Appl Biochem Biotechnol (2011) 163:186–194
DOI 10.1007/s12010-010-9027-8

Redesign the α/β Fold to Enhance the Stability of Mannanase Man23 from *Bacillus subtilis*

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Received: 19 January 2010 / Accepted: 25 June 2010 /
Published online: 18 July 2010

Abstract In this work, we engineered the α/β fold of mannanase Man23 based on its molecular structure analysis to obtain more stable variants. By introducing 31 single-site mutations in the α/β fold and shuffling them, the incorporation of four mutations (K178R, K207R, N340R, and S354R) displayed a good balance between high activity and stability at higher temperature and broader pH. This quartet variant was characterized by an almost threefold increased activity and a sevenfold increased stability compared to native mannanase Man23. Our results suggest that such work is safe to increase our target protein stability with no loss of activity.

Keywords Mannanase · Protein stability · Rational design · α/β Fold

Mannanase, an extracellular enzyme, has hemicellulase activity or the activities of both hemicellulase and cellulase [24]. It is widely used in many areas including food processing, cattle industry, and paper making, as well as textile printing and dyeing [9, 25]. It is especially involved in weakening plant tissues by degrading mannan polymers in the cell walls [21]. Mannanases are glycoside hydrolases that are a widespread group of enzymes, due to the complexity of the carbohydrates structures. Glycoside hydrolases from various sources are classified into different families based on their amino acid sequence similarities, and mannanases are mainly assigned to either glycoside hydrolase family 5 or 26 according to their sequence comparison [18].

A kind of mannanase, termed Man23, belonging to GH26 family and having high activity, was secreted from *Bacillus subtilis* B23 which was screened in our lab. What we

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have known about mannanase Man23 is that the sites of H129, E159, H190, E191, W196, F197, W198, and W199 are active sites and mutations at the sites of H129, H190, and W198 increased their activity 3.5-, 2.2-, and 3.8-fold, respectively. Besides the catalytic efficiency, for industrial and laboratory settings, it is necessary to make a protein more stable in certain abnormal situations, such as a higher temperature or acidic/alkaline conditions. Herein, we would like to further study how to get a balance between the catalytic efficiency and the stability of mannanase Man23.

In order to obtain a more stable protein under extreme or near extreme conditions of pH, temperature, or solvent composition which has hampered biocatalyst implementation, a number of approaches have been taken to develop highly stable proteins for biocatalysis applications. This has been done firstly by isolating and expressing novel proteins with exceptional stability [14, 15] and in recent years by the more popular method of engineering proteins by rational design to get higher stability. In this work, we improved the rational design for mannanase Man23 according to the molecular structure analysis. Based on a knowledge of protein stability, we designed 31 single-site variants and then shuffled them in tandem to obtain a balance variant with high activity and better stability. Our study provides insight into the integration of structure and function and has led to the design and construction of stable and active mannanase Man23.

Materials and Methods

Predicting and Analyzing the Structure

The secondary structure and 3D structure modeling were predicted by submitting amino acid sequences to the Swiss Model server (http://swissmodel.expasy.org/) [20] using mannanase Bcman from *B. subtilis* as a template [1]. The visualization and analysis of structures were performed using Swiss-PDB Viewer software.

Cloning Gene and Constructing Single-Site Mutations

The coded gene of mannanase Man23 was cloned from *B. subtilis* B23 using the primers P1 (5′-ATGCCTACTAAGT-3′) and P2 (5′-TGATTCAGCTATCTGTG-3′). After being linked with vector pBS-T, the gene was transformed into TOP10 competent cells [2]. According to the site-directed mutagenesis kit manual, the single-site mutations were introduced into the plasmid pBS-T-*man23* using pairs of reverse primers. The constructed single-site variants were S58T, N54D, G62A, G63A, S109T, G121A, G122A, S137T, I144L, P155R, G160A, K178R, G181A, K207R, R211H, K220R, G231A, G254A, P270R, S274T, G275A, M279T, S294T, G298A, S299R, N307Q, Q311E, N340R, S341R, E347H, and S354R, respectively.

Shuffling Single-Site Variants

Through PCR reaction, pBS-T-*man23* and its single-site variants were added the digestion site by the primers P3 (5′-CGCGGATCCATGCCTACTAAGT-3′, *Bam*H I) and P4 (5′-CGGAATTCTGATTCAGC TATCTGTG-3′, *Eco*R I). When shuffling the single-site variants, the PCR product from pBS-T-*man23* was used as a template, while the products from variants were used as the parent DNA [23]. After the latter was digested with DNase I, the digestion fragments were reassembled through a PCR cycling program [12]. The reassembled mixture

along with primers P3 and P4 was amplified to the full-length genes with the template gene. PCR was carried out under the following conditions: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s; 55 °C for 30 s; 72 °C for 3 min, then 72 °C for 10 min, 4 °C thereafter.

Transforming and Expressing the Gene

The genes with digestion sites *Bam*H I and *Eco*R I were linked with vector pHY-p43 and transformed into *Brevibacillus brevis* for expression.

B. brevis was cultured in T2 medium overnight at 37 °C with agitation at 200 rpm. Cultures were added to 5 ml T2 medium by the 1% incubation amount and continued to culture at 37 °C till the late stage of logarithmic growth. *B. brevis* was recovered and washed with 5 ml 50 mmol/l Tris–HCl (pH 7.5), then resuspended in 5 ml 50 mmol/l Tris–HCl (pH 8.5), and cultured at 37 °C. One hour later, *B. brevis* were re-collected and resuspended in 0.5 ml TP medium. At this time, 100 μl solution of pHY-p43-*man23* mixing in buffer (TE/TP = 1:1) and 1.5 ml PEG solution were added into TP medium sequentially. The mixtures were incubated at 37 °C for 10 min. *B. brevis* was again recovered, then resuspended in 1 ml MT medium, and agitated at 37 °C for 30 min. After antibiotics were added into MT medium, the cultivation was continued for another 2 h.

Determining the Enzyme

Protein concentration was measured using the Bradford assay [5]. The Mannanase activity assay was improved from the method of monitoring the release of reducing sugar [16, 17]. One unit of enzyme activity was defined as the amount of enzyme liberating 1 μmol mannose per minute at 50 °C and pH 6.8. The activity formula was as follows:

$$\text{Mannanase activity}(\text{U/ml}) = 5.56 C_{\text{e}} V_{\text{de}} / V_{\text{je}} V_{\text{s}} t$$

5.56 1.0 mg gram molecule of mannose, micromole
C_{e} Mannose content calculated according to optical density (OD) value and standard curve, milligram
V_{de} Metered volume of enzyme, milliliter
V_{je} Volume of enzyme added to the substrate solution, milliliter
V_{s} Volume of substrate solution, milliliter
t Time, minute

$$\text{Specific activity of mannanase (units per milligram)} = 5.56 C_{\text{e}} V_{\text{de}}^2 / C_{\text{p}} V_{\text{je}} V_{\text{s}} t$$

C_{p} Protein content calculated according to the OD value and standard curve, milligram

Determining Optimal Temperature and pH

The optimum temperature was evaluated by using the standard activity assay at 30, 40, 50, 60, 70, and 80 °C. The assay was performed at the optimal pH 6.8, which was previously determined. The buffer was 50 mmol/l phosphate buffer.

The optimum pH was evaluated by using the standard assay method with the 50 mmol/l phosphate buffer. The assay was performed at 50 °C but at different pH.

Determining Thermostability and Kinetic Parameters

Thermostability was measured by incubating the samples for 20 min at different temperatures and then plotting the mannanase activity versus the incubation temperature. The half-lives of the samples were calculated from the slope of the denaturation curves obtained by linear regression of log(mannanase activity at 80 °C) versus incubation time.

Moreover, thermal denaturation curves and the temperature of the midpoint of the transition (T_m) were determined by monitoring the change in the CD value at 220 nm [13]. Mannanase samples were dissolved in 50 mmol/l phosphate buffer (pH 6.8), and the protein concentration was 0.2 mg/ml. The temperature was raised at a rate of 0.5 °C/min from 20~90 °C. The temperature of the transition midpoint (T_m) was calculated from the curve fitting of the resultant CD values versus the temperature on the basis of least-squares analysis [6]. The entropy change of unfolding at T_m(ΔS_m) and the enthalpy change of unfolding at T_m were calculated by van't Hoff analysis. The difference of the free energy change between unfolding of the mutants and that of the wild type at the T_m of the wild type ($\Delta\Delta G_m$) was calculated by the relationship given by Becktel and Schellman [4], i.e., $\Delta\Delta G_m=\Delta T_m \Delta S$ (wild type), where ΔT_m is the change in T_m of the mutants relative to the wild type and ΔS_m (wild type) is the entropy change of the wild-type at T_m.

Statistical Analysis

Data were presented as the mean±standard error of the mean. Results were compared with the analysis of variance and Fisher's protected least-significant difference tests, with a significance of $P<0.05$.

Results and Discussion

Structure Characteristics of Mannanase Man23

Like other kinds of mannanase known, mannanase Man23 that belongs to GH26 displays a $(\beta/\alpha)_8$ fold catalytic module characteristic. From the results provided by the Swiss Model server, 127 amino acid residues form the α-helix and β-strand (Fig. 1) and rest residues organize loops linking helixes and strands.

The active sites located on the loops are all buried in the TIM barrel and the $(\beta/\alpha)_8$ folds sustain strongly tertiary structures of mannanase Man23. Now we tend to believe these folds are important to protein stability [19]. From a body of researches, the mesophilic and hyperthermophilic homologues have a common basic stability afforded by the conserved protein core. The highly conserved core is already quite optimized for stability, even in mesophilic enzyme [7]. Moreover, the mutations in the protein core have the possibility to create the spatial interference or introduce the unfavorable strains. For these reasons, more effective mutation would be found in the less conserved areas of the protein.

As we know, mechanisms of protein stability are so complicated that it is a challenge to find out potential sites responsible for its stability. Fortunately, the growing body of experimental results threw lights on the factors enhancing protein stability. Some promising factors that include hydrogen bonds, ion pairs, and hydrophobic surface were taken into

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TARGET    25      HTVYPVNP NAQQTTKDIM NWLAHLPNRS ENRVMSGAFG GYSDVTFSMT
2qhaA     1       htvspvnp naqqttktvm nwlahlpnrt enrvlsgafg gyshdtfsma

TARGET                       hhhhhhh hhh    h        sssssss ss    sss h
2qhaA                        hhhhhhh hhh    h        sssssss ss    sss h


TARGET    73    EENRLKNATG QSPAIYGCDY GRGWLETADI TDTIDYSCNS SLISYWKSGG
2qhaA     49    eadrirsatg qspaiygcdy argwletani edsidvscns dlmsywkngg

TARGET          hhhhhhhh       sssssss ss          hhsss   hhh hhhhhhhh
2qhaA           hhhhhhhh       sssssss sssss       hhsss   hhh hhhhhhh


TARGET    123   LPQVSLHLAN PAFPSGNYKT AISNSQYKNI LDPSTVEGKR LEALLSKIAD
2qhaA     99    ipqislhlan pafqsghfkt pitndqykki ldsstaegkr lnamlskiad

TARGET          sssss                      hhhhhhh h     hhhhh hhhhhhhhhh
2qhaA           sssss            sss       hhhhhhh h     hhhhh hhhhhhhhhh


TARGET    173   GLTQLKNQGV TVLFRPLHEM NGEWFWWGLT GYNQKDNERI SLYKELYKKI
2qhaA     149   glqelenqgv pvlfrplhem ngewfwwglt synqkdneri slykqlykki

TARGET          hhhhhhhh     ssss                          hhhh hhhhhhhhhh
2qhaA           hhhhhhhh     ssss                          hhhh hhhhhhhhhh


TARGET    223   YRYMTETRGL DNLLWVYSPD ANRDFKTDFY PGSSYVDITG LDAYFTDPYA
2qhaA     199   yhymtdtrgl dhliwvyspd anrdfktdfy pgasyvdivg ldayfqdays

TARGET          hhhhhhh      ssssssss                    sss sssss
2qhaA           hhhhhhh      ssssssss         hh         sss sssss


TARGET    273   ISGYDEMLSL KKPFAFAETG PSGNIGSFDY AAFINAIRQK YPQTAYFLTW
2qhaA     249   ingydqltal nkpfaftevg pqtangsfdy slfinaikqr ypktiyflaw

TARGET             hhhhhh    ssssssss ss          hhhhhhhhh       sssss
2qhaA              hhhhhh    ssssssss ss          hhhhhhhhh       sssss


TARGET    323   DEQLSPAANQ GAQSLYQNSW TLNKGEIWNG GSLTPIAE
2qhaA     299   ndewspavnk gasalyhdsw tlnkgeiwng dsltpive-

TARGET                       hhhhhh  s ss     sss    sss
2qhaA                        hhhhhh  s ss     sss    sss
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Fig. 1 The second structure of mannanase Man23 based on the template 2qhaA. Modeling based on template 2qhaA (1.45 A). Sequence identity 70.536% and *e* value 5.03395e−143. *2qhaA* chain A of mannanase Bcman from *B. subtilis*

account when engineering mannanase Man23. We designed mutations on 31 sites in or next to the folds and investigated how the introduced mutations would affect the mannanase Man23 stability at higher temperature and broader pH. These sites were given here above in "Gene cloning and constructing single-site mutations."

Determination and Analysis of Mannanase Man23 Variants

Based on researches in recent years, it is more popular that mutations occur on multiple sites or a certain domain, since multiple mutations would give an added effect as compared with single mutations. In our work, two generations of mutations—single-site mutations and their shuffled multiple mutations—were utilized to engineer the target enzyme.

Even though some experiments argued a few hyperthermophilic enzymes would combine local flexibility with high overall stability [10, 22], some other experiments still support the incompatibility in activity and stability [8, 11]. We need more studies to get a better understanding of the degree of incompatibility or compatibility between the conformational rigidity and protein activity. Meanwhile, we hope to get a good variant balance.

From the final circle of shuffling mutations, a quartet termed mannanase M0710 was screened out with excellent balance of activity and stability. This quartet variant contained the mutated sites of K178R, K207R, N340R, and S354R in tandem. As compared with native mannanase Man23, the activity of M0710 increased by almost threefold (Fig. 2), and the activity efficiency increased by 10.8-fold (Table 1). Meanwhile, the optimal catalysis temperature increased by 15 °C (Fig. 2), the half-life at 80 °C increased by sevenfold (Table 1), T_m value increased by 10.5, and $\Delta\Delta G_m$ increased by 5.2 kcal/mol. Somewhat surprisingly, the enzyme was improved in pH tolerance as well (Fig. 3).

The variant M0710 was modeled using the X-ray structure of 2qhaA as a template to understand the structural mechanism for changes on the stability. After analyzing parameters on mutation sites, we concluded that increasing the hydrogen bonds is a major change on K178R, K207R, and N340R (Fig. 4), while the molecular surface is modified on S354R.

The hydrogen bond is an important contributor to protein stability, so there is no doubt that those new hydrogen bonds added on K178R, K207R, and N340R promote the stability, and $\Delta\Delta G_m$ increased by 2.51, 1.09, and 2.41 kcal/mol, respectively. Even though no new

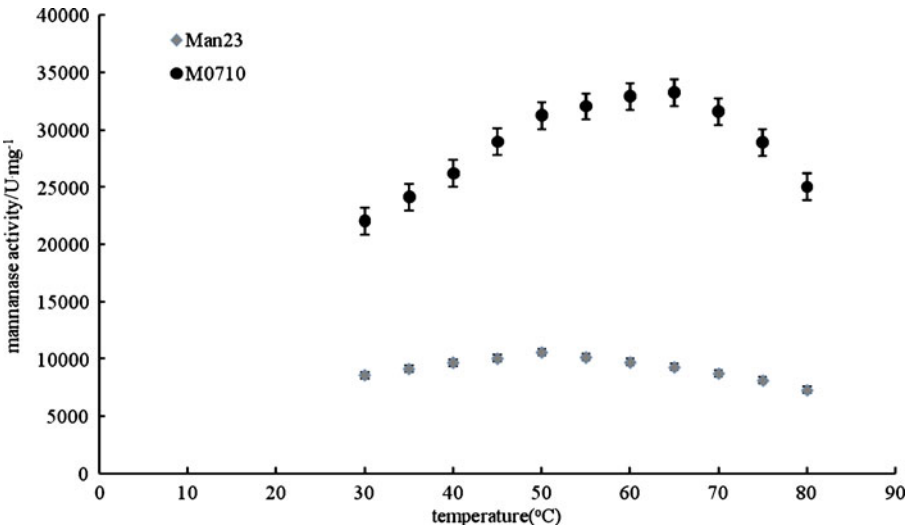

Fig. 2 Catalytic activity of M0710 and Man23 at different temperatures; 0.9 ml of 0.5 g/100 ml glucomannan (prepared in 50 mmol/l phosphate buffer, pH 6.8) was mixed with 0.1 ml of 1 mg/ml enzyme solution and incubated at 30, 35, 40, 45~75, and 80 °C for 3 min. After that, the reaction was terminated by boiling water bath. The content of the reducing sugar was determined using the DNS method

Table 1 Kinetic parameters and HL at 80 °C of M0710 and Man23.

Enzyme	K_m/(mg L^{-1})	K_{cat}/s^{-1}	HL (min)
Man23	8.3±0.4	5.2±0.0	1.5
M0710	0.8±0.0	56.2±0.1	10

hydrogen bonds appear on S354R, we can distinguish tiny modification around a surface cavity. As compared with the R group of serine, arginine has a much longer R group that can fill up the cavity near the site 354. Otherwise, all of these four sites have been displaced by arginine. Because the arginine residue has one fewer methylene group than lysine and high pK_a, Arg more easily develops less unfavorable contacts with the solvent, and at the same time, it also easily maintains ion pairs and a net positive charge at elevated temperatures [7]. In particular, the introduction of arginine on the sites 340 and 354 strengthens the ion pair networks. From this side, ion pair network is another critical mechanism to improve the stability of mannanase Man23.

Compared with the increasing number of papers discussing the adaption of protein to temperature, fewer papers discussed the adaption of protein to pH. Some results demonstrated that it proved evolutionarily less costly, more flexible, and much safer to make the protein surface adapt to the solvent conditions and interaction partners than to make the active sites [3]. We speculate that increasing the net charges and strengthening the ion pair networks outside of the active sites, including the protein surface, possibly have better buffer effects on changes in active site pK_a values. To some extent, mutations for adapting to high temperature would improve the protein rigidity to tolerate a broader pH.

In our work, all the mutations were designed on the sites in or next to helixes and strands, which are non-catalytic sites. Because we have already known which loops the active sites are located in, these mutations introduced here tend to have little interference

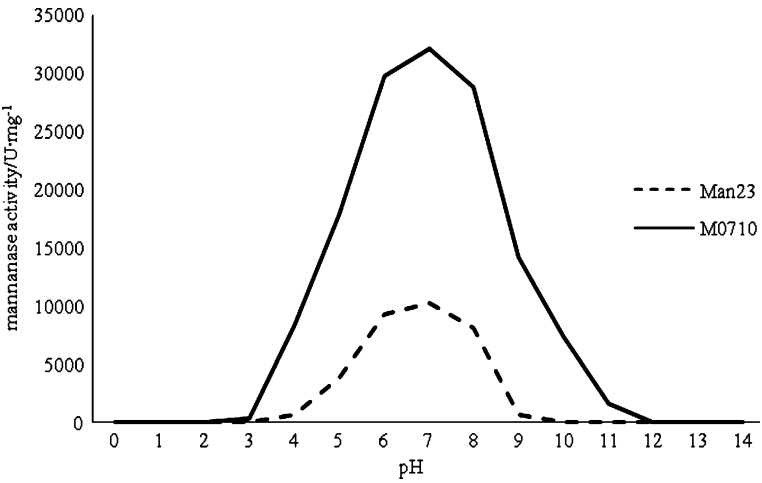

Fig. 3 Catalytic pH range of M0710 and Man23; 0.9 ml of 0.5 g/100 ml glucomannan (prepared in 50 mmol/l phosphate buffer) was mixed with 0.1 ml of 1 mg/ml enzyme solution and incubated at 50 °C for 3 min with different pH of 2, 3, 4, 5, 6~10, 11, and 12. Boiling water bath terminated the reaction, and then the content of the reducing sugar was determined using the DNS method

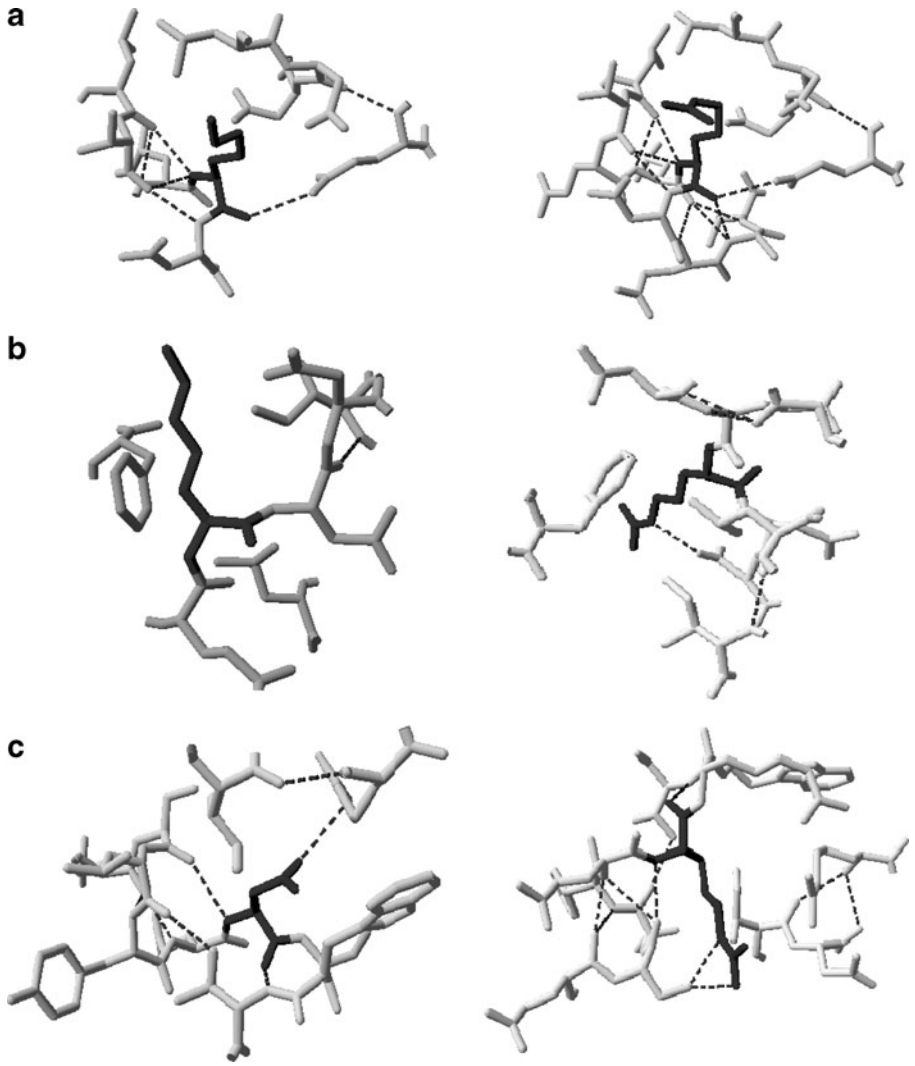

Fig. 4 Mapping of hydrogen bonds onto the site 178, 207, and 340 in mannanase Man23 and its variant

with the active center. More importantly, the mutations that can interact to build a strong network are energetically more effective than the equivalent number of isolated mutations.

It has been demonstrated that enzyme function is maintained as long as native-like conformational mobility and stability is preserved [26]. The strategy in this paper is a safe way to be compatible in activity and stability.

Conclusion

The findings in this paper suggest that the multiple mutations in or near the helixes and strands have contributed to the increased stability of our target protein at a higher

temperature and a broader pH. At the same time, these mutations are safe to provide a balance with protein activity, without any loss of activity.

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