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Substrate-Binding Mode of Bacterial Chitosanases

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Mode of substrate-binding of chitosanases from *Streptomyces* sp. N174 (N174 chitosanase) and *Bacillus circulans* MH-K1 (MH-K1 chitosanase) was examined by site-directed mutagenesis and physicochemical techniques, including thermal unfolding, fluorescence spectroscopy, and X-ray crystallography. Asp57 located at the central portion of the binding cleft of N174 chitosanase was mutated to asparagine and alanine (D57N and D57A), and the relative activities of the mutated enzymes were 72 and 0.5% of that of the wild type, respectively. Thermal unfolding experiments in the presence of $(GlcN)_n$ clearly indicated the importance of Asp 57 for substrate binding. Kinetic analysis of $(GlcN)_6$ degradation catalyzed by N174 chitosanase suggested that Asp57 is most likely to participate in the substrate binding at subsite -2 through hydrogen bonding as well as electrostatic interaction. On the other hand, for MH-K1 chitosanase, we focused our attention on Tyr148 and Lys218, which are located at the bottom of the binding cleft and at the flexible loop forming the edge of the binding cleft, respectively. These residues were mutated to serine (Y148S) and proline (K218P), respectively, and the enzymatic activities of Y148S and K218P were found to decrease to 12.5 and 0.16% of the wild type. When $(GlcN)_3$ binding ability to the chitosanase was evaluated from the change in tryptophan fluorescence intensity, the binding abilities of Y148S and K218P were found to be reduced from that of the wild type by 1.0 and 3.7 kcal/mol of binding free energy, respectively. The crystal structure of K218P revealed that the main chain and side chain structures of the loop comprising Lys218 are affected by the mutation. Thus, we concluded that the flexible loop

comprising Lys218 plays an important role in substrate binding, and that the role of Tyr148 is less important but significant, probably due to stacking interaction.

Key words: chitosanase, substrate binding, fluorescence, thermal unfolding, X-ray crystallography

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