Effect of Family 22 Carbohydrate-Binding Module on the Thermostability of Xyn10B Catalytic Module from *Clostridium stercorarium*

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A family 22 carbohydrate-binding module (CBM22) from *Clostridium stercorarium* Xylanase10B raised the optimum temperature of the xylanase, but in the remaining activity of heating test, apparently the catalytic module alone showed higher remaining activity. Differential scanning calorimetry showed that CBM22 conferred resistance to thermal unfolding of the enzyme and prevented the enzyme from refolding after thermal unfolding.

**Key words:** carbohydrate-binding module; xylanase; thermostability; differential scanning calorimetry (DSC)

*Clostridium stercorarium* is a thermophilic non-cellulosomal plant cell wall degrading bacterium. It produces many cellulases and xylanases. The gene encoding for one of these enzymes, xylanase10B from *C. stercorarium* F-9, was cloned and the expressed enzyme was characterized.1) This enzyme was classified into family 10 glycoside hydrolase according to sequence similarity. It is composed of two family 22 carbohydrate-binding modules (CBM), a family 10 catalytic module (CM), a family 9 CBM, and two repeats of surface layer homologous modules consecutively from the N-terminus. It hydrolyzes xylan, carboxymethyl cellulose, and lichenan. Lichenase, β-1,3,1,4-glucanase activity was lost on removal of CBM22, indicating that the CM played not only a binding role, but also the role of determinant of substrate specificity.2) CBM22 was originally identified as a thermostabilizing module, because the removal of CBM22 caused a fall in the optimum temperature of enzymes in some cases of thermophilic and mesophilic enzymes.3) Subsequently, this module was classified as one of the CBMs binding to xylan and other substrates.4) In this study, we investigated the effect of CBM22 on the thermostolerance of Xyn10B from *C. stercorarium*. In Fig. 1, a schematic diagram of Xyn10B is shown. Two kinds of recombinant proteins (CBM-CM and CM) were expressed in *Escherichia coli*. The CBM-CM and the CM were composed of a CBM22 and a catalytic module, and only a catalytic module, respectively. The coding region was amplified with a pair of synthetic oligonucleotide primers; primer 1, 5'-GGGGCCATGGCACCATAACCTTTGAGGACCAA-3' (*Nco*I site underlined), and primer 2, 5'-GGGGAGATCTATATTCTCATATGTCCGG-3' (*Bgl*II site underlined), from pMA-5.1 as a template DNA.5) It included the full length gene of Xyn10B. The amplified DNA fragment was digested with *Nco*I and *Bgl*II restriction enzymes and ligated at the *Nco*I-*Bgl*II sites of pQE-60T (Qiagen, Hilden, Germany), yielding plasmid pCBM-CM. The expression vector for CM protein was reported previously,2) and the expression and purification of these proteins were described previously.2) The final preparations showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

To determine the optimum temperatures, these enzymes were incubated with 1.5% (w/v) oat-spelt xylan (Fluka, Buchs, Switzerland) as a substrate for 10 min at various temperatures (50°C to 90°C) in 20 mM sodium phosphate buffer (pH 8.0). Reducing sugars released from the substrate were measured with the 3,5-dinitrosalicylic acid reagent.5) The CBM-CM and the CM showed optimum temperature at 75°C and 65°C respectively (Fig. 2). The enzyme having CBM22, the CBM-CM, reacted at a higher temperature, indicating that CBM22 raised the optimum temperature. Subsequently, thermostability was tested. The enzymes were incubated at 70 to 90°C in 20 mM sodium phosphate buffer (pH 8.0) without the substrate for 10 min. After incubation, the enzyme solutions were cooled on ice for 10 min, and then the remaining enzyme activities were assayed at 60°C with oat-spelt xylan as substrate. The CBM-CM began to lose activity from 74°C and lost it...
completely at 84°C, while the CM was still active at 84°C, and kept 40% of its activity even at 90°C. This result was contrary to our expectation that the CBM-CM was more stable than the CM. To elucidate this reverse result, the proteins were subjected to differential scanning calorimetry (DSC).\(^6\) Thermal unfolding of the proteins was measured by highly sensitive DSC, VP-DSC (MicroCal, Northampton, MA) at a scan rate of 1 K min\(^{-1}\). The protein concentrations were 100 μg/ml. All runs were carried out in 20 mM sodium phosphate buffer (pH 8.0). Figure 2C shows DSC traces of the thermal unfolding of the CBM-CM and the CM. Single endothermic peaks were observed at about 80°C and 74°C respectively. These results are consistent with the data for optimum temperature. The presence of CBM had a positive effect on protein thermal unfolding, i.e., CBM22 had a thermostabilizing effect. After completion of the initial DSC scanning up to 90°C, the sample solutions were cooled to 20°C in a calorimeter cell and immediately rescanned to check the reversibility of the unfolding reaction. The reversibility of the CM was not full, but the endothermic peak reappeared at same temperature, i.e., the CM was refolded after thermal denaturing. No peak was observed in the case of CBM-CM (a\(^0\) and b\(^0\) in Fig. 2C). These results indicate that the CBM-CM could not be refolded after the thermal unfolding process, while the CM could be refolded. The rescanning after thermal unfolding indicated why the CM was more stable in the remaining activity test. After incubation at a higher temperature, the enzyme preparation was cooled, then the enzyme activity was measured. In this cooling step, the CM was refolded again in active form, while the CBM-CM became denatured and did not refold. After incubation, the enzyme solution was not cooled and assayed immediately. In this case, the CM activity was lost at 80°C (Fig. 2B). The cooling step was essential for refolding the CM. Since the term “thermostability” applied to enzymes often leads to confusion, both the thermal activity and the reversibility of thermal unfolding should be checked as indicated above. We are presently attempting to elucidate the reason for the CBM22 thermostabilizing effect.

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**References**

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