Molecular Orbital Theory on Cellulolytic Reactivity Between pNP-Cellooligosaccharides and β-Glucosidase from Cellulomonas uda CS1-1

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Abstract A β-glucosidase with the molecular mass of 160,000 Da was purified to homogeneity from cell extract of a cellulolytic bacterium, Cellulomonas uda CS1-1. The kinetic parameters (Km and Vmax) of the enzyme were determined with pNP-cellooligosaccharides (DP 1-5) and cellobiose. The molecular orbital theoretical studies on the cellulolytic reactivity between the pNP-cellooligosaccharides as substrate (S) molecules and the purified β-glucosidase (E) were conducted by applying the frontier molecular orbital (FMO) interaction theory. The results of the FMO interaction between E and S molecules verified that the first stage of the reaction was induced by exocyclic cleavage, which occurred in an electrophilic reaction based on a strong charge-controlled reaction between the highest occupied molecular orbital (HOMO) energy of the S molecule and the lowest occupied molecular orbital (LUMO) energy of the hydronium ion (H3O+), more than endocyclic cleavage, whereas a nucleophilic substitution reaction was induced by an orbital-controlled reaction between the LUMO energy of the oxonium ion (H2O+) protonated to the S molecule and the HOMO energy of the H2O2 molecule. A hypothetical reaction route was proposed with the experimental results in which the enzymatic acid-catalyst hydrolysis reaction of E and S molecules would be progressed via SN1 and SN2 reactions. In addition, the quantitative structure-activity relationships (QSARs) between these kinetic parameters showed that Km has a significant correlation with hydrophobicity (logP), and specific activity has with dipole moment, respectively.

Keywords: Cellulolytic reactivity, Cellulomonas uda CS1-1, β-glucosidase, pNP-β-cellooligosaccharides, MO theory

β-Glucosidases are widely distributed in animals, plants, fungi, and bacteria. They hydrolyze cellobiose to glucose in the final step of cellulose degradation. Because the cellobiose acts as an inhibitor of other cellulase enzymes such as endo-β-1,4-glucanase and exo-β-1,4-glucanase [14], they play important roles to remove the inhibitory effect of cellulolytic enzymes.

Enzyme reactions are biologically catalyzed by a variety of ionizable groups functioning as electrophiles, nucleophiles, or general acid/base catalysts [3, 15, 16]. As a result, the pKa values of one or a few key ionizable groups within its active-site cleft are employed as the factors to define the experimental and theoretical functions of these catalytically essential groups or to establish the pathway of a given enzyme reaction. For example, a comparison of active sites of endoglucosidasises reveals a striking correlation with these pKa values, in that the residue hydrogen bonded to the general acid/base catalyst is asparagine in an alkaline pH circumstance, whereas it is aspartic acid in those with a more acidic pH optimum [29].

The application of quantitative structure-activity relationships (QSARs) analyses on enzymatic reactions with substrate (S) molecules have provided another theoretical basis for understanding the enzymatic reactivities between enzyme (E) and S molecules [9]. The QSAR has also rendered rapid progress on the knowledge of protein structures available for synthesizing and designing more effective new enzymes [13]. The studies on QSAR analyses of the compounds relevant to the enzymatic reaction were initiated with fibrinolytic activity by Hansch and his coworkers [11, 16], in which they identified that the hydrophobicity (logP) of carbonic acids has a positive correlation with the fibrinolytic ability. When the E-S complexes are being formed in the hydrolysis reaction of glycosidic bond combined with phenoxy group substituted to glucose by emulsin (β-glucosidase), the hydrophobic interaction increases and desorption of the phenoxy moiety from the surface of enzymes in a step of the catalytic reaction is hindered by the increased hydrophobic effect [10].

A number of studies on hydrolysis reactions of the glycosidic bond caused by certain enzymes have been
conducted until now [4, 5, 10, 20, 25, 29]. However, studies on molecular orbital (MO) theory and QSARs relevant to the reactivity of enzymatic hydrolysis attributed by β-glucosidase for β-1,4-glycosidic linkages within cellooligosaccharides are rare.

In this work, the physical and kinetic characterizations of β-glucosidase purified from *Cellulomonas uda* CS1-1 are reported. Furthermore, we describe the results on the enzymatic hydrolysis reactivity between the β-1,4-glucosidic bond in pNP-β-cellooligosaccharides (DP 1–5) and the β-glucosidase enzyme by means of the application of frontier molecular orbital (FMO) interaction theory [8] and QSAR methodology [18] with the obtained kinetic parameters.

**Materials and Methods**

**Organism and Culture Condition**

*Cellulomonas uda* CS1-1 (KCTC 1371) was grown on Dubos' salt [31] solution supplemented with 0.05% yeast extract and 10 mM glucose as the carbon source to purify β-glucosidase from cell extract. The cultivation was carried out in a Quickfit FV5L fermenter for 2 days at 30°C.

**Purification of β-Glucosidase**

The purification of a β-glucosidase from cell extract of *Cellulomonas uda* CS1-1 was performed by means of successive chromatography on Ultro-gel Ac34, DEAE-Sephadex A-50, and hydroxyapatite. A 5-ml aliquot of the enzyme solution, which was collected from cell extract by 30–80% ammonium sulfate saturation, was applied to a column (1.8×100 cm; bed volume, 220 ml) of Ultro-gel Ac34. The elution was carried out with 20 mM phosphate buffer, pH 6.8, at a flow rate of 15 ml h$^{-1}$. A 5-ml aliquot of active fractions from the preceding step was applied to a column (2.3×30 cm; bed volume, 130 ml) of DEAE-Sephadex A-50 with exchange capacity of 3.5±0.5 milliequivalent per gram, which was equilibrated with 50 mM Tris-HCl buffer, pH 7.5. Elution of the column was performed with a linear salt gradient of 250 ml of 0.5 M sodium chloride in the same buffer. The flow rate was adjusted to 15 ml h$^{-1}$. A 5-ml aliquot of the enzyme from the preceding step was applied to a column (1.8×25 cm; bed volume, 50 ml) of hydroxyapatite, which was equilibrated with 40 mM phosphate buffer, pH 6.8. Elution of the column was performed with 50 ml of 40 mM phosphate buffer, pH 6.8, and 50 ml of 0.4 M phosphate buffer by a linear gradient and the flow rate was adjusted to 15 ml h$^{-1}$.

**Assay of β-Glucosidase Activity**

β-Glucosidase activity was determined with p-nitrophenyl-β-D-glucopyranoside (pNPG) as the substrate. Enzyme solution (1 ml) was added to the preincubated solution at 40°C for 10 min, which contained 1.5 ml of McIlvaine buffer, pH 6.4, and 0.5 ml of 5 mM pNPG. After incubation for 20 min at 40°C, the enzyme activity was stopped by adding 2 ml of 1 M sodium carbonate. The absorbance of the yellow color that developed from the p-nitrophenolate anion was monitored at 400 nm (ε=18.3 mM$^{-1}$ cm$^{-1}$) and compared with a standard curve constructed using p-nitrophenol (pNP). The enzyme activity toward cellbiose was assayed by the glucose oxidase/peroxidase method [24].

**Estimation of Protein and Molecular Mass**

Protein content was determined by the method of Lowry et al. [21] with bovine serum albumin as a standard. The enzyme proteins of each purification step were identified by native- or SDS-PAGE using a vertical slab gel (Model AE-6640, Atto Co., Japan) at 10% of acrylamide concentration. The molecular mass was determined by SDS-PAGE using the method of Laemmli [19].

**Hydrolysis Products of pNP-Cellooligosaccharides**

To estimate the reaction products of β-glucosidase obtained during degradation of p-nitrophenol-cellooligosaccharides (pNPG$_n$, n=2–5, Seikagaku Kogyo Co., Japan) as substrates, the chromatographic hydrolysis products, pNPG$_n$, pNPG$_2$, pNPG$_3$, pNPG$_4$, and pNPG$_5$ released from pNPG$_n$ were separated by HPLC on a Develosil ODS-5 column (150×4.5 mm ID, Nomura Chemical Co., Japan). Two hundred μl of reaction mixtures that contained 2 units of enzyme and 0.2 mM of substrates in a 50 mM McIlvaine buffer, pH 6.5, were incubated at 40°C with time interval ranging from 0.1 to 12 h, and then the reaction was stopped by placing the tube in a boiling water bath for 15 min. Aliquots (10 μl) of reaction mixture were applied to the column and the reaction products were eluted by a linear gradient of 0.3% butanol in 10 mM McIlvaine buffer (A), pH 3.8, and 0.7% butanol in the same buffer (B) at 1.5 ml min$^{-1}$ and monitored spectrophotometrically at 300 nm.

**Kinetic Constants**

The hydrolysis products of pNPG$_n$ (n=2–5) with β-glucosidase from *C. uda* CS1-1 were measured using HPLC analysis. Michaelis constants ($K_m$; mM), maximum velocities ($V_{max}$; mM min$^{-1}$ mg$^{-1}$ protein), and specific activities (SA; mM min$^{-1}$ mg$^{-1}$ protein) of the enzyme were determined by calculating the molar ratios of the reaction products. The values of pNPG and cellbiose were estimated by the assay conditions described in Methods.

**Physicochemical Parameters and MO Functions**

The most stable conformers of three-dimensional structure were determined by minimizing the p-nitrophenol (pNP) group substituted molecules to the anomeric C$_1$-atom in β-.
cellooligosaccharides \([\beta-(\text{Glc})_n, \beta-(\text{Glc})_3]\) linkage to the 1,4-\(\beta\)-glycosidic bond as substrate (S). The 21 physicochemical parameters of the most stable conformers, which are the STEERIMOL parameters (Å) \((L, B_1, B_2)^a\), hydrophilicity \((\log P)^{[28]}\), dipole moment \((DM)\), lipole moment \((LM)\), molecular volume \((\text{Å}^3)\), molecular surface area \((\text{Å}^2)\), and polar surface area \((\text{Å}^2)\) \([10]\) etc., were calculated and then the 2D-QSAR models for the enzymatic hydrolysis were derived using the TSAR program (Ver. 3.3) \([1]\). And also the values of MO function such as HOMO (highest occupied molecular orbital) energy \((eV)\), LUMO (lowest occupied molecular orbital) energy \((eV)\), net charge, and atomic orbital \((AO)\) coefficient were also calculated using the AMI method \([7]\), which is a semiempirical molecular orbital theory in MOPAC \([27]\).

**RESULTS AND DISCUSSION**

**Purification of \(\beta\)-Glucosidase**

A \(\beta\)-glucosidase from the cell extract of *Cellulomonas uda* CS1-1 was purified to homogeneity by means of successive chromatography on Ultrogel Ac34, DEAE-Sephardex A-50, and hydroxyapatite. As summarized in Table 1, the specific activity and purification factor of the purified enzyme were 164 units/mg and 17 folds as compared to the crude enzyme. The molecular mass was identified as 160,000 Da by SDS-PAGE (Fig. 3A).

Table 1. Summary of purification steps for \(\beta\)-glucosidase from *C. uda* CS1-1.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification factor (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>410</td>
<td>3,900</td>
<td>9.6</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate (0.3–0.8 satn.)</td>
<td>286</td>
<td>3,620</td>
<td>16.2</td>
<td>93</td>
<td>1.7</td>
</tr>
<tr>
<td>Ultragel Ac 34 AF-I (Fr. Nos. 15–28)</td>
<td>150</td>
<td>3,250</td>
<td>19.6</td>
<td>83</td>
<td>2.0</td>
</tr>
<tr>
<td>DEAE A-50 D-I (Fr. Nos. 25–36)</td>
<td>30</td>
<td>2,100</td>
<td>70</td>
<td>54</td>
<td>7.3</td>
</tr>
<tr>
<td>Hydroxyapatite H-II (Fr. Nos. 15–28)</td>
<td>10</td>
<td>1,640</td>
<td>164</td>
<td>42</td>
<td>17.0</td>
</tr>
</tbody>
</table>

\(\beta\)-Glucosidase activity was determined with \(p\)-nitrophenyl-\(\beta\)-glucoside as the substrate at 400 nm.

**Kinetics of the Purified \(\beta\)-Glucosidase**

The kinetic studies of the purified \(\beta\)-glucosidase were performed with various substrates. When the \(K_m\) values for \(p\)NPG and cellobiose were estimated using the Michaelis-Menten equation \([23]\), typical Michaelis-Menten kinetics were observed by plotting the rate of enzyme activity against \(p\)NPG at the concentration of 125 \(\mu\)M–10 mM, and cellobiose at 5 mM–8 mM (data not shown). In addition, by the Lineweaver-burk plots, \(K_m\) values for \(p\)NPG and cellobiose were 1.33 and 0.24 mM, and \(V_{max}\) were 65.6 and 6.0 mM in vitro \(mg^{-1}\) protein (Table 2), respectively. Although the affinity on \(p\)NPG was lower than cellobiose, the value of \(V_{max}\) was higher (than that). This means that the enzyme has high turnover for the \(p\)NPG substituted with the \(p\)-nitrophenyl group rather than cellobiose as substrate. In the case of kinetic parameters for \(p\)NP-cellooligosaccharides (DP 2-5), the \(K_m\) values for \(p\)NPG \(_a\), \(p\)NPG \(_b\), and \(p\)NPG \(_c\) were calculated as 1.60, 0.32, 0.021, and 0.011 mM, and \(V_{max}\) values for each substrate were 0.14, 0.25, 0.63, and 2.63 mM in vitro \(mg^{-1}\) protein, respectively (Table 2). The hydrolysis rate of \(p\)NP \(_a\) appeared to exceed nearly four-folds that of \(p\)NP \(_b\) and ten-folds that of \(p\)NP \(_c\).

**MO Functions and Stability of Molecule**

Enzymatic reactions of S molecules, \(p\)NP-cellooligosaccharides (DP 1-5), caused by the \(\beta\)-glucosidase (E) that cleaves a cellulose into glucose units, are general acid catalytic hydrolysis induced by glutamic acid (Glu) (or aspartic acid).

Table 2. Michaelis constant, specific activity, and physicochemical parameter of \(p\)NP-cellooligosaccharides and cellobiose by \(\beta\)-glucosidase.

<table>
<thead>
<tr>
<th>(S)</th>
<th>(K_m^{a})</th>
<th>(V_{max}^{b})</th>
<th>(SA^{c})</th>
<th>(\log P^{d})</th>
<th>(DM^{e})</th>
<th>(L_m^{f})</th>
<th>LM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(p)NPG (_a)</td>
<td>1.33</td>
<td>65.6</td>
<td>164(-1.36)</td>
<td>0.253</td>
<td>3.106</td>
<td>6.426</td>
<td>11.25</td>
</tr>
<tr>
<td>(p)NPG (_b)</td>
<td>1.60</td>
<td>0.14</td>
<td>0.90</td>
<td>-0.964</td>
<td>5.663</td>
<td>10.183</td>
<td>21.80</td>
</tr>
<tr>
<td>(p)NPG (_c)</td>
<td>0.32</td>
<td>0.25</td>
<td>7.50</td>
<td>-2.181</td>
<td>8.211</td>
<td>22.477</td>
<td>28.05</td>
</tr>
<tr>
<td>(p)NPG (_d)</td>
<td>0.02</td>
<td>0.63</td>
<td>11.20</td>
<td>-3.398</td>
<td>9.669</td>
<td>21.324</td>
<td>38.07</td>
</tr>
<tr>
<td>(p)NPG (_e)</td>
<td>0.01</td>
<td>2.63</td>
<td>22.50</td>
<td>-4.614</td>
<td>14.697</td>
<td>24.889</td>
<td>45.00</td>
</tr>
<tr>
<td>cellobiose (_b)</td>
<td>0.24</td>
<td>6.00</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^{a}\)mM, \(^{b}\)mmM min\(^{-1}\) mg\(^{-1}\), \(^{c}\)mM min\(^{-1}\) mg\(^{-1}\), \(^{d}\)dipole moment, \(^{e}\)total length (Å) of substrate molecule, \(^{f}\)molecular volume (Å\(^3\)) of substrate molecule, \(^{g}\)the values for \(V_{max}\) and SA are units min\(^{-1}\) mg\(^{-1}\), \(^{h}\)The extrapolated value in parentheses was calculated according to the model (II) in Table 6. ND: not determined. The presented values for \(K_m\) and \(V_{max}\) were averages of triplicate experiments.
Table 3. Total energies, HOMO and LUMO energies (e.v.) of pNP-β-(glc)₃, (3), and their protonated forms (A–F) by the MOPAC–AMI calculation method.

<table>
<thead>
<tr>
<th>Type</th>
<th>HOMO</th>
<th>LUMO</th>
<th>ΔE&lt;sub&gt;HOMO-LUMO&lt;/sub&gt;</th>
<th>Total E&lt;sup&gt;h&lt;/sup&gt;</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-10.5696</td>
<td>-6.6033</td>
<td>-3.9663</td>
<td>41.635</td>
<td>1</td>
</tr>
<tr>
<td>A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-12.0016</td>
<td>-5.9026</td>
<td>-6.0990</td>
<td>48.599</td>
<td>5</td>
</tr>
<tr>
<td>B&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-12.7590</td>
<td>-5.0569</td>
<td>-7.7021</td>
<td>53.277</td>
<td>7</td>
</tr>
<tr>
<td>C&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-12.6005</td>
<td>-3.9500</td>
<td>-8.6505</td>
<td>43.406</td>
<td>2</td>
</tr>
<tr>
<td>D&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-12.6006</td>
<td>-4.0359</td>
<td>-8.5647</td>
<td>45.489</td>
<td>3</td>
</tr>
<tr>
<td>E&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-12.0773</td>
<td>-4.9506</td>
<td>-7.1224</td>
<td>47.186</td>
<td>4</td>
</tr>
<tr>
<td>F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-11.7218</td>
<td>-4.8491</td>
<td>-6.8727</td>
<td>50.085</td>
<td>6</td>
</tr>
</tbody>
</table>

1, Total energy=-14.546 kcal/mol, HOMO=-10.290 eV, LUMO=-4.407 eV; 2, total energy=-25.931 kcal/mol, HOMO=-10.188 eV, LUMO=-1.297 eV.
*Neutral molecule (3), the protonated form to the O<sub>4</sub><sup>δ</sup>, O<sub>5</sub><sup>δ</sup>, O<sub>6</sub><sup>δ</sup>, and O<sub>7</sub><sup>δ</sup> atoms.*

Table 4. Net atomic charges of various constitutional atoms in pNP-β-(glc)₃, (3), and their protonated forms (A–F) by the MOPAC–AMI calculation method.

<table>
<thead>
<tr>
<th>Type</th>
<th>β-(glc)₁</th>
<th>β-(glc)₂</th>
<th>β-(glc)₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₅</td>
<td>-0.2207</td>
<td>-0.1236</td>
<td>-0.2935</td>
</tr>
<tr>
<td>C₁</td>
<td>-0.0218</td>
<td>0.1434</td>
<td>-0.2758</td>
</tr>
<tr>
<td>O₈</td>
<td>0.2436</td>
<td>0.1478</td>
<td>-0.0738</td>
</tr>
<tr>
<td>C₄</td>
<td>-0.2547</td>
<td>-0.1226</td>
<td>-0.2684</td>
</tr>
<tr>
<td>O₉</td>
<td>-0.2606</td>
<td>0.1194</td>
<td>-0.2636</td>
</tr>
<tr>
<td>C₅</td>
<td>-0.2479</td>
<td>0.1230</td>
<td>-0.2780</td>
</tr>
<tr>
<td>O₁₀</td>
<td>-0.2572</td>
<td>0.1267</td>
<td>-0.2702</td>
</tr>
</tbody>
</table>

*Neutral molecule (3), the protonated form to the O<sub>4</sub><sup>δ</sup>, O<sub>5</sub><sup>δ</sup>, O<sub>6</sub><sup>δ</sup>, O<sub>7</sub><sup>δ</sup>, and O<sub>8</sub><sup>δ</sup> atoms.*
and the LUMO energy of $H_2O^+$ will occur more in superiority than a nucleophilic reaction by the orbital-controlled reaction between the LUMO energy of 3 (N) and the HOMO energy of $H_2O^+$. However, in the case of the reaction between $SH^+$ (C) and $H_2O$, since the energy difference ($\Delta E_{\text{LUMO}} - \Delta E_{\text{HOMO}}$) between HOMO of $SH^+$ (C) and LUMO of $H_2O$ is larger than the energy difference ($\Delta E_{\text{LUMO}} - \Delta E_{\text{HOMO}}$) between LUMO of $SH^+$ (C) and HOMO of $H_2O$, a nucleophilic reaction by the charge-controlled reaction pursuant to a strong interaction between the LUMO energy of 3 (N) and the HOMO energy of $H_2O$ would occur much faster.

**AO Parameters and Cleavage Pattern**

AO coefficients (2pz) of HOMO energy for various constitutional atoms were calculated in Table 5, and nodal properties based on AO coefficients are represented in Fig. 2, to investigate the cleavage pattern on the linkages of around the oxygen atoms in the oxonium ion $SH^+$ (A–F) and the S molecule 3 (N). From the apparent results, it would be expected that the cleavage possibility of those linkages is very high because of an existence of a node between C-O bonds in the N molecule. In particular, $SH^+$ (C), which is produced by protonation of the stable $O_2$ atom, indicated that the cleavage does not occur in the second linkage of the $\beta$-1,4-(C$_1$-O$_1$-C$_2$) glycosidic bond but in the C$_1$-O$_1$ bond. This corresponded well with the experimental results in Table 2. In case of each $SH^+$, which was produced by protonation reaction to one of six oxygen atoms in the S molecule 3 (N), the cleavage pattern of the C-O bond could be explained with the nodal properties based on AO coefficients as the following. First, in the $O_2$-$C_1$-$O_1$ bond, N and B-F cleave totally all C-O bonds but A does not, and in the $C_2$-$O_2$-$C_1$-$O_1$ bond, N and A cleave all C-O bonds but B does not. C cleaves only the $C_1$-$O_1$ bond, but D-F do only the $C_1$-$O_1$ bond in contrast to C. In the case of the $C_2$-$O_2$-$C_1$-$O_1$ bond, N and A-C cleave all C-O bonds, D cleaves the $C_1$-$O_1$ and C$_1$-$O_1$ bond, E does not cleave all the bonds, and F

![Fig. 1. FMO interaction between S molecule 3 (N: neutral molecule; C: protonated form to the $O_2$ atom, $SH^+$) and $H_2O$ (or water molecule) in the enzymatic hydrolysis of substrate 3, by the MOPAC (AMI) calculation method.](image1)

![Fig. 2. Nodal properties based on AO coefficients (HOMO, 2pz) of the S molecule 3, by the MOPAC-AMI method. N, Neutral molecule; C, protonated form to the $O_2$ atom, $SH^+$; the bonds of bold parts indicate the $\beta$-1,4-glycosidic bond.](image2)

| Table 5. Atomic orbital (AO) coefficients (HOMO) of various constitutional atoms (2pz) in pNP-$\beta$-(glc)$_1$, (3), and their protonated forms (A–F) by the MOPAC-AMI calculation method. |
|---|---|---|---|---|---|
| Type | $O_1$ | $C_1$ | $O_2$ | $C_2$ | $O_3$ | $C_3$ | $O_4$ | $C_4$ |
| $N^+$ | -0.1956 | 0.1883 | -0.1056 | -0.0451 | 0.0549 | -0.0079 | 0.0148 | 0.0051 | -0.0060 | 0.0034 | -0.0032 |
| $A$ | -0.0011 | -0.0005 | 0.0060 | -0.0134 | 0.0189 | -0.0200 | 0.0142 | -0.0224 | 0.1453 | -0.0301 | 0.0322 |
| $B^+$ | -0.0006 | 0.0008 | -0.0003 | -0.0002 | -0.0040 | -0.0045 | -0.0225 | 0.0284 | -0.0652 | 0.0049 | -0.0316 |
| $C'$ | 0.2302 | -0.0876 | 0.0345 | -0.0024 | -0.0018 | -0.0016 | 0.0022 | 0.0008 | -0.0006 | 0.0001 | -0.0002 |
| $D'$ | -0.3577 | 0.0470 | -0.0656 | -0.0105 | 0.0172 | 0.0003 | 0.0002 | -0.0016 | 0.0007 | 0.0004 | -0.0001 |
| $E'$ | -0.2473 | 0.0714 | -0.0921 | -0.0041 | 0.0014 | 0.0006 | 0.0002 | -0.0001 | 0.0001 | 0.0001 | 0.0001 |
| $F'$ | -0.3648 | 0.0189 | -0.0495 | -0.0019 | 0.0001 | 0.0006 | -0.0002 | -0.0001 | 0.0001 | 0.0001 | 0.0001 |

*Neutral molecule (3), **protonated form to the $O_2$, $O_3$, $O_4$, $O_5$, $O_6$, $O_7$, and $O_8$ atoms.*
cleaves only the C$_4$-O$_5$ bond. These results suggest that the cleavage pattern of C-O bonds will be different according to the position of an oxygen atom on which the protonation occurs. Experimentally, the cleavage pattern of β-glucosidase was similar to that of the endo-1,4-glucanase that cleaves cellulose to glucose units randomly, rather than the exo-1,4-glucanase from _C. uda_ CS1-1 that cleaves cellobiose units [31], suggesting the exo-1,4-glucanase has uncompetitive inhibition reactivity.

Enzymatic Hydrolysis Reactions

The plausible pathway of the enzymatic acid-catalyst hydrolysis reaction between E and S molecules is proposed in Scheme 1, based on the MO functions and FMO interaction. In the step of the initiation reaction, E acts as a proton donor in general acid catalysis by Glu (or Asp) of the reaction site in the E receptor [3], whereas water molecules act as proton transfer agents, respectively.

The fast protonation reaction to the oxygen atom of the β-1,4-glycosidic bond occurs by H$_3$O$^+$ produced from the acidic catalysis reaction. The first step of the acidic hydrolysis reaction is the formation of SH$,^+$ in which exocyclic cleavage by the electrophilic charge-controlled reaction occurs mainly, because the compound protonated to the oxygen atom of the β-1,4-glycosidic bond is more stable than that of the oxygen atom in the 1,5-ether linkage of the pyranose ring (Table 3). In addition, since endocyclic cleavage by the nucleophilic orbital-controlled reaction in which SH$^+$ is produced by protonation with H$_3$O$^+$ to the 1,5-ether linkage of pyranose occurs in the minority, the water molecules act as a nucleophile to the carbonium ion produced by the cleavage of the C-O bond in SH$.^+$ The related previous research reported that all cellulolytic reactions including exocyclic (or endocyclic) cleavage is progressed by way of the carbonium ion [2]. Since both exocyclic and endocyclic cleavage reactions produce secondary carbonium ion, the reaction will be taken by competing with a unimolecular SN$_1$ reaction and a SN$_2$ reaction, which is a bimolecular nucleophilic substitution reaction to C$_1$ atom. Therefore, a series of the reaction progresses in that in the case of exocyclic cleavage, the C$_1$-O bond in the pyranose molecule is cleaved and thereafter the linkage is formed again, and in the case of endocyclic cleavage, the C$_1$-O bond of the β-1,4-glycosidic bond is cleaved.

Relationships Between Structure and Reactivity

QSARs are important tools to understanding the relationships between structure and reactivity of active compounds exhibiting certain biochemical activities. To investigate the QSAR on $K_m$ and SA values obtained from the cellulolytic reaction between E and S, $K_m$ and SA, and the physicochemical parameters of pNPG$_7$-pNPG$_5$ are listed in Table 2, in which the data relating to pNPG$_5$ values were not used because these values have no significant correlations with various kinds of the physicochemical parameters. The results showed that the more a number of glucose unit (β-Glc as a continuous chain) increases, the more the $K_m$ value also increases. This means that it is easy to produce an E-S complex by increasing an affinity force between E and S, and the phenomenon was also identical for $V_{max}$ and

**Scheme 1.** Plausible enzymatic hydrolysis pathway of pNP-substrates by β-glucosidase from _C. uda_ CS1-1.

R= p-nitrophenoxy group.
The 2D-QSAR models (I)–(III), which were induced to investigate the structure-activity relationships between E and S molecules, and those statistical parameters are summarized in Table 6. When the relationships on $K_m$ value, MR (cm$^3$/mol), and logP constant [28] were examined, the weighting factor of the MR constant, which is a duplicate parameter that has correlations with both the steric factor and dispersion force, was very low. Because it is difficult to decide whether an interaction between the S molecule and β-glucosidase would be dependent on dispersion (MR>0) or steric (MR<0) effect [20], model (I) suggests that the $K_m$ constant is dependent on logP, but the influence is minor. Moreover, as the correlations of length (L) and width (B) of the substituents in which the pNP parent was attached to the substituents, β-(Glc)–β-(Glc)$_n$, have been analyzed with $K_m$ constant, respectively, only the length of the substituents did not have a significant correlation. Even though the effect is low, the total length ($L_{MT}$) of S molecules with pNP had a good correlation ($r$=0.940), as shown in model (II). The phenomenon could be explained with the reason that the L and B$_n$ of S molecules are flexible by various factors of the glucose units with pNP, and the pNP parent with its great hydrophobicity acts as an element to increase the affinity between the E and S molecules. As shown in Fig. 3, the SA of enzyme-like model (III) had a significant correlation for the DM of S molecules. These results suggest that the SA and $K_m$ of β-glucosidase relating to cellulolytic reactivity would be influenced by the $L_{MT}$ of the S molecule, including the DM and logP of S molecules.

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**References**


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**Table 6. Statistical results of the best 2D-QSAR models with $K_m$, $V_{max}$, and specific activity of pNPG, ($n$=1–5).**

<table>
<thead>
<tr>
<th>Model</th>
<th>$K_m$</th>
<th>$K_m$</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r$</td>
<td>5 ($n$=1–5)</td>
<td>5 ($n$=1–5)</td>
<td>4 ($n$=2–5)</td>
</tr>
<tr>
<td>S$^r$</td>
<td>1.781</td>
<td>2.008</td>
<td>245.26</td>
</tr>
<tr>
<td>F$^d$</td>
<td>10.700</td>
<td>22.120</td>
<td>1250.03</td>
</tr>
<tr>
<td>r$^e$</td>
<td>0.884</td>
<td>0.940</td>
<td>0.998</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.412±0.294</td>
<td>2.130±0.341</td>
<td>−12.199±0.680</td>
</tr>
</tbody>
</table>

| Weighting factor | logP: 0.35±0.160 | L$_{MT}$: −0.086±0.060 | DM: 2.377±0.160 |

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<sup>a</sup>Statistical values and weighting factors, <sup>b</sup>number of used compound, <sup>c</sup>means square, <sup>d</sup>F-value, <sup>e</sup>correlation coefficient.