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Engineering of multiple arginines into the Ser/Thr surface of *Trichoderma reesei endo*-1,4-β-xylanase II increases the thermotolerance and shifts the pH optimum towards alkaline pH

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We studied the effects of increase in the number of surface arginines on the enzyme activity and stability of *Trichoderma reesei endo*-1,4-β-xylanase II. The number of arginines was increased in two mutant series. The first set contained six arginines on different sides of the protein surface. These arginines had no significant effect on the thermostability. However, the optimal pH region became narrower. Another series of five arginines was engineered into the 'Ser/Thr surface', formed of part of the double-layered β-sheet located on one side of the 'right-hand-like' xylanase. These mutations shifted the activity profile to the alkaline region by ~0.5–1.0 pH units. In addition, the arginines on the Ser/Thr surface increased the enzyme activity at high temperature, although the enzyme stability in the absence of substrate decreased significantly at 50–55°C. In the presence of the substrate, the thermostability increased 4–5-fold at 60–65°C. Thus, the substrate neutralized the destabilizing effect of Ser/Thr surface arginines and revealed a stabilizing effect of the same mutations. The stabilizing effect of arginines at high temperatures was seen clearly only when five arginines were introduced into the Ser/Thr surface.

Keywords: *endo*-1,4-β-xylanase/family 11/ pH-dependent activity/protein surface arginines/ thermostability

Introduction

Several studies have shown that surface arginines have a role in protein stability (Mrabet *et al.*, 1992; Vogt *et al.*, 1997). Replacement of lysine with arginine has been shown to increase the thermostability of many proteins. Increasing the number of arginines on the protein surface also appears to be involved in the adaptation of enzymes to highly alkaline pH (Shirai *et al.*, 1997).

Endo-1,4-β-xylanases hydrolyze β-1,4-linked xylopyranose chains found in xylan, one of the most abundant polysaccharide fibers in nature. Xylanases are used for biomass modification in pulp bleaching, baking and manufacture of animal feed (Viikari *et al.*, 1994; Prade, 1996). In family 11 xylanases (EC 3.2.1.8.; previously family G), two large β-pleated sheets and one α-helix form a structure that resembles a partly-closed right hand (Törrönen and Rouvinen, 1997). The stability of family 11 xylanases has been improved by several mutations at different positions in the protein structure (Arase *et al.*, 1993; Wakarchuk *et al.*, 1994; Sung *et al.*, 1998; Georis *et al.*, 2000; Sung and Tolan, 2000; Turunen *et al.*, 2001). In this study, we used *Trichoderma reesei* xylanase II (XYNII) (Tenkanen *et al.*, 1992), which shows optimal enzyme activity at pH 5–6, as a model to study how a systematic increase of arginines on the protein surface affects the pH optimum, thermostability and enzyme activity at high temperature.

Materials and methods

Expression vectors and production of xylanase

We used two clones of *T.reesei* xylanase II (XYNII). The first was the pALK143 expression construct (ROAL, Rajamäki, Finland; Turunen *et al.*, 2001), in which XYNII was expressed under the *Bacillus amyloliquefaciens* α-amylase promoter. Eleven amino acids of the *T.reesei* XYNII prosequence were present in this construct (Saarelainen *et al.*, 1993). The xylanase expressed from pALK143 was secreted into the medium under the control of the α-amylase signal sequence. Secondly, XYNII was expressed under the pKKtac vector (VTT, Espoo, Finland), in which protein secretion is governed by the pectate lyase (pelB) signal sequence (induction by 1 mM IPTG). The wild-type XYNII was expressed in higher quantities from pKKtac than from pALK143. The R series mutations were introduced to XYNII via the pKKtac vector and the ST series mutations via pALK143.

Generation of XYNII mutants

In planning the mutations, Swiss-PdbViewer (http://www.expasy.ch/spdbv/) (Guex and Peitsch, 1997) was used as a tool to examine the XYNII structure (1xyp). The mutations were generated by polymerase chain reaction (PCR), in which the mutations were introduced into the oligonucleotide primers as described elsewhere (Turunen *et al.*, 2001). The mutant clones were grown on agar plates containing xylan (birchwood xylan; Sigma, Steinheim, Germany) coupled to Remazol-brilliant blue, in which the xylanase activity is indicated by white halos around the positive colonies (Biely *et al.*, 1985).

Enzyme assay

Xylanase activity was determined by measuring the amount of reducing sugars liberated from 1% birchwood xylan (Bailey *et al.*, 1992). The activity determination in standard conditions was carried out at pH 5 and 50°C, with a reaction time of 10 min. Citrate-phosphate buffer (50 mM) was used in the xylanase assays at pH 3.0–7.5. Properties of the mutant xylanases were tested using the *Escherichia coli* culture broth as the source of xylanases. When the xylanase activity was very low in the *E.coli* culture broth, the enzyme was concentrated by 65% ammonium sulfate precipitation followed by dialysis against 25–50 mM citrate-phosphate buffer, pH 6. Inactivated *E.coli* culture broth or citrate-phosphate buffer was used to dilute the enzyme for activity and stability measurements. Bovine serum albumin (0.1 mg/ml) was included in the time-dependent hydrolysis experiments (Figure 5).

Results

The mutants

The sites on the protein surface to be replaced by arginines were mainly selected on the basis that the site in question is

Table I. Arginine mutants of *T.reesei* XYNII

Mutant	Amino acid changes
Introduction of arginines to different sides of XYNII (R series)	
R1	K58R
R2	K58R, A160R
R3	K58R, A160R, N97R
R4	K58R, A160R, N97R, N67R
R5	K58R, A160R, N97R, N67R, T26R
R6	K58R, A160R, N97R, N67R, T26R, A132R
Introduction of arginines to the Ser/Thr surface (ST series)	
ST1	S186R
ST2	S186R, N67R
ST3	S186R, N67R, T26R
ST4	S186R, N67R, T26R, Q34R
ST5	S186R, N67R, T26R, Q34R, S40R
ST6	S186R, N67R, T26R, Q34R, N69R
ST7	S40R

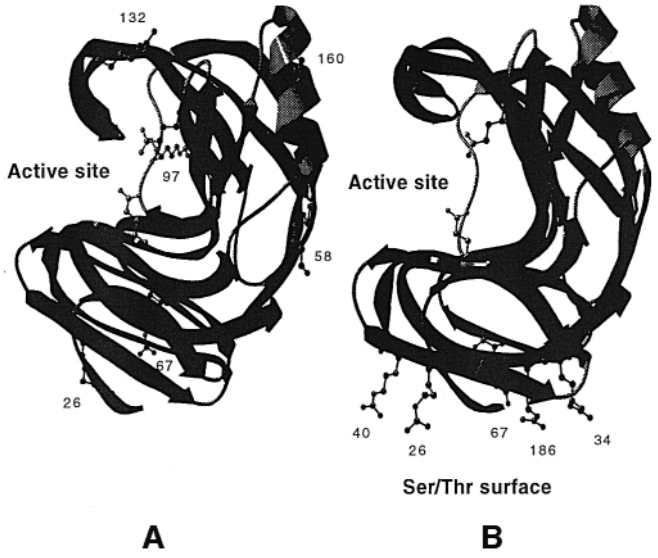

Fig. 1. The structure of *T.reesei* XYNII and the amino acid positions changed to arginines are shown (**A**) for the R6 mutant and (**B**) for the ST5 mutant. The β-strands are shown in blue (and violet), the α-helix in green and the catalytic glutamates in the active site in green. The pictures of the enzyme structures were created by Swiss-PdbViewer.

occupied by a charged amino acid (often arginine) in some members of the family 11 xylanases. Two mutation series were performed: the R series and the ST series (Table I). The mutations in the R series were located on different sides of the protein, whereas the mutations in the ST series were located only on the Ser/Thr surface (Figure 1). The Ser/Thr surface of the wild-type XYNII does not contain any charged residues. The wild-type XYNII contains altogether six arginines and four lysines.

Arginines on different sides of the protein surface

The engineered arginines in the R series did not increase the thermostability, except for the mutation K58R (mutant R1), which increased the half-life of XYNII at 55°C (pH 6) from ~5 min to 10–20 min. R1 was more stable than wild-type XYNII and the other mutants over a wide pH range (pH 5–9) (results not shown). A surprising finding was that the other R series mutants (R2–R6) generally had a similar stability than the wild-type XYNII (results not shown). Introduction of several

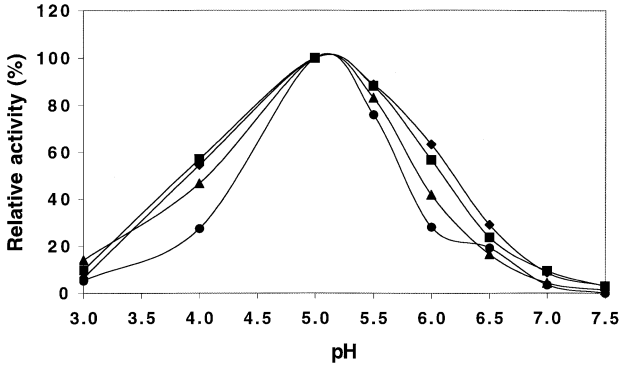

Fig. 2. pH-dependent activity profile of the R series mutants. The xylanase activity was measured by 30 min incubation at 60°C. Symbols: wild-type XYNII (diamonds), R4 (squares), R5 (triangles), R6 (circles).

Table II. The half-lives (min) of the ST series mutants in the absence of substrate at 50°C, and in the presence of substrate at 60 and 65°C

Substrate mutant	Absent 50°C[a]	Present	
		60°C[b]	65°C[b]
WT	64	26	3.1
ST1	55	n.d.	n.d.
ST2	44	n.d.	n.d.
ST3	22	14	3.0
ST4	7	24	4.4
ST5	7	125	12
ST6	15	87	16

[a]The half-lives without substrate were determined at 50°C (pH 6).
[b]The half-lives in the presence of substrate were determined from time-dependent hydrolysis experiments performed at 60 and 65°C (pH 5). n.d., not determined.

arginines on different sides of XYNII made the pH-dependent activity profile narrower (Figure 2). The R series mutants had essentially the same temperature optimum (maximum 1–2°C elevation in R4–R6) than the wild-type XYNII (not shown).

Arginines on the Ser/Thr surface

When the arginines were introduced into the Ser/Thr surface (Figure 1), the effect was much different from that in the R series mutants. The thermostability in the absence of substrate decreased with an increasing number of arginines (Table II). When four to five arginines were introduced into the Ser/Thr surface, the half-life decreased from ~60 to ~7–15 min at 50°C, whereas one or two arginines on the Ser/Thr surface had little effect on the stability.

The arginines had a clear effect on the pH-dependent activity profile. When the xylanase assay was done in the standard conditions (10 min incubation at 50°C), the introduction of five arginines into the Ser/Thr surface (ST5 or ST6) shifted the activity profile to the alkaline region by 0.5–1.0 pH units (Figure 3). The pH optimum of the mutants ST5 and ST6 was at pH 6–7, whereas that of the wild-type XYNII was at pH 5–6. Three or four arginines on the Ser/Thr surface also shifted the pH optimum upwards, but to a smaller extent than five arginines (Figure 3; ST3 not shown). The pH-activity profile produced with 30 min incubations at 60°C showed an upward shift in the activity profile of 0.5 pH units in ST5 and ST6 compared with the wild-type XYNII (results not shown). Such a shift at high temperature with prolonged incubation indicated that in addition to the pH-dependent activity profile

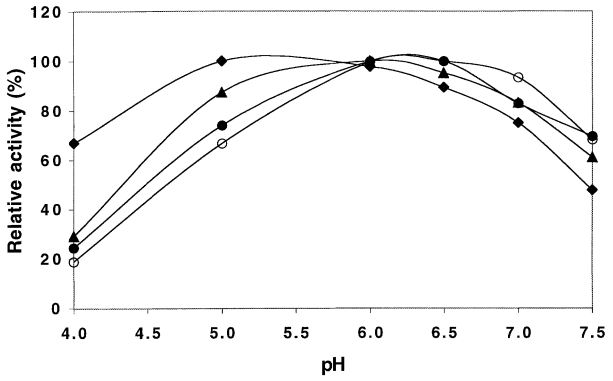

Fig. 3. pH-dependent activity profiles of the ST series mutants. The xylanase activity was measured by 10 min incubation at 50°C. Symbols: wild-type XYNII (diamonds), ST4 (triangles), ST5 (closed circles), ST6 (open circles).

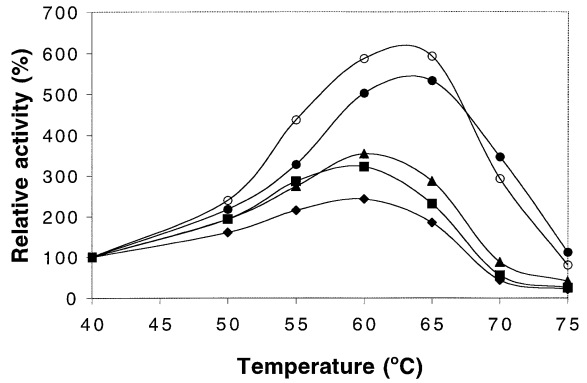

Fig. 4. Activity of the ST series mutants as a function of temperature. The enzyme activity was measured by 10 min enzyme incubation at each temperature (pH 5). The activity at 40°C is shown as 100%. Symbols: wild-type XYNII (diamonds),ST3 (squares), ST4 (triangles), ST5 (closed circles), ST6 (open circles).

(Figure 3), the pH-stability region in the presence of the substrate had also shifted to a more alkaline pH.

A surprising finding was that despite decreased thermostability in the absence of the substrate, the arginines on the Ser/Thr surface considerably increased the enzyme activity at elevated temperature. The apparent temperature optimum in the 10 min assay increased by ~5°C when five arginines (ST5 and ST6) were engineered into the Ser/Thr surface (Figure 4). Four arginines (ST4) had only a small effect. The location of the fifth arginine (S40R in ST5 and N69R in ST6) did not have a significant effect on the increase in the temperature-dependent activity.

The hydrolysis experiments revealed that the five-arginine mutants (ST5 and ST6) had an increased thermostability in the presence of the substrate. When the hydrolysis of xylan was followed as a function of time, the mutants ST5 and ST6 retained their activity at 65°C and pH 5 remarkably better than the wild-type XYNII (Figure 5). Although the wild-type XYNII showed some activity at 65°C, the activity was rapidly lost during the prolonged reaction. In contrast, the ST5 and ST6 mutants digested xylan fibers continuously during the 90 min hydrolysis experiment (Figure 5). The mutants with three (ST3) or four arginines (ST4) did not exhibit improved stability in the presence of the substrate at 65°C compared to the wild-type XYNII (Figure 5; Table II). The R series mutant, R6, showed no increase in stability in the hydrolysis experiment at 60°C (results not shown).

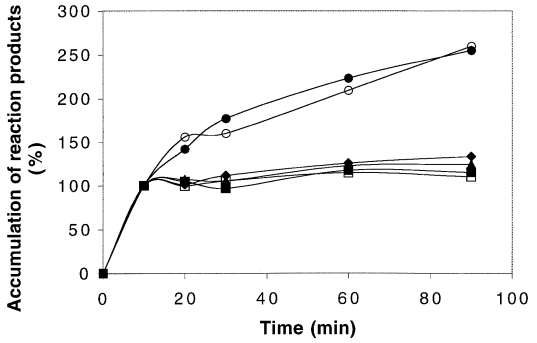

Fig. 5. Hydrolysis of 1% xylan by the ST series mutants as a function of time at 65°C. To compare different enzymes, the activity detected after 10 min enzyme reaction was set as 100%. Symbols: wild-type XYNII (diamonds), ST3 (closed squares), ST4 (triangles), ST5 (closed circles), ST6 (open circles) and ST7 (open squares).

Table III. Arrhenius activation energy (E_a) and apparent temperature optima (T_{opt}) of selected ST series mutants

Mutant	E_a (kJ/K/g mol)[a]	T_{opt} (80%) (°C)[b]
WT	57.2	53–64
ST3	61.9	53–64
ST4	66.5	56–65
ST5	77.3	58–68
ST6	81.1	56–67

[a]Arrhenius activation energies were determined from the temperature-dependent activity curves.
[b]T_{opt} (80%): an estimated temperature region in which the enzyme shows >80% of its maximal activity in a 10 min reaction.

The half-lives in the presence of the substrate could be determined from the time-dependent hydrolysis curves (Figure 5). The results (Table II) showed that the stability of the five-arginine mutants (ST5 and ST6) at 60 and 65°C was four to five times higher than that of the wild-type XYNII.

The Arrhenius activation energy was determined from temperature-dependent activity curves (temperature range 40–55°C) (Doran, 2000). The activation energy clearly increased when five arginines were introduced into the Ser/Thr surface (Table III). The level of increase was ~40%. The four to six arginines in the R series increased the Arrhenius activation energy only by 10–20% (not shown). The higher Arrhenius activation energy indicates that the catalytic activity was decreased at these temperatures in the mutant enzymes compared to the wild-type XYNII. The temperature-dependent activity profiles show that elevation of the temperature increased the activity of ST mutants more than that of the wild-type XYNII (Figure 4).

In conclusion, the increase of enzyme stability at high temperature by the introduction of arginines into the Ser/Thr surface is dependent on the number of arginines; five arginines are required, whereas four arginines had only a very small effect. The exact position of the arginines on the Ser/Thr surface appeared not to be critical for the effect. The increased apparent temperature optima of ST5 and ST6 appeared to be a result of increased thermostability, and changes in the Arrhenius activation energy also affected the response of the mutant enzymes to increased temperature.

Discussion

Several studies indicate that there is a correlation between protein stability and the number of arginines on the protein

surface. The comparison of mesophilic proteins and their thermophilic counterparts has revealed that thermophilic proteins have, on average, a higher arginine content on the protein surface (Argos *et al.*, 1979; Vogt *et al.*, 1997). At the same time the amount of lysine was observed to decrease, thus increasing the Arg/Lys ratio. This result is supported by experimental mutagenesis studies in which Lys→Arg mutations stabilize the protein structure (Cunningham and Wells, 1987; Mrabet *et al.*, 1992). It has been proposed that an important reason for the increased stability is stronger hydrogen bonding of the large guanidinium group of arginine with nearby polar groups (Mrabet *et al.*, 1992; Borders *et al.*, 1994). In the majority of protein families, an increased number of hydrogen bonds correlates with increased thermostability (Vogt *et al.*, 1997). Involvement in the ion pair networks is another possible factor responsible for the increased thermostability conferred by arginine, although other explanations can also be found in specific cases (Mrabet *et al.*, 1992).

In addition to their effects on protein stability, electrostatic interactions also have a role in the adaptation of proteins to functioning at low or high pH. Some acidic xylanases (PDB codes: 1BK1, 1ukr), which function optimally at low pH have an increased number of acidic residues and tend to avoid basic residues on the Ser/Thr surface (Krengel and Dijkstra, 1996; Fushinobu *et al.*, 1998). There is a similar correlation between the number of acidic residues and low pH optimum in pepsin, which also functions in the acidic pH range (Sielecki *et al.*, 1990). Sequence comparisons of bacterial serine proteases revealed that adaptation to highly alkaline conditions involves a decrease in the number of acidic amino acids and lysine and an increase in arginine and neutral hydrophilic amino acids (Masui *et al.*, 1994; Shirai *et al.*, 1997). In particular, the Arg/Lys ratio increased with increasing activity at alkaline pH (Masui *et al.*, 1994). In a subtilisin-family of serine proteases, the arginine residues were localized to a hemisphere of the protein structure (Shirai *et al.*, 1997).

The family 11 xylanases can be active at acidic or alkaline pH even without charged amino acids on the Ser/Thr surface. The acidic *T.reesei* xylanase I (PDB code: 1xyn) and the xylanase (1QH7) from a strictly alkaliphilic *B.agaradhaerens* do not contain any charged amino acids on the Ser/Thr surface (Törrönen and Rouvinen, 1995; Sabini *et al.*, 1999). However, the catalytic core of *B.agaradhaerens* xylanase contains many ion pairs, including two arginines (altogether eight) and five lysines (altogether nine) more than that of *T.reesei* XYNII, which could be involved in the functioning at high alkaline pH.

In our study, we tested how a systematic increase in the amount of arginines on the protein surface affects the stability and functional properties of *T.reesei* xylanase II (XYNII). The *T.reesei* XYNII has low thermostability and is inactivated rapidly above 50°C, a process that involves conformational changes (Jänis *et al.*, 2001; Turunen *et al.*, 2001). In the R series mutants, six arginines were introduced into different sides of XYNII, and in the ST series five arginines were introduced into the Ser/Thr surface, which forms a major part of the outer surface of the double-layered β-sheet and contains a large number of serines and threonines (Figure 1). The active site is located in a large canyon on the inner side of the partly closed structure formed of the double-layered β-sheet. Two active site glutamates are responsible for the catalytic activity, one functioning as a nucleophile and the other as an acid/base catalyst (for review see Törrönen and Rouvinen, 1997).

The introduction of arginines into different sides of the protein surface in the R series did not have any significant effect on the thermostability. The major effect was that the optimal region of the pH-dependent activity became narrower than in the wild-type XYNII. The introduction of arginines into the Ser/Thr surface resulted in a shift of the pH profile to alkaline pH by 0.5–1.0 pH units. A clear shift in the pH-dependent activity was observed when four or five arginines were introduced into the Ser/Thr surface. These results indicate that the Ser/Thr surface of family 11 xylanases has a role in determining the functional properties of the catalytic site. However, an increase in the number of arginines is not in itself sufficient to cause an acidic enzyme to function in highly alkaline conditions.

The effect of the Ser/Thr surface arginines on thermostability was complex. These arginines decreased the thermostability in the absence of the substrate. However, in the presence of the substrate the 5-fold arginine mutations on the Ser/Thr surface considerably increased the enzyme activity at elevated temperature. The hydrolysis experiments performed as a function of time showed that the presence of five arginines on the Ser/Thr surface increased the thermostability. The half-life in the presence of substrate was increased four to five times after the introduction of five arginines. Thus, when the destabilizing effect of the mutations was neutralized by the substrate, a clear stabilizing effect of the Ser/Thr arginines was revealed. The increased apparent temperature optimum appears to be mainly a result of the increased thermostability.

The location of arginines on the Ser/Thr surface appeared not to be critical, since the same effect was seen when the fifth arginine was located at two different sites. The dramatic effect of the fifth arginine indicates that the formed positive net charge on the Ser/Thr surface could be mainly responsible for the effects on enzyme properties. The engineering of arginines into the Ser/Thr surface did not create any ion pairs. In *B.agaradhaerens* xylanase (1QH7), the number of basic residues is high in other regions of the protein, indicating that the Ser/Thr surface is not the only region suitable for the introduction of arginines. In conclusion, the straightforward increasing of arginines on different sides of the protein surface may not alter enzyme properties in a desired way. It is evident from our study that local introduction of arginines on a specific protein surface can be a more successful approach.

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