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Construction of Fusion Protein XYNAB and Improvement of its pH and Thermal Stability from *Aspergillus niger* 400264

Xinran Li*, Weijia Zhang*, Xi Li, Qian Wu, Hui Xu, Dairong Qiao, Yi Cao and Yu Cao**

Microbiology and Metabolic Engineering Key Laboratory of Sichuan Province, College of Life Science, Sichuan University, Chengdu, Sichuan - 610064, P.R. China.

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In order to improve the pH and thermal stability of xylanase, the fusion enzyme gene (*xyn*AB) was constructed by *Aspergillus niger* SCTCC 400264 xylanase genes (*xyn*A and *xyn*B). Based on *Aspergillus niger* SCTCC400264 xylanase genes *xyn*A (FJ785738.1) and *xyn*B (FJ772090.1), the fusion expression vector pET-32a-*xyn*AB was constructed with an oligopeptide of seven amino acids (GlyGlyGlySerGlyGlyGly) introduced between XYNA and XYNB. After pET-32a-*xyn*AB transformed into *E. coli* BL21 competent cells, the fusion enzyme was expressed for activity detection by 3, 5-dinitrosalicylic acid (DNS) method and further characterized. The enzyme activities of *xyn*A, *xyn*B and *xyn*AB were 16.58U/mg, 1201.7U/mg and 47.3U/mg, respectively. There appeared two peaks of the optimum temperature of XYNAB, the first peak value was 50ºC, the same with XYNA and the second one was 35ºC, the same with XYNB. The optimum pH of XYNAB was 4.0, which compromised the properties of two single enzymes whose optimum pH were 3.0 and 5.0, respectively. When incubated at 80 ºC for 5min, it retained 35% of enzyme activity, showing higher thermostability than XYNA but lower than XYNB. XYNAB had a good stability at pH ranging from 2.0 to 10.0. The effect of metal ions on it was basically the same as XYNA, XYNB. The enzyme activity of XYNAB was strongly increased by K^+ and Mg^{2+} but inhibited by Cu^{2+}, Fe^{3+} and Mn^{2+}. The enhanced difunctional XYNAB fusion enzyme was gained in this paper. According to the conformation of XYNAB, the gap of XYNB for binding substrate was mostly blocked or covered by the whole XYNA, which resulted in XYNB not binding with substrate completely and so XYNAB showed the whole of the XYNA activity and part of XYNB.

Key words: *Aspergillus niger*; Xylanase; Fusion Enzyme; pH stability; Thermal stability.

Xylan is a kind of polymeric pentose which is widely found in plants and accounts for approximately 35% of the dry weight of the cell[1]. It is also the second most abundant renewable resource after cellulose in nature[2]. Belonging to the families of hemicellulose, xylan is a polymer of xylopyranose whose backbone is β-D-xylopyranose composed of β-1, 4-glycosidic bonds with L-arabinose as branches consisting of α-1, 2 and α-1, 3-glycosidic bonds[3].

As a typical acid-base affinity reaction, the complete degradation of xylan requires β-1,4-endo-xylanase, β-1,4-exo-xylanase and β-xylosidase (side-chain hydrolase) to work together[4]. In terms of the cleavage pattern, endo-xylanases, key enzymes in the hydrolysis process, can hydrolyze xylosidic bands from the inner backbone and produce xylooligosaccharides, xylobiose and so on, which can largely reduce viscosity. Exo-xylanases, on the other hand, can only function at the end of the main chain and therefore have a less effect on the reduction of viscosity than the former. Besides, β-xylosidases

* To whom all correspondence should be addressed.
E-mail: geneium@scu.edu.cn
*Xinran Li & Weijia Zhang contributed equally to this work.

can cleave the end of xylooligosaccharides and produce xylose[5, 6, 7].

Xylanases, a group of enzymes degrading β-1,4-xylosidic bands in xylan, are classified into families F/10 and G/11 based on the analysis of amino acid sequence and hydrophobic cluster[8, 9]. Among them, xylanases in families G/11 have a high specificity to xylan, with xylose as the main enzymolysis product. Due to the small catalytic area (about 20kDa), it is easy for them to contact with substrate and therefore brings important application in feed industry.

When two active proteins are constructed into fusion protein, it requires both to form normal three-dimensional conformation in order to keep their respective activities or better play their bioactivities, during which the linker peptide plays a crucial role. There can't be too many α-helixes or β-sheets in the composition of the linker peptide sequence or it will restrict the activity of the fusion protein. The OsmC-region (osmotically induced protein family) of the two-domain esterase EstO from the psychrotolerant bacterium *Pseudoalteromonas arctica* has been shown to increase thermolability. Thermostability of XynB-OsmC is significantly changed in comparison to wild-type XynB. The OsmC-fusion variant showed an apparent decrease in thermostability between 40 and 45ºC, while both proteinsare highly instable at 50ºC [10]. A coexpression system for the extracellular production of phytase and xylanase was established in *Pichia pastoris*. The genes for each enzyme were fused in-frame with the a-factor secretion signal and linked by the 2A-peptide-encoding sequence. Each enzyme was expressed extracellularly as individual functional proteins. The specific activities of 2A-expressed phytase (PhyA-2A) and 2A-expressed xylanase (XylB-2A) were 9.3 and 97.3U/mg, respectively[11]. The xynA was fused with the hyperosmotically inducible periplasmic protein of *E. coli*, OsmY. The purified fusion enzyme exhibited the highest activity at 65 ºC and pH 6.0 [12].

Two xylanase gene *xyn*A and *xyn*B cloned from *Aspergillus niger* SCTCC400264 [13] belong to family G/11. The optimum temperature of XYNA and XYNB were 35 ºC and 55 ºC and the optimum pH of them were 3.0 and 5.0, respectively. In terms of the affinity to substrate, XYNA was better than XYNB while the latter was obviously superior to the former in thermostability[14]. Ultimately, the difference in the nucleotide sequence which leads to diverse space-conformations of proteins results in different characterizations of enzymes. However, XYNA and XYNB coding region shared only 52.5% and 51.5% similarity in the nucleotide sequence and the amino acid sequence, respectively. Generally, sequences of xylanases in the same family should have high homology and close affinity. However, the homology of two isozyme genes XYNA and XYNB from the identical *Aspergillus niger* was not high, suggesting that there probably exists distinct evolutionary approaches or catalytic mechanisms between two genes. In view of their respective advantages in the enzymatic characterization, a fusion enzyme of XYNA and XYNB was constructed and expressed and its catalytic characterization was further explored in this paper.

MATERIALS AND METHODS

Vectors and Strains

pMD19-T-XynA , pMD19-T-XynB pET32a(+) , *E.coli* JM109 and *E.coli* BL21 were all stored in this lab.

Enzymes and chemicals

DNA polymerase, T4 ligase, restriction endonucleases *EcoR* I ,*Xhol* I, *BamH* I and *Bgl* II, pMD 19-T Vector, TaKaRa PCR Amplification Kit, ExTaq™ PCR Kit and Gel Extraction Kit were all purchased from TaKaRa Dalian, China. The primers for PCR were synthesized by Invitrogen, China.

METHODS

Construction of the fusion expression vector

The vectors were amplified from pMD19-T-XynA and pMD19-T-XynB by PCR primer of the SenseA (5'GGAATTCAGTGCCGGTATCAACTACGTG3', *EcoR*I) and the AntienseA (5'CGGGATCCACCACCAGAGGAGATCGTGACACTGG3', *BamH*I), the SenseB (5'GAAGATCTGGTGGTGGTTCGACCCCGAGCTCGACCGGCGAGAA3', *Bgl*II) and the AntisenseB (5'CCGCTCGAGTTACTGAACAGTGATGGAGGAAGA3', *Xhol*I), respectively. The volume of PCR reaction system was 50 µL containing 5 µL 10x buffer, 1 µL template, 1µL(0.1 g/L) primer, 0.5 µL LA Taq polymerase, 1µL

5 mmol/L dNTP and 40.5 µL sterile water. The PCR cycle was designed as following: denaturing at 94 ºC for 30 s, annealing at 55 ºC for 30 s, extension at 72 ºC for 1 min, total 30 cycles. The PCR products extracted from the 0.8% agarose gel were purified by UNIQ-10 column and digested with *Bgl* I and *Xhol* I, *Bgl* II and *Xhol* I, respectively. Two kinds of restriction fragments were purified by UNIQ-10 column and then ligated with pET-32a(+) vector prepared by *EcoRI* and *Xho/I* digestion. After the product transformed into *E. coli* BL21 competent cells, transformants were cultured in the LB medium containing 50 mg/L kanamycin overnight. Then the fusion expression clone pET-*xyn*AB was screened from single colonies by streak cultivation. The resulting plasmids were sequenced by Invitrogen, China for verifying the correctness of the fusion sequence.

Induced expression and purification of the recombinant protein

The transformant strain *E. coli* BL21(DE3)-pET-32a-*XynAB* was cultured in a 3-ml LB medium supplemented with 50µg/mL ampicillin. When the optical density value reached 0.6~0.8, IPTG was added to a final concentration of 0.3mM to induce protein expression, and the transformant was further cultured at 30 ºC for 4h. Cells were harvested by frozen centrifugation, resuspended in PB buffer and then disrupted by sonication. Total protein was analyzed by SDS-PAGE to detect the expression of interest protein. After cell lysis buffer was centrifuged, the protein in the supernatant was harvested and further loaded onto Ni affinity chromatography column. The concrete method was operated as described by the handbook of protein purification from Qiagen. Protein concentration was determined by Bradford method using bovine serum albumin (BSA) as the calibration standard[5].

Definition and determination of xylanase activity

One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1µmol of reducing sugar as xylose per minute. The formula for calculating enzymatic activity was as following:

$$U = C*V*N/Mr/t$$

U: the enzymatic activity of samples.
C: the concentration of xylose obtained by the standard curve;
V: the volume of the enzyme solution;
N: the dilution multiple of purified enzyme;
Mr: the molecular weight of xylose (150.13g/mol)
t-reaction time(10min)

Xylanase activity was measured by 3,5-dinitrosalicylic acid(DNS) method[15]. Briefly, in a glass test tube, 0.2mL of the enzyme solution was mixed in 1.8 mL buffer (pH 5.0) containing 1% birchwood xylan and incubated at 50ºC for 10 min. Then 2.0mL of DNS reagent solution was added and reaction mixture was boiled for 7min. After cooled to room temperature, the mixture was diluted to 10 mL with water and then shaken well. The blank control was performed by adding 0.2mL diluted water instead of the enzyme solution. The subsequent steps were performed as described above and the absorbance of samples was measured with the blank control as reference.

Determination of xylanase initial reaction rate and kinetic parameters

The *K*m and *V*max kinetic parameters of xylanase were determined in citric acid-Na_2HPO_4 buffer containing various amounts of xylan ranging from 0.25%-2% under optimum temperature and pH**.** The Michaelis-Menten equation was rewritten as 1/V=*K*m/*V*max×1/[S], 1/*V*max using the method described by Lineweaver-Burk. Different [S] was chosen to measure the relevant V and the reciprocal plot was made according to 1/v and 1/[S], based on which the values of *K*m and *V*max were calculated.

Determination of optimum temperature and thermostability

The optimum temperature for xylanase activity was determined at temperatures ranging from 30 ºC to 70 ºC at an interval of 5ºC at pH 5.0 for 10min. The themostability assay was performed by incubating the recombinant enzyme at 65ºC and 85ºC for 2 to 60min and measuring the residual enzyme activity under optimum reaction condition before quick cooling.

Determination of optimum pH and pH stability

The optimum pH for xylanase activity was determined in different buffer solutions with pH ranging from 2.0 to 10.0 at an interval of 1.0 at 55ºC for 10min. The pH stability was performed by incubating purified enzyme in buffer solutions with different pH for 30min and measuring the residual enzyme activity under optimum reaction condition.

Effect of metal ions

The effect of metal ions on xylanase activity was determined at 37 ºC for 30 min by

adding various metal ions, i.e. Co^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, K^{+}, Mg^{2+}, Ca^{2+} and Fe^{2+} into purified enzyme at a final concentration of 1 mmol/L and measuring the residual enzyme activity under optimum reaction condition.

Prediction of the protein tertiary structure

The prediction of the protein tertiary structure was performed by SWISS-MODEL (http://swissmodel.expasy.org/).

RESULTS

Induced expression and purification of the *Aspergillus niger* XYNAB fusion protein

The *xynAB* gene was successfully expressed in the *E.coli* BL21(DE3). There appeared an apparent target band of 60kDa on SDS-PAGE, which matched with the estimated molecular weight of the *xyn*AB ORF without signal peptide (40.33kDa) with the His-tag (21kDa). Additionally, the protein was expressed in the supernatant with good solubility which was helpful for subsequent purification.

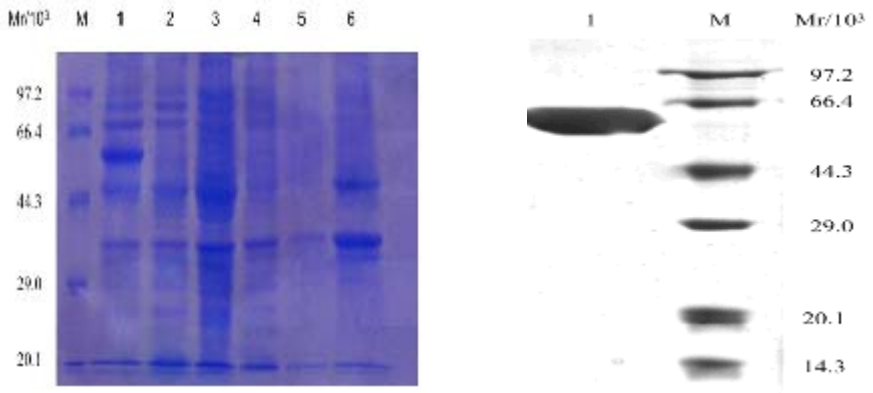
After recombinant protein XYNAB purified by Ni affinity chromatography column, a single target band appeared on SDS-PAGE (Fig. 2.) which met the requirements of further characterization of enzyme. The concentration of *xyn*AB recombinant protein was 6 mg with a total volume of 20ml measured by Bradford Coomassie Brilliant Blue method and the protein would be further used for enzymatic activity assay and characterization.

Specific activity and kinetic parameters of the *Aspergillus niger* XYNAB

The specific activity of the fusion enzyme XYNAB was higher than the single enzyme XYNA, while far lower than that of the single enzyme XYNB and not higher than the sum of specific activities of two single enzymes (Table 1). However, in terms of *Km*, the affinity to substrate of XYNAB was apparently higher than those of two single enzymes. In addition, the catalytic activity of XYNAB was significantly better than XYNA while there was still a large gap between XYNAB and XYNB.

Table 1. Specific activity and kinetic parameters of the fusion enzyme XYNAB in comparison with the single enzyme XYNA and XYNB

Characterization	XYNAB	XYNA[13]	XYNB[13]
*K*m(mg/ml)	10.68	13.8	18.7
*V*max(μmol/mg.min)	72.63	43.66	1666.7
Specific activity(U/mg)	47.3	16.58	1201.7



A: M: Marker; Lane1: supernatant of pET32a-*xyn*AB; Lane2: supernatant of pET32a; Lane3: supernatant of pET32a-*xyn*AB uninduced; Lane4: precipitation of pET32a-xynAB; Lane5: precipitation of pET32a; Lane6: precipitation of pET32a-*xyn*AB uninduced.
B: M: Marker; Lane 1: XynAB, 200 mmol/L Imidazole elution

Fig. 1. Expression (A) and purification (B) of the *xyn*AB fusion protein confirmed by SDS-PAGE

Optimum pH and pH stability of the *Aspergillus niger* XYNAB

The optimum pH of the fusion enzyme XYNAB was 4.0 (Fig. 2A) and that of the single enzyme XYNA and XYNB were 3.0 and 5.0, respectively. Therefore, the optimum pH of XYNAB compromised the properties of two single enzymes. Additionally, the residual enzyme activity of XYNAB was more than 75% at pH ranging from 2.0 to 10.0, which showed good pH stability generally.

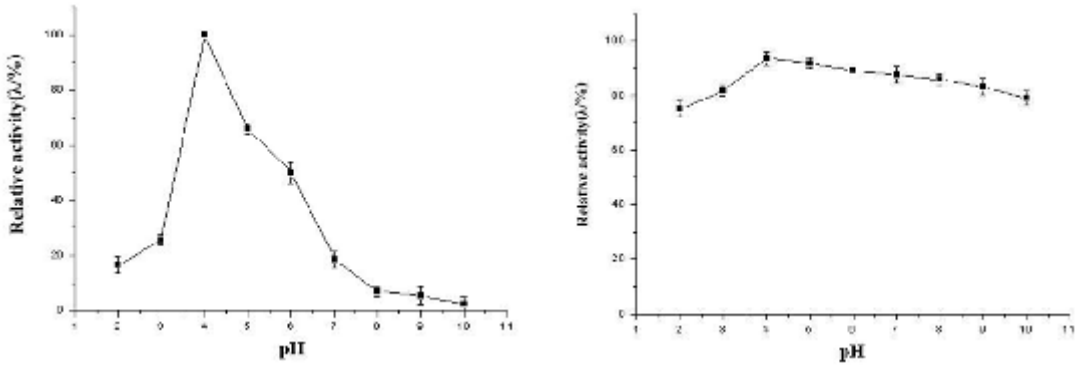


Fig. 2. Optimum pH (A) and pH stability (B) of the *Aspergillus niger* XYNAB

Optimum temperature and thermostability of the *Aspergillus niger* XYNAB

There were two peaks of the optimum temperature of the *Aspergillus niger* XYNAB (Fig. 3), the first and highest one was 50ºC and the second was 35ºC. Correspondingly, the optimum temperature of the fusion enzyme XYNAB almost simultaneously reflected that of two single enzymes XYNA and XYNB which were 35ºC and 55ºC, respectively.

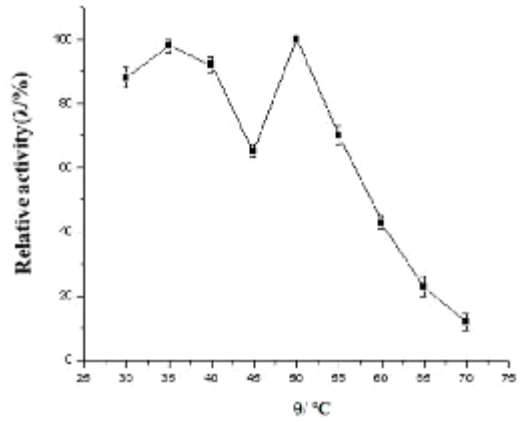


Fig. 3. Optimum temperature of the *Aspergillus niger* XYNAB

When incubated at 65ºC for 5min, the *Aspergillus niger* XYNAB retained 53% of its initial activity while for 10min it retained only 22% (Fig. 4A). When heated at 80ºC for 5min, the residual enzyme activity of XYNAB was 35% while for 10min that was only 5% (Fig. 4B) . In terms of the general thermostability, XYNAB was similar to XYNA but largely lower than XYNB[13].

Effect of metal ions on the enzyme activity of the *Aspergillus niger* XYNAB

The results of XYNAB treated with various metal ions (Table 5) showed that the activity of XYNAB was strongly increased by K^+ and Mg^{2+}, followed by Ca^{2+} and Fe^{2+}, while it was not much affected by Zn^{2+} and Co^{2+}. Oppositely, the activity of XYNAB was inhibited by Cu^{2+} , Fe^{3+} and Mn^{2+}.

DISCUSSION

Design of the linker sequence in the fusion of *xyn*A and *xyn*B

The common amino acids in linker are non-polar hydrophilic glycine (Gly) and serine (Ser) with simple structure and small size. At present, the linker peptide containing Gly-Ser is one of the most commonly reported linker peptides, especially the linker $(Gly_4\ Ser)_3$ which is widely used in the construction of fusion protein due to its appropriate

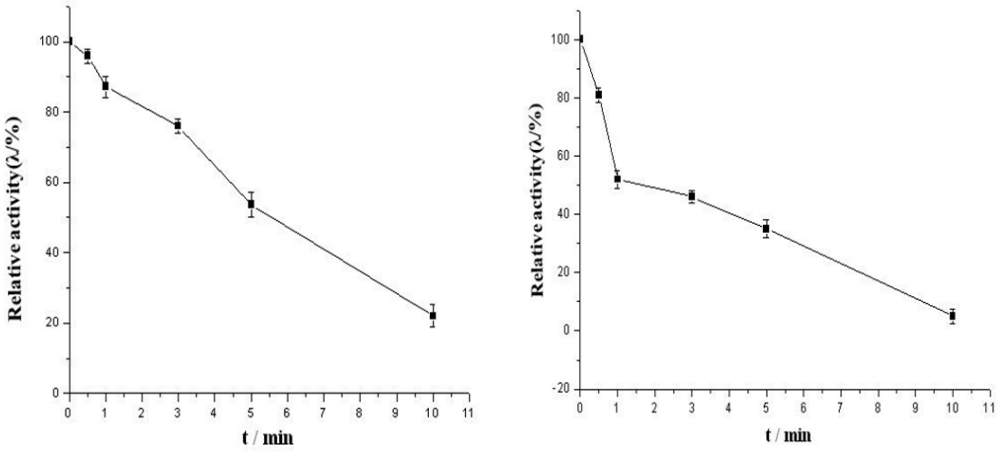


Fig. 4. Thermostability of the *Aspergillus niger* XYNAB

amino acid length, hydrophobicity and extensibility that can keep functional proteins good stability and bioactivity[16].

The flexible peptides (GGGGS)n (n≤3), the α-helical peptides (EAAAK)n (n≤3) and two other peptides were used as linkers to construct biofunctional fusions of β-glucanase (Glu) and xylanase (Xyl) for improved catalytic efficiencies of both moieties. The peptide linker (GGGGS)2 resulted in the best fusion, whose catalytic efficiency had a net increase of 326% for the Glu and of 43% for the Xyl. The two moieties of a fusion with the linker (EAAAK)3 also showed net increases of 262 and 31% in catalytic efficiency[17]. The linker for the construction of fusion gene in this paper was $Gly_3SerGly_3$. The result showed that the affinity to substrate of XYNAB was apparently higher than two single enzymes XYNA and XYNB, however, the enzyme activity was merely 185% higher than the single enzyme XYNA but far lower than that of the single enzyme XYNB. Consequently, in order to achieve a good characterization of higher specific activity than the sum of that of two single enzymes, some improvements can be made as following: 1) in the process of constructing fusion gene, *xynB* can be put in front of *xynA* so that the catalytic site of XYNB which has better enzymatic characterization is not likely to be covered, maybe it will lead to a better result. 2) when the linker to designed, the number of nucleotides can be properly increased, the peptide length can be longer or the linker α-helical peptides (EAAAK)n (n≤3) can be used to appropriately increase the spatial distance between *xynB* and *xynA* to avoid the steric hindrance.

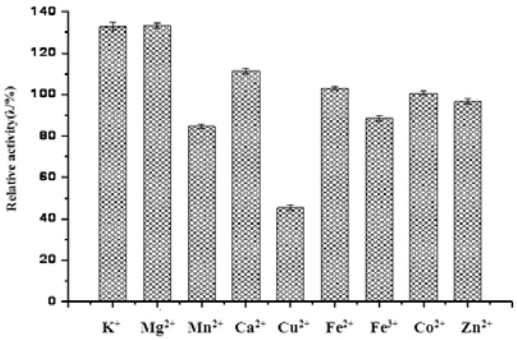


Fig. 5. Effect of metal ions on the enzyme activity of the *Aspergillus niger* XYNAB

Effect of the affinity to substrate of the *Aspergillus niger* XYNAB

On the whole, the tertiary structure of xylanases in the family G/11 appears a sandwich structure with one α-helix and two β-sheets which form a huge gap for binding polymeric xylan[19]. The bioinformatics analysis results showed that (Fig. 6) two single enzymes *xynA* and *xynB* were ligated by the linker peptide consisting of seven amino acids and the tertiary structure of both was typical β-sheet appearing r-hand which was similar to the crystal structure of the xylanase I in the *Aspergillus niger* family G/11[20]. Ligated by the linker peptide, XYNA and XYNB were constructed into fusion

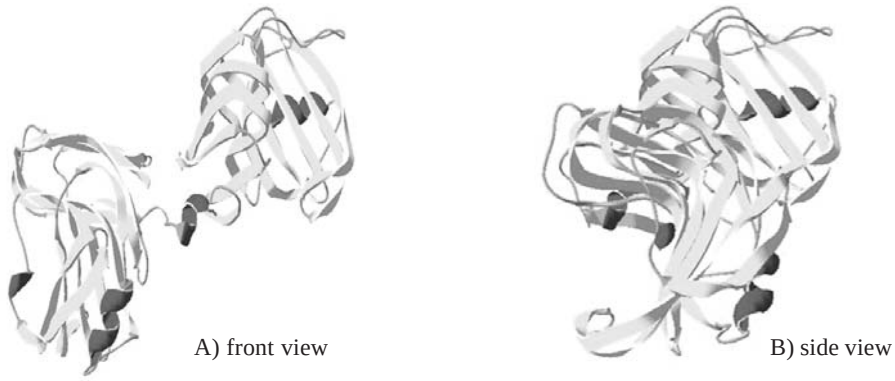


Fig. 6. Prediction of the tertiary structure of the *Aspergillus niger* isozyme fusion gene xynAB

protein XYNAB. In consideration of the characterization of XYNAB which was biased towards and a little better than XYNA and the locus of *xynA* in front of *xynB* when constructing the fusion gene, it was probably that the gap of XYNB for binding substrate was mostly blocked or covered by the whole XYNA, which resulted in XYNB not binding with substrate completely. Thus, XYNAB showed only the whole of the XYNA activity but relatively little of XYNB. Simultaneously, the value of *Km* reflects the ability of enzyme binding to substrate. The *Km* of XYNAB was 10.68, lower than 13.8 of XYNA, suggesting that the binding ability to substrate of XYNAB was increased just a little compared to that of XYNA and on the other hand supporting our conclusion.

Effect of the pH and thermal stability of the *Aspergillus niger* XYNAB

There were two peaks of the optimum temperature of the *Aspergillus niger* XYNAB which were 50ºC and 35ºC, respectively, in accord with the optimum temperature of two single enzymes XYNA and XYNB[13]. The thermostability of XYNAB was basically in line with that of XYNA but different in the change law. When incubated at 65ºC and 85ºC, the thermostability of XYNA declined linearly while that of fusion enzyme dropped rapidly after incubated at 85ºC for 1min and then declined slowly, which was similar to the change law of XYNB at 85ºC. It was probably that the conformation of XYNAB had some changes under high temperature and then the covered gap of XYNB for binding substrate was exposed, thereby the role of XYNB could be played to some extent.

The optimum pH of two single enzymes XYNA and XYNB were 3.0 and 5.0, respectively and that of XYNAB was 4.0, which compromised the properties of two single enzymes. Additionally, XYNAB at pH ranging from 2.0 to 10.0 showed good stability. Similar cases were also found in the research of the characterization of *Aspergillus sulphureus xyn*AB fusion enzyme[18]. The optimum pH of *Aspergillus sulphureus* XYNAB was 4.4 and the relative enzyme activity retained more than 75% at pH ranging from 2.4 to 5.4. When incubated at pH 5.4, XYNA only retained 50%, while at pH 2.4 the residual enzyme activity of XYNB was less than 50% and XYNAB retained about 40% activity at pH 6.4 which was nearly neutral. The broadening of XYNAB pH scope might be due to the summation of activities of two enzymes.

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