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In silico Approach in Designing Xylanase for Biobleaching Industry

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Abstract: The use of hemicellulolytic enzymes has recently attracted considerable interest as a substitute for chlorine chemicals in pulp bleaching. The challenges in incorporating enzyme to the bleaching system are to have a stable and active xylanase at high temperatures and higher alkaline pH conditions. In this study, we have used computational methods to analyze the structural factors responsible for the activity and themostability of Bacillus circulans xylanase which have been identified as one of the promising xylanase source to treat the pulp before bleaching it through the conventional bleaching sequences. Simulated point mutation shows that arginine substitution potentially increased the number of hydrogen bond; correlate with the xylanase activity and some changes in amino acid sequence specifically to the α-helix and β-sheet appeared to be promising in improving thermostability of xylanase. This in silico method results can be used to develop new efficient xylanase for pulp bleaching industry..

Key words: *Bacillus circulans* xylanase, *in silico* mutagenesis, enzyme mutation, point mutation, thermostable xylanase

INTRODUCTION

Enzyme application nowadays has become important and widely used by the industries as it is a safe and environmental friendly besides having more advantages compared to the chemical reagents. But because it comes or originates from the natural sources, it has some drawbacks that limit the abilities of it. Enzymes have it own temperature range, pH range as well as the substrate specificity. These properties make it unique and narrow the function of enzyme itself.

Xylanases are enzymes that catalyze the degradation of xylan, the main component of hemicellulose (Honda *et al*., 2001). Their biotechnological applications are of interest to the animal-feed, food-processing andpulp-and-paper industries (Shibuya *et al*., 2000) . In particular, xylanase has been found to be effective in reducing chlorine dosage requirements in the Kraft pulp-bleaching process (Viikari *et al*., 1994) that use the D-E-D-E-D sequence with chlorine dioxide (D stage) were carried out with alkali extraction stage (E). *Bacillus Circulans* Xylanase (BCX) is a 20.4 kDa endo-(1, 4)-beta-xylanase belonging to the family 11 of glycosidases [4] believed to be suitable for pulp bleaching industry as it is small in size. This enzyme catalyzes the hydrolysis of the polysaccharide xylan, a major constituent of plant biomass. Bcx and several homologous xylanases from *Aspergillus*, *Bacillus*, *Thermomyces* and *Trichoderma* sp. have been characterized extensively in terms of structure and enzymology (Torronen and Rouvinen, 1995; Schlacher *et al*., 1998). It has 185 amino acid residues. The structure contains two important catalytic residues which are glutamatic acid at position 78 and 127. These two catalytic residues are believed to play a big role in the catalysis of the enzyme.

In terms of economical overview, in pulp industries (Suurnakki *et al*., 1996), the use of xylanase to degrade xylan is more beneficial compared to use of chemical processes which are expensive and cause environmental pollution. Application of xylanases together with other bleach agents, such as oxygen and hydrogen peroxide in pulp industry has been extensively investigated and projections of a totally chlorine-free pulp technology have been put forward (Srinivasan and Rele, 1999). As the industry demand a competitive and profitable process, the modification of xylanase Bcx enzyme to improve its properties can give a big impact to the industries as it widens the ability of this specific enzyme.

Much study has been done to engineer enzymes to achieve the desirable state through mutation (Otten *et al*., 2004; Vukulenko *et al*., 1999; Schmidt *et al*., 2003; Gray *et al*., 2001). In this study, we use the Computer

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Aided Mutation (CAM) to suggest mutations to xylanase Bcx enzyme with the objective of improving the thermostability. Different strategies are carried out which include alteration to α-helix and β-sheet composition, lysine substitution and double mutation to the active site.

APPROACH AND METHODS

General preparation: The crystal structure of xylanase bcx with ligands β-D-xylopyranose and 1,2-Deoxy-2-Fluoro-xylopyranose (1BVV) was extracted from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (Berman *et al*., 2000). The ligands confirmed in the OCA browser-database for protein structure/function derived from Ligand Protein Analysis (LPC) interface (Sobolev *et al*., 1999).

Mutational studies: Simulated mutations were carried out by using CAChe WorkSystems Pro (Ver. 7.5.0.85 2006). Structure before and after mutations were optimized using augmented molecular mechanics (MM3) (Allinger *et al*., 1989) until it reaches the convergence threshold of 0.001 kcal mol^{-1}. Multiple points of mutation were carried out to *Bacillus circulans* xylanase.

Analysis: The mutated models and the original model were compared to observe the changes reflecting the mutagenesis. Final energy score of MM3 and the hydrogen bond formations are the main scope of observation.

RESULTS

The wild type structure 1BVV analyzed with three mutational strategies; (i) lysine mutation with arginine (Turunen *et al*., 2002) at positions K40R, K95R, K99R, K135R and K154R, (ii) α-helix and β-sheet set of mutations (Villiarias and Querol, 2006), which involve 18 points of mutations. For α-helix the mutation done at position 150 where Val substituted with Ala, V150A. In β-sheet where Ala mutated with Gly done at positions 18, 55, 59, 142 and 170 and the rest of â-sheet mutation involve in substitution of Val to Ile at position 16, 19, 28, 37, 38, 57, 81, 82, 98, 168, 182 and 184 and (iii) double mutation in the active site region that involve N35D and Q127E.

The energy of the final structure calculated using augmented molecular mechanics MM3 for all three mutant structures as a comparison to the wild type structure of xylanase bcx as shown in Table 1. Hydrogen bond formations were observed in overall structures as the mutagenesis involve randomly throughout the structure, not focusing on certain region or active site (Table 2).

Table 1: Energy of the final structure calculated using MM3

Structure	Energy score (kcal mol^{-1})
Wild type	-1146.27
Lysine mutation with arginine	-1316.57
α-helix and β-sheet set of mutation	-1420.14
N35D, Q127E	-1479.38

Table 2: Total hydrogen bond in overall structures

Structure	Total hydrogen bond
Wild type-1BVV	428
Lysine mutation with arginine	433
α-helix and β-sheet set of mutation	476
N35D, Q127E	479

DISCUSSION

The mutations designed to meet the objective of improving the thermostability of xylanase Bcx to suit the demand of biobleaching industry. Results reported herein are pre-results that suggest the potential of point mutations to meet the objective. Analysis starts with the target of minimizing the energy of the final structure as a subject of comparison between the structure before and after simulated mutation took place. The lower the energy score reflect the better conformation and stability of the structure. From Table 1, all three mutational approaches shown approximately 300 kcal mol^{-1} improvement compared to the wild type structure. This indicates that the mutations took place offer better structure conformation and in addition to that, no destruction observed to the structures; all α-helix and β-sheet conserved as it is.

The analysis followed by hydrogen bond observations. One classic and also pioneering work in thermostability studies is that of Vogt *et al*. (1997). These authors performed a statistical test upon 16 different families of proteins that each contained at least one thermophile. In 80% of the cases studied, there was a clear correlation with the thermostable character of the family in question found: the number of hydrogen bonds and the polar surface fraction related to the number of hydrogen bonds to water molecules are increased in thermophiles in comparison to mesophiles. In other word, it suggests that if the hydrogen bond can be increased, the thermophilicity of the structure may also increase.

Lysine mutation with arginine: Several studies have shown that arginines have a role in protein stability (Vogt *et al*., 1997; Mrabet *et al*., 1992). Replacement of Lys with Arg has been shown to increase the thermostability of many proteins. The comparison of mesophilic proteins and their thermophilic counterparts has revealed that thermophilic proteins have, on average, a higher Arg content (Vogt *et al*., 1997). At the same time amount of Lys was observes to decrease. This result

is supported by experimental mutagenesis studies in which Lys➔Arg mutations stabilize the protein structure (Mrabet *et al*., 1992; Cunningham and Wells, 1987). In this result, we demonstrated that the Lys➔Arg mutagenesis managed to form additional hydrogen bond throughout the structure and believed to give an impact to the thermostability of xylanase bcx. It has been proposed that an important reason for the increased stability is stronger hydrogen bonding of the large guanidinium group of Arg with nearby polar groups (Mrabet *et al*., 1992; Borders *et al*., 1994).

α-helix and β-sheet set of mutation: In designing thermostable enzyme, the common strategy, from both a theoretical and an experimental point of view, involved the comparison of the structures and amino acid compositions of mesostable and thermostable protein. This is certainly a logical approach to the problem if one considers that it may constitute a set of strategies designed by nature to produce sturdier molecules capable of thriving under harsh conditions. In the world of protein engineering this has produced empirical procedures that have yielded relative good results. Present designed mutations took the advantage of various researches done on this area as reported by Villiarias and Querol (2006). By structural comparison, substitution on α-helix and β-sheet that involve Val➔Ala, Gly➔Ala and Val➔Ile mutagenesis is closed to thermostable structure. The α-helix and β-sheet alterations resulted in additional 48 hydrogen bond formation. The energy of the final structure shown an improvement of 273.88 kcal mol^{-1} compared to the wild type. Both observations give some insight that these mutations may improve the thermostability of xylanase bcx as it offers a better stable structure and remarkable new hydrogen bond formations.

Double mutations of N35D, Q127E: In this approach, double mutations carried out in the active site region defined to be 5.5 Å around the ligand. Simulated mutations of Asn➔Asp and Gln➔Glu were done at position 35 and 127, respectively. It is observed that 51 additional hydrogen bonds formed compared to 428 hydrogen bonds in wild type structure. The strategy is to substitute the uncharged polar amino acid of Asn and Asp with charged polar amino acid Gln and Glu. The improvement of the energy of the final structure about 333.11 kcal mol^{-1} is in parallel to the addition of hydrogen bond. As in the majority of protein families, increased number of hydrogen bond correlates with increased of thermostability (Vogt *et al*., 1997), these double mutations have a good potential to produce a thermostable xylanase Bcx.

CONCLUSION

As biobleaching industry demand highly stable xylanase at high temperature, the designing of new xylanase enzyme that can suit the industrial requirement are the main interest. *Bacillus circulans* xylanase that reported to study well in biobleaching industry if thermostably improved and it is much more favorable. Even though there are many factors correlated with thermostability, the hydrogen bond is still the most relevant factor reported by researchers. It is believed that, the strong hydrogen bond is a key factor behind the thermostable enzyme. Those three strategies of mutations are proven *in silico* have a great potential to produce thermostable xylanase Bcx. Nevertheless, further investigation need to be done to study the stability of those mutant enzyme at high temperature.

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