.

Fig. 3. Reverse-phase HPLC of the Peptides from the Native Lysozyme (a), and from the Lysozyme Treated
with the Binary Modification (b).
Tryptic digest was applied on a column of ODS T120A (4.6 x 250 mm, Tosoh), and eluted with the linear
gradient system of 0.1% trifluoroacetic acid (solvent A) and 60% acetonitrile in solvent A (solvent B). Each
peak was identified by amino acid analyses referring to the Canfield’s nomenclature of tryptic peptides of hen
egg white lysozyme.15 The arrow with a broken line indicates the position at which the peptide T9 from the
native lysozyme should be eluted.
tide T9 (Trp62-Trp63-Cys64-Asn65-Asp66-Gly67-Arg68) of the native lysozyme disappeared but three peaks (b1, b2, and b3) which can be detected with absorbance at 365 nm appeared. The main peak with the absorbance at 365 nm (b1) was analyzed by Edman
Fig. 4. Gel-filtration Profile of the Products from the Incubation of (GlcNAc)₅ with the Native Lysozyme (a) and with the Lysozyme Treated with the Binary Modification (b).

Initial concentrations of the lysozyme and (GlcNAc)₅ were 0.03 mM and 0.1 mM, respectively. The enzymatic reaction was done in 10 mM sodium acetate buffer (pH 5.0) and at 50°C. The reaction mixture was applied to a column of TSK-GEL G2000PW (7.5 x 600 mm, Tosoh), and was eluted with distilled water. The roman numerals indicate the polymerization degree of the reaction products, respectively. Peaks 1, 2, and 3 were assigned to (GlcNAc)₃₉, (GlcNAc)₃₆, and (GlcNAc)₃₃, respectively, by computer simulation.

degradation, and was identified as X-Trp-CmCys-Asn-Asp-Gly-Arg. X indicates an unknown spot on TLC of DABTH-derivatives and should be the nitrophenylsulfenylated Trp residue, considering from the absorption spectrum of the peptide which had a maximum at 365 nm. Although two minor peaks (b2 and b3) were identified as peptide T9 by Edman degradation analysis, a Cys residue was detected instead of CmCys in amino acid analyses of the peptides. This indicated that the two minor peaks were the peptide T9 derived from insufficiently carboxymethylated lysozyme. From these results, Trp 62 in the Asp 101-modified lysozyme was identified to be selectively nitrophenylsulfenylated as in the case of the native lysozyme nitrophenylsulfenylation.¹⁰}
Experimental time-courses of the reaction catalyzed by the modified lysozyme

Figure 4 shows the gel filtration profiles of the reaction products from the incubation of the lysozyme and (GlcNAc)$_3$. (GlcNAc)$_2$ and (GlcNAc)$_3$ were predominantly produced by the modified lysozyme, while the native lysozyme gave the comparable amounts of the products, GlcNAc and (GlcNAc)$_{2,3,4}$. As seen from the figures, the amount of each transglycosylation product produced by the modified lysozyme that was eluted earlier than the initial substrate (GlcNAc)$_3$ was larger than that produced by the native lysozyme. In order to obtain the values of the reaction parameters for the modified lysozyme, the reaction was done with higher enzyme concentrations, and the experimental time-courses shown in Fig. 5(a) were obtained.

Data-fitting

Time-courses of the lysozyme-catalyzed reaction were calculated to reproduce the experimental time-courses shown in Fig. 5(a). In our previous papers, it was found that the nitrophenylsulfenylating of Trp 62 inhibited the sugar residue bindings to subsites A, B, and C with increasing the binding free energy changes by 1.7 kcal/mol, 0.3 kcal/mol, and 2.7 kcal/mol, respectively, and that Asp 101 modification with glucosamine inhibited the bindings with increasing the values by 1.1, 0.0, and 2.5 kcal/mol for the corresponding subsites. Thus, the calculations were done assuming that the binary modification of Asp 101 and Trp 62 increases the binding free energy change by 3.0, 0.3, and 5.0 kcal/mol, respectively. The calculated time-courses were quite different from the experimental; the overall reaction rate was much lower than that of the experimental and the amounts of GlcNAc and (GlcNAc)$_3$ were scarcely produced in the calculated time-courses (data not shown). This indicated that the binary modification of Asp 101 and Trp 62 does not result in the sum of the increases in the binding free energy changes caused by the individual modifications of Asp 101 and Trp 62.

Calculations were further repeated by changing the values of $\Delta G_A$, $\Delta G_B$, and $\Delta G_C$. The calculated time-courses which gave the smallest value of the cost function (Eq. (2)) were finally obtained with $\Delta G_A = +0.2$, $\Delta G_B = -2.7$, and $\Delta G_C = -2.4$ kcal/mol. These values are shown in Fig. 5(b). The sugar residue binding to subsite A was successfully suppressed by the binary modification, and the suppression at subsite A led to the enhancement of transglycosylation activity as expected from the computer simulation.

Simulation of the gel filtration profiles

To analyze the composition of the transglycosylation products, the theoretical profiles of the gel filtration were calculated for each of the reaction time. Figure 6(a) shows the theoreti-
Fig. 6. Simulation of the Gel Filtration Profile for the Native Lysozyme (a) and for the Lysozyme Treated with the Binary Modification (b).

Roman numerals are the polymerization degree of the reaction products. \( t_i \) values for (GlcNAc)\(_n\), used for the calculations were 120.0 \((n=1)\), 109.5 \((n=2)\), 103.2 \((n=3)\), 98.7 \((n=4)\), 95.2 \((n=5)\), 92.3 \((n=6)\), 89.9 \((n=7)\), 87.7 \((n=8)\), 85.9 \((n=9)\), and 84.2 \((n=10)\) min.

cal profiles for the native lysozyme-catalyzed reaction with (GlcNAc)\(_3\). The profile for the transglycosylation products exhibited a pattern similar to the experimental profiles shown in Fig. 4(a). From the theoretical profiles, the peak 1 in Fig. 4(a) was found to consist mainly of (GlcNAc)\(_3\). Figure 6(b), which shows the theoretical profiles for the modified lysozyme, also exhibited the same aspect as the experimental one shown in Fig. 4(b). Thus, the peaks 2 and 3 in the transglycosylation products shown in Fig. 4(b) were assigned to (GlcNAc)\(_8\) and (GlcNAc)\(_6\), respectively, from the theoretical profiles.

Discussion

It has been recognized that hydrolases, such as carbohydrolases and proteases, often contain some extent of synthetic activity, which can be used for the production of useful compounds. For example, the synthetic activities of trypsin and lysozyme have been used for the synthesis of biologically active peptides and useful oligosaccharides, respectively.\(^{16, 17}\)

Although several types of organic solvents are often used for enhancement of the synthetic activity of the enzymes, chemical modification methods are also available. Since a number of
methods for chemical modification of lysozyme were designed by many investigators, it is possible to enhance the synthetic activity, transglycosylation, of lysozyme by these modification methods.

Computer simulation of the lysozyme-catalyzed reaction was done by changing the values of binding free energy changes, and suggested that the inhibition of the sugar residue binding to subsite A causes a considerable increase in the transglycosylation activity. In the experiments according to this suggestion, the binary modification of Asp 101 and Trp 62 in the lysozyme molecule decreased the affinity of the sugar residue at subsite A and enhanced the transglycosylation activity. The inhibition of sugar residue binding to subsite A should increase the frequency of the oligosaccharide binding to subsites E and F and lead to the enhancement of the transglycosylation. The use of the modified lysozyme is considered to be convenient for the synthesis of a useful oligosaccharide.

From the gel filtration profiles, the composition of the transglycosylation products was estimated by computer simulation. The modified lysozyme predominantly produced (GlcNAc)_8 as the transglycosylation product. Since the initial substrate (GlcNAc)_3 prefers to bind to the modified lysozyme occupying subsites B, C, D, E, and F, the complex of the modified lysozyme and (GlcNAc)_3, in which the C_1 carbon of the reducing end residue is converted to carbonium ion intermediate, should be formed by the splitting of the β-1,4-glycosidic linkage located between subsites D and E. Then, the lysozyme-catalyzed transfer reaction of the (GlcNAc)_3 intermediate to the

Fig. 7. Model Scheme for the Lysozyme-catalyzed Reaction of Chitooligosaccharide. Meanings of the notations are summarized in Table I.
initial substrate \((\text{GlcNAc})_5\) should lead to the enhanced production of \((\text{GlcNAc})_8\). This suggests that \((\text{GlcNAc})_5\) as a transglycosylation acceptor can bind to subsites E and F only with the first and second GlcNAc residues from the nonreducing end of the oligosaccharide.

In the computer simulation (Fig. 1), the degradation rate of the initial substrate, \((\text{GlcNAc})_5\), was accelerated by subsite A modification. Nevertheless, the rate decreased considerably in the reaction catalyzed by the lysozyme treated with the binary modification in comparison with the native lysozyme reaction (Fig. 5(a)). The binding free energy change at subsite C of the modified lysozyme was estimated to be \(-2.4\ \text{kcal/mol}\), while that of the native lysozyme was \(-5.0\ \text{kcal/mol}\). The decrease in the sugar residue affinity at subsite C in the modified lysozyme should considerably decrease the rate of the initial substrate degradation. However, the individual modification of Asp 101 or Trp 62 also caused a similar level of decrease in the affinity at subsite C. This suggests that it is quite difficult to predict the effect of binary modification on the binding subsites from the data on the individual modifications. By making a further investigation about the lysozyme treated with the binary modification, it may be possible to obtain significant information about the binding subsites of lysozyme. This problem will be reported in the succeeding paper.

Appendix

Mathematical model for the lysozyme-catalyzed reaction.

As described in refs. 13 and 14, a reaction scheme for the simulation of the lysozyme-catalyzed reaction was constructed as Fig. 7. The meanings of the notations in the model scheme were summarized in Table I. Let \(S_n\), \(M_n\), and \(I_j\) be the total concentration of \((\text{GlcNAc})_n\), concentration of free \((\text{GlcNAc})_n\), and concentration of \((\text{GlcNAc})_n^+\), respectively. These variables may be represented by

\[
S_n = M_n + \sum_{i=-1}^{n+3} C_{n,i} + \sum_{j=1}^{p-1} B_{n,j}, \quad n = 1, 2, \ldots, p
\]

\[
I_j = A_j + \sum_{n=1}^{p} B_{j,n}, \quad j = 1, 2, \ldots, p-1
\]

From Fig. 7 the differential equations for these variables may be written as

\[
\frac{dS_i}{dt} = k_{+2} A_i - k_{-1} \sum_{j=1}^{p} B_{j,i} + k_{+1} \sum_{l=2}^{p} C_{i,l-1}
\]

**Table 1. Schematic Representation of the Notations in Fig. 7**

<table>
<thead>
<tr>
<th>Notations</th>
<th>Schematic representation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>M_n</td>
<td>Free chito-oligosaccharide</td>
</tr>
<tr>
<td>C_{n,i}</td>
<td>1:1 lysozyme-oligosaccharide complex</td>
</tr>
<tr>
<td>A_i</td>
<td>Carbonium Intermediate-lysozyme complex</td>
</tr>
<tr>
<td>B_{i,j}</td>
<td>Complex of A_i and j-mer saccharide</td>
</tr>
</tbody>
</table>

* \(n, i, j\) represent the polymerization degree.
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\[
\frac{dS}{dt} = \frac{k_2A_n - k_1}{t} \left( \sum_{m=1}^{n-1} B_{m,n-m} - \sum_{j=1}^{p-1} B_{j,n} \right)
+ k_1 \left( \sum_{i=1}^{p} C_{i,j-n} - \sum_{l=1}^{n-1} C_{n} \right),
\]
\[
n = 2, 3, \ldots, p - 1
\]
\[
\frac{dS_2}{dt} = \left( \frac{dS}{dt} \right)_{n=p} + k_1 \sum_{n=p+1}^{p-1} B_{m,n-m}
\]
\[
\frac{d}{dt} = -k_2A_n + k_1 \sum_{n=p}^{p-1} C_{i,j-n} - \sum_{l=1}^{n-1} C_{n} \]
\[
n = 1, 2, 3, \ldots, p - 1
\]

where \(k_{-1}, k_{-1}, \) and \(k_{-2}\) represent the rate constants for the cleavage of the glycosidic linkage, for the regeneration of glycosidic linkage, and for the hydration, respectively. By introducing the binding constants, the amounts of complexes \(C_n\) and \(B_{j,n}\) are approximately expressed by

\[
C_n = K_n E^* M_n
\]
\[
B_{j,n} = K_{*j} A_j M_n
\]

the binding constants \(K\) and \(K^*\) are calculated from the binding free energy change at each binding subsite according to the relationship,

\[
K_{X \rightarrow Y} = \exp\left( -\Delta G_{X \rightarrow Y} / RT \right)
= \exp\left( -\left( \Delta G_{X} + \Delta G_{M_n} \right) / RT \right)
= \exp\left( -\left( \Delta G_{X} + \sum_{i=1}^{p} \Delta G_{Y} + \Delta G_{M_n} \right) / RT \right)
\]

where \(X \sim Y\) indicates that the sequential subsites X to Y in an arbitrary region in all of the subsites (A, B, C, D, E, and F) are participating in the binding of oligosaccharide. The conservation equation for the total amounts of substrate and enzyme may be written as

\[
\sum_{n=1}^{p} nS_n + \sum_{n=1}^{\nu} nI_n = \sum_{n=1}^{\nu} nM_n
\]
\[
E + \sum_{n=1}^{p} \sum_{i=1}^{n+1} C_{n,i} + \sum_{j=1}^{n+1} I_j = E^*
\]

where the subscript 0 indicates the initial or total concentration of the respective species.

The variables \(C_{n,i}\) and \(B_{j,n}\) in Eqs. (3), (4), and (8) can be eliminated with substitution of Eq. (6). Then, we have

\[
S_n = M_n + \sum_{j=1}^{p+1} K_{*j} A_j M_n,
\]
\[
n = 1, 2, \ldots, p
\]
\[
A_j = l_j \left( 1 + \sum_{n=1}^{p} K_{*j} M_n \right),
\]
\[
j = 1, 2, \ldots, p - 1
\]
\[
E = \left( E^* - \sum_{j=1}^{p+1} l_j \right) \left( 1 + \sum_{n=1}^{p+1} K_{n} M_n \right)
\]

Furthermore, \(A_j\) and \(E\) in Eq. (9) can be eliminated using Eqs. (10) and (11). Thus, \(S_n\) can be calculated by

\[
S_n = M_n + \sum_{j=1}^{p+1} K_{*j} \left[ \left( E^* - \sum_{j=1}^{p+1} l_j \right) \left( 1 + \sum_{n=1}^{p+1} K_{n} M_n \right) \right] \left( 1 + \sum_{n=1}^{p+1} K_{*n} M_n \right)
\]
\[
\times \left( 1 + \sum_{n=1}^{p+1} K_{*n} M_n \right),
\]
\[
n = 1, 2, \ldots, p
\]

Finally, Eqs. (5), (6), (8), (10), (11), and (12) are simultaneously and numerically solved to obtain the time-courses.

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