Thermal Unfolding of the Starch Binding Domain of *Aspergillus niger* Glucoamylase

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A fragment of the starch-binding domain (SBDF) of *Aspergillus niger* glucoamylase was prepared using recombinant DNA techniques, and its thermal unfolding was investigated by high-sensitivity differential scanning calorimetry (DSC). Thermal unfolding of SBDF was found to be reversible at pH 7 as expected from a DSC study of the whole enzyme molecule [Tanaka A. et al., J. Biochem., 117, 1024–1028 (1995)] but not reversible at acidic region. Numerical analysis of the DSC curves showed that the denaturation was two-state, and some of the SBDF molecules were oligomeric (average degree of oligomerization was 1.2) at pH 7. It was suggested that the denaturation temperature of SBDF was lower than that of the starch-binding domain in the whole enzyme molecule by about 4.5 degree (decrease in the Gibbs energy change was 5.3 kJ mol⁻¹) indicating a possibility that the starch-binding domain is stabilized by glycosylation of the domain itself, or by the highly glycosylated linker region.

Key words: DSC; glucoamylase; *Aspergillus niger* starch-binding domain; thermal unfolding

Glucoamylase [EC3.2.1.3] hydrolyzes starch to produce glucose. *Aspergillus niger* glucoamylase is a glycoprotein that consists of 616 amino acid residues. There are two major domains in the protein molecule1–4) forming a dumb-bell shape, as schematically shown in Fig. 1 (top); one is the N-terminal side catalytic domain, which consists of approximately 470 amino acid residues, and the other is the C-terminal side starch binding domain with 110 amino acid residues. These two major domains are linked by a highly O-glycosylated linker region. Structure of these domains are elucidated in detail by X-ray crystallography and NMR.5–12) Commercial preparation of the enzyme involves an isozyme that lacks the starch binding domain. To discriminate between these two species, the whole enzyme molecule is designated to be G1 and the isoform without the starch binding domain to be G2 (Fig. 1). G2 hydrolyzes maltooligosaccharides identically with G1, but can hydrolyze starch much less effectively than G1 can.13)

In our preceding studies using adiabatic differential scanning calorimetry (DSC),13) the following points were made: (1) Thermal unfolding of the catalytic domain is irreversible while that of the starch-binding domain is reversible.14) (2) These two domains unfold independently in the G1 molecule. (3) The starch-binding domain does not affect unfolding parameters of the catalytic domain and linker region. (4) The catalytic domain may consist of three units for unfolding,8) which were physically identified by Coutinho and Reilly15) by the method of Tsai and Nussinov.16)

For this study, we tried to prepare a fragment protein of the starch binding domain (110 amino acid residues; Thr507-Arg616) using recombinant DNA techniques and observe the thermal unfolding of the fragment using a differential scanning calorimeter to analyze the unfolding mechanism of the fragment and detect possible difference(s) in the unfolding between the starch-binding

![Fig. 1. Schematic Representation of the Domain Structure of *A. niger* glucoamylase.](image)

The whole enzyme molecule G1 consists of the catalytic domain and the starch-binding domain (SBDF). An isozyme G2 lacks the starch-binding domain. Fragment protein of SBDF prepared in this study is tentatively designated as "SBDF."
domain in the G1 protein and its detached form (fragment). This study will be the first of the series of calorimetric studies of wild type and engineered SBDs, aiming to obtain a base to improve the physicochemical properties of industrially used glucoamylase.

In this paper, especially for the DSC section, we tentatively use the term “SBDf” to designate the isolated fragment of the starch-binding domain with 110 amino acid residues to discriminate from the starch-binding domain (SBD) as a part of the G1 molecule (Fig. 1).

**Materials and Methods**

*Glucoamylase G1*. Glucoamylase from *A. niger* was purchased from Sigma Chemicals Co. Isozyme G1 was purified from the commercial preparation using the method described earlier.\(^1\)\(^2\)\(^3\)

*Preparation of SBDf*. The fragment of the starch binding domain, SBDf, was prepared as follows: Chromosomal DNA of *A. niger* IFO4066 was prepared by the method of Raeder and Broda.\(^1\)\(^7\) The gene coding the starch-binding domain was amplified by polymerase chain reaction (PCR) using Ampli Taq Gold (PE Applied Biosystems) with the primers 5′-AAGGATCCAGCTGAGGAAAGGACCTCCGTAGCACTCCACC-3′, which encodes the Factor Xa cleavage site, SBD from *A. niger* glucoamylase, and an additional *BamHI* site for cloning in pBluescript II SK-(Stratagene) and pQE30 (Quiagen) vectors and 5′-AAAAGCTTCTACGGCCAGGTGCTAGCTAC-3′, which included an intrinsic stop codon and a *HindIII* site. The amplified fragment was digested with *BamHI* and *HindIII* and ligated into the *BamHI-HindIII* site of pBluescriptII SK- to yield pAnSBD1. *Escherichia coli* JM109 was transformed with the plasmid by electroporation (Gene Pulser, Bio-Rad). Positive clones were selected using color selection on Luria-Bertani (LB) plate medium containing isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. White colonies were screened by PCR using the primers described above. The presence of the SBD gene was confirmed by DNA sequencing using the Dye-terminator cycle sequencing kit and the 310 PRISM capillary sequencer (PE Applied Biosystems). The cloned gene encoding SBD was found to be identical to that of the glucoamylase G1.\(^2\)\(^8\)\(^9\) The *BamHI-HindIII* fragment from pAnSBD1 was subcloned into the *BamHI-HindIII* sites of the expression vector pQE30. The resulting plasmid, pQESBD1, was used to transform *E. coli* JM109. Plasmid DNA preparation, electrophoresis of DNA fragments and other basic DNA manipulations were done using routine procedures.\(^2\)\(^0\) DNA fragments were isolated from agarose gels by using a Gene cleanII kit (BIO101).

**Expression in *E. coli* and purification.** *E. coli* JM109 harboring pQESBD1 was grown at 37°C on LB broth with ampicillin (100 µg/ml). When the absorbance at 600 nm of culture reached 0.5, IPTG was added to the culture at a final concentration of 0.1 mM, and the cultivation was continued overnight at 22°C, preventing the aggregation of SBDf protein into inclusion bodies. The cells were harvested by centrifugation at 1,600 × g for 10 min and resuspended in 50 mM sodium phosphate buffer (pH 7.6) containing 0.3 M sodium chloride and disrupted by sonication. The cell debris were removed by centrifugation at 18,000 × g for 20 min. The cell-free extract obtained was used for purification of SBDf. NiNTA agarose (Qiagen) was used for purification in accordance with the supplier’s protocol. Each of the fractions was put on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) apparatus. The fraction containing SBDf was dialyzed against Factor Xa reaction buffer (20 mM Tris-HCl, 0.1 M sodium chloride, 2 mM CaCl₂, pH 8.0).

After overnight dialysis, the SBDf fraction was incubated with Factor Xa (New England BioLabs) at room temperature for 1 day to remove the His-tag region. The proteolytic mixture was run on SDS-PAGE, and the SBDf band in the gel was transblotted to a polyvinylidene difluoride membrane. After being electroblotted, the membrane was washed and stained. The stained SBDf band was cut out and put on a Protein sequencer 476A (PE Applied Biosystems). The resulting N-terminal sequence indicated the proteolysis for removing His-tag was complete. The mixture was put on a Hitrap Q Sepharose ion exchange column (Pharmacia) equilibrated with 50 mM Tris-HCl (pH 8.0). The proteins absorbed were eluted with a linear gradient of sodium chloride (0 to 1 M) in the same buffer. The eluted fractions were tested with SDS-PAGE, and SBDf fractions were collected. After the fraction was dialyzed against distilled water, the final preparation was obtained as a freeze-dried powder. SDS-PAGE was done by the method of Laemmli.\(^2\)\(^1\)

Protein concentrations were calculated using the absorption coefficient values at 280 nm of 1.37 × 10⁻² g⁻¹ cm⁻¹ M⁻¹ for G1 and 3.05 × 10⁻⁴ g⁻¹ cm⁻¹ M⁻¹ for SBDf, and molecular weights of 82,500 (G1) and 12,100 (SBDf), respectively.

**Differential Scanning Calorimetry.** Thermal unfolding of the proteins was measured by a high sensitivity differential scanning calorimeter, VP-DSC, of MicroCal Inc. at a scan rate of 1 K min⁻¹. Unless otherwise mentioned, the protein samples were dialyzed against 20 mM phosphate buffer (pH 7.0) at 4°C using a dialysis tube with cutoff at molecular weight of 3,500, and the final dialysate was used as a reference solution for the DSC scans. The instrumental base lines were measured with sample and reference cells filled with the buffer. The observed DSC data were analyzed after the deduction of the instrumental base line. The DSC curves were analyzed by a least-squares curve fitting method based on the theories outlined by Privalov and Khechinashvili\(^2\)\(^2\) and by Sturtevant\(^2\)\(^3\) with base lines drawn according to the method by Takahashi and Sturtevant.\(^2\)\(^4\) The standard deviation (S.D.) of the calculated values from the observed data was expressed as a relative value to the maximal value of the excess specific heat cₘₐₓ.
Results and Discussion

Preparation of SBD

The cloned SBD gene using PCR was sequenced and then found to be identical to that of glucoamylase G1 that was isolated from A. niger. We confirmed that pAnSBD1 did not contain any mutations in the coding region amplified by PCR. SBD with the His-tag was purified from the cell-free extract by a Ni-NTA column. After digestion with factor Xa, SBD was purified with the Hi-trapQ ion exchanger. The final product gave a single band on SDS-polyacrylamide gel electrophoresis. The N-terminal amino acid sequence of SBD was TSXTPTFAVA, indicating that the proteolysis occurred exclusively at the carboxyl side of the arginine residue in the factor Xa recognition site. The proteolysis for removing His-tag was complete. Although the third residue could not be identified, a strong peak with the retention time 7.34 min appeared. The peak was deduced to be the C residue. These results indicate that SBD is a polypeptide from T507, just as we designed. Typically 10 mg of pure SBD was obtained from 4 liters of E. coli culture broth.

Thermal unfolding of SBD

Figure 2 shows DSC traces of the thermal unfolding of SBD thus obtained observed at three different pHs. These DSC traces of SBD, a fragment of the G1 molecule, were similar to those observed with other small globular proteins, such as T4 lysozyme and Staphylococcal nuclease.

At pH 7, a single endothermic peak was observed at around 53°C. In the pre-transition region, a fairly steep slope of the base line (temperature dependence of the apparent heat capacity of the protein in the native state) was seen, averaging 4.5×10⁻² J g⁻¹ K⁻², but that of the post-transition region, which is the temperature dependence of the apparent heat capacity of the denatured state of the protein, was close to zero. This indicates that Δc_p, the heat capacity change of the protein upon denaturation, is a function of temperature. After the completion of the initial DSC scan up to 78°C, the SBD solution was cooled to 25°C in the calorimeter cell and immediately rescanned to check the reversibility of the unfolding reaction. Judging from the shape of the two DSC traces (cf. "pH 7" and its "Rescan" in Fig. 2), and the thermodynamic parameters described later, it is reasonable to conclude that the thermal unfolding of SBD is fully reversible, as was expected from the previous DSC studies of glucoamylase G1. Even when the rescan was repeated 3 times, the first (initial) and the

### Table

<table>
<thead>
<tr>
<th>Scan</th>
<th>Protein</th>
<th>pH</th>
<th>Conc. (mg/ml)</th>
<th>t_(1/2) (°C)</th>
<th>ΔH_molar (J g⁻¹)</th>
<th>ΔH_molar/ΔH_cal</th>
<th>Δc_p at t_(1/2) (J g⁻¹ K⁻¹)</th>
<th>S.D. (%)</th>
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<tr>
<td>A</td>
<td>SBD</td>
<td>7.0</td>
<td>0.35</td>
<td>52.3</td>
<td>28.5</td>
<td>32.8</td>
<td>0.42</td>
<td>1.4</td>
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<tr>
<td>B</td>
<td>SBD</td>
<td>7.0</td>
<td>0.48</td>
<td>52.7</td>
<td>33.8</td>
<td>1.12</td>
<td>0.27</td>
<td>0.80</td>
</tr>
<tr>
<td>C</td>
<td>SBD</td>
<td>7.0</td>
<td>0.94</td>
<td>52.5</td>
<td>26.5</td>
<td>1.28</td>
<td>0.42</td>
<td>0.99</td>
</tr>
<tr>
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<td>SBD</td>
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<td>1.94</td>
<td>52.9</td>
<td>30.0</td>
<td>1.21</td>
<td>0.47</td>
<td>0.91</td>
</tr>
<tr>
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<td>SBD</td>
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<td>52.6</td>
<td>30.2</td>
<td>1.23</td>
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<tr>
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<td>7.0</td>
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<td>33.6</td>
<td>1.05</td>
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<td>21.0</td>
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<td>1.3</td>
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<td>1.54-11.90</td>
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<td>4.82±0.13</td>
<td>0.98±0.03</td>
<td>0.06±0.14</td>
<td>3.3±1.2</td>
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</tbody>
</table>

* Parameters are as follows: t_(1/2) temperature at which 50% of the protein molecules are denatured, ΔH_molar specific enthalpy, ΔH_molar van’t Hoff enthalpy, Δc_p molar calorimetric enthalpy (ΔH_molar × molecular weight).

Reheat of scan A.

Reheat of scan B.

G1 after heat treatment. Values of the parameters are average±standard deviation of 7 observations.

Value of "joules per gram of G1" and of "joules per gram of SBD in G1", respectively (see text). The latter value may be compared with that for SBD.
fourth scans gave essentially the same DSC traces.

The specific enthalpy of the unfolding reaction, \( \Delta h_{\text{cal}} \), was roughly evaluated from the area of each DSC trace to be approximately 30 J g\(^{-1}\). The molar calorimetric enthalpy \( \Delta H_{\text{cal}} \) is then calculated from \( \Delta h_{\text{cal}} \times \text{molecular weight} = 360 \text{kJ mol}^{-1}\).

For the simple two-state unfolding N=\( \rightleftharpoons \)D, where N is the native state of the protein and D the denatured state, the van’t Hoff enthalpy \( \Delta H_{\text{vH}} \) is given by the following equation;

\[
\Delta H_{\text{vH}} = 4RT_n\ln c_{\text{max}} / \Delta h_{\text{cal}}
\]

(1)

where \( R \) is the gas constant and \( T_n \) is the absolute temperature at which the excess specific heat reaches its maximal value \( c_{\text{max}} \). The \( \Delta H_{\text{vH}} \) value thus obtained is about 440 kJ mol\(^{-1}\). The ratio \( \Delta H_{\text{vH}} / \Delta H_{\text{cal}} \approx 1.2 \) suggests that the unfolding reaction is a little more complicated than the simple two-state.\(^{20}\)

The DSC data were then numerically analyzed. The adjustable parameters for the unfolding reaction are \( t_{1/2} \) (in degrees Celsius) at which the unfolding is half-completed (\( [D]/[N]=1 \) in monomeric units), \( \Delta h_{\text{cal}} \) the specific enthalpy at \( t_{1/2} \), and the ratio \( \Delta H_{\text{vH}} / \Delta H_{\text{cal}} \). The values of these parameters thus obtained are listed in Table. In the table, the values of \( \Delta G_{\text{cal}} \) at \( t_{1/2} \) are also listed. Figure 3 is an example of the curve analysis, where closed circles refer to the observed DSC data and the solid line is a theoretical curve drawn using the values listed in the Table. Good agreement between the observed and the calculated values was seen with the standard deviation of 0.9\%. The ratio \( \Delta H_{\text{vH}} / \Delta H_{\text{cal}} \approx 1.2 \), was slightly greater than unity, suggesting that a part of the protein molecules is oligomeric (most probably dimeric) in the native state and/or the denatured state.*

**pH dependence**

The thermal denaturation of SBDF was also observed at pH 2.4 (20 mm glycin buffer) and at pH 4.5 (optimum pH of the enzyme activity; 20 mm acetate buffer), as shown in Fig. 2. At pH 4.5, the denaturation started at around 60°C, but, after the DSC trace reached around the peak, a huge exothermic signal was observed most probably due to the precipitation of the protein.\(^{**}\)

The peak temperature was at least 13 degree higher than that at pH 7. Reheating of the sample showed no endothermic peak. At pH 2.4, the DSC trace was similar to that obtained at pH 7 with 5.5-degree higher peak temperature. Although no conspicuous precipitation was seen after the initial scan at pH 2.4, reheating of the sample showed no endothermic peak. Thus SBDF is more stable at around pH 4.5 than at pH 7 or 2.4 in terms of the denaturation temperature, but it is more stable at neutral pH in terms of the reversibility of the denaturation. The DSC trace at pH 2.4 was analyzed and the results are also listed in the Table. The ratio \( \Delta H_{\text{cal}} / \Delta H_{\text{cal}} \) was 2.9, suggesting that SBDF is oligomeric at the native state and/or the denatured state.

**Thermal denaturation of G1 after heat treatment and comparison with SBDF**

In the preceding studies, it was shown that the rescan of a G1 solution after the initial DSC scan reflected the thermal unfolding of SBD, since the unfolding of SBD was reversible but the rest of the G1 molecule unfolded irreversibly on the first scan.\(^{13,14}\) Figure 4 (solid line) shows the DSC trace of G1 after the solution was heated up to 75°C, at which the denaturation is completed, and immediately cooled down. Reheating of the sample gave almost the same DSC trace.\(^{13}\) A small hump was seen at around 43°C, and the slope of the base line after the completion of the unfolding was negative, both of which were reproducible.

In Fig. 4, to compare the intrinsic contribution of SBD with SBDF, the DSC trace of G1 after heat treatment was numerically magnified vertically by a factor of 6.82 (=82500/12100), because one gram of G1 contains approximately 1 g × (12100/82500)=0.147 g of SBD. It is thus necessary to magnify the specific heat by a factor of 6.82 to give the specific heat by “joules per gram of SBD,” if the rescans reflects the unfolding of SBD only.

The DSC trace of G1 after the heat treatment was analyzed by the procedure described above and the results are also listed in the Table. The ratio \( \Delta H_{\text{cal}} / \Delta H_{\text{cal}} \) was close to unity, indicating that the unfolding is the simple two-state and there is no oligomerization under the experimental conditions used. The value of \( \Delta H_{\text{cal}} \), when expressed by joules per gram of SBD as observed above, 32.8 J g\(^{-1}\), is similar to that of SBDF (30.2 J g\(^{-1}\)). A remarkable difference was seen in \( t_{1/2} \); the \( t_{1/2} \) of G1 af-

* If we can take the difference in \( t_{1/2} \) for the SBDF concentration of 0.35 mg/ml and 1.94 mg/ml (52.3 and 52.9°C, respectively; see Table) seriously, the oligomerization may be mainly at the native state.

** At pH 4.5, G1 solution also precipitated on the first heating, which may be due, at least partly, to the pH used being close to the isoelectric point of G1 (4.0).\(^{15}\)
DSC Studies of Starch Binding Domain of Glucoamylase 2131

Fig. 4. Comparison of the DSC Traces of G1 after Heat Treatment (Solid Line) and SBDF (Dashed Line).

Observed at pH 7. Excess specific heat of G1 after heat treatment was expressed as ‘per gram of the starch-binding domain in the G1 molecule’ (see text).

Heat treatment was approximately 4.5°C higher than that of SBDF under the experimental conditions used.

The apparent destabilization of SBDF compared with SBD is given by $\Delta G_f = \Delta G_f(SBDF) - \Delta G_f(SBD)$, which is the change in the standard Gibbs free energy $\Delta G_f$ of denaturation at the temperature of half-denaturation of SBDF. Since $\Delta G_f(SBDF) = 0$ at this temperature, $\Delta G_f$ is given from the Gibbs-Helmholtz equation as follows:

$$\Delta G_f(\text{at } T_F) = \Delta H_f \frac{T_S - T_F}{T_S} - \Delta C_P \left( T_S - T_F + T_F \ln \frac{T_S}{T_F} \right)$$

where $T_S$ and $T_F$ are the absolute temperature of half-denaturation of SBD and SBDF, respectively, $\Delta H_f$ is the molar denaturation enthalpy of SBD at $T_S$, and $\Delta C_P$ is the denaturational heat capacity change of SBD at $T_S$. $\Delta G_f$ is calculated to be 5.25 kJ mol$^{-1}$ at 52.5°C.

Wang et al. reported that glycoproteins were destabilized by deglycosylation. The apparent decrease in thermostability of SBDF described above may be due to SBDF prepared in this study being deglycosylated simple protein while native SBD is glycosylated. It is also possible that the destabilization derives from lack of the highly $O$-glycosylated linker region, which is considered to protect glucoamylase from thermal denaturation.

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