Protein Modeling and Engineering Increased Pepsin Susceptibility into a Heat-Tolerant Variant of *Escherichia coli* Phytase

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Abstract

Mutagenesis of recombinant proteins to increase resistance to heat denaturation (i.e., thermal stability) often results in a parallel increase in resistance to proteases (i). Although thermal stability of a protein is a desirable property for many industrial uses, proteins that are stable to proteases such as pepsin, in simulated human gastric fluid (SGF), are perceived as potentially allergenic. Directed molecular evolution of the native *Escherichia coli* (*E. coli*) phytase to generate a more thermo-tolerant phytase also resulted in 3.5 fold increased stability to simulated gastric digestion (ii). With the goal of reducing SGF stability, we have rationally engineered a thermally stable variant of *E. coli* phytase to show that its susceptibility to pepsin can be modified without causing undesirable changes to its catalytic and physical properties that are critical for its use as an industrial enzyme.

Various strategies were tested to improve susceptibility of the phytase protein to pepsin digestion. Disulfide linkage(s) were mutated to increase the possibility of protein unfolding. A crystal structure and full-dimensional model of phytase was used to engineer in susceptible pepsin cleavage sites at strategic locations on exposed loops of the folded protein such that proteolysis could more readily proceed without the need for complete unfolding of the protein In this second approach new pepsin cleavage site(s) were created by substitution mutations and pre-existing, but weaker cleavage-site(s) were mutated to form more labile cleavage sites. Finally, short peptides containing pepsin cleavage sites were inserted into the exposed loops of the phytase. To evaluate the effectiveness of above strategies, phytase variants were expressed in an *E. coli* host and assessed for SGF-stability, specific activity, pH activity-profile and thermal stability. These biochemical screenings demonstrated that for more than half of the variants, the time required for complete pepsin digestion was reduced to a few minutes (2 to 25 minutes), compared to more than 60 minutes for the original SGF resistant phytase. Yet many of these variants preserved all or most of their desired functional properties. Thermal stability remained comparable (+/- 5^{o}C) to the original phytase and the pH activity-profile remained unaltered for most, while the catalytic activity was improved in half of these variants. This protein engineering methodology used a thermo-tolerant molecule as the template protein. The inherent stability of the template molecule may have been a contributing factor that allowed it to retain its desired catalytic properties while withstanding any deleterious consequences of mutagenesis.

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