Computational design-based molecular engineering of the glycosyl hydrolase family 11 B. subtilis XynA endoxylanase improves its acid stability

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Rational protein engineering was applied to improve the limited stability of the glycosyl hydrolase family 11 (GH11) endo-β-1,4-xylanase from Bacillus subtilis under acidic conditions. Since the pH dependence of protein stability is governed by the ionisation states of the side chains of its titrable amino acid residues, we explored the strategy of changing pH-stability profiles by altering pKₐ values of key residues through in silico designed mutations. To this end, computational predictions and molecular modelling were carried out using the recently developed pKD software package. Four endoxylanase variants, in which the pKₐ values of either Asp4 and Asp11 or His149 were targeted to shift downwards through incorporation of three to five point mutations, were generated and recombinantly expressed in the cytoplasm of Escherichia coli. All four mutants showed considerably increased functional stability at acid pH levels. They retained ~30–70% and ~75–95% of their activity after incubation at pH 3 and 4, respectively, in comparison with only ~23% and ~57%, respectively, for the wild-type enzyme under the experimental conditions. No acidophilic adaptation of the catalytic activity had occurred. In addition, their functional stability and catalytic activity profiles under different temperature and ionic strength conditions were significantly altered. These findings contribute to general understanding of the molecular mechanisms governing the pH-dependent stability of GH11 proteins, and hence they can be applied to enhance the stability and effectiveness of many GH11 endoxylanases used in industry today.

Keywords: endo-β-1,4-xylanase/in silico protein design/mutagenesis/pH-stability

Introduction

Endo-β-1,4-xylanases (EC 3.2.1.8, endoxylanases) are key enzymes in the degradation of heteroxylan, the predominant hemicellulose in the cell walls of plants and the second most abundant polysaccharide on earth. These glycoside hydrolases break down the heteroxylan backbone by catalysing the hydrolysis of internal β-1,4-bonds. Owing to the abundance of heteroxylan in plant material, endoxylanases are increasingly used as improvers and processing aids in a wide range of biotechnological processes such as industrial bread-making, feed production, biobleaching of pulps in paper manufacturing and bioconversion of agricultural residues to fuel ethanol (Subramaniyan and Prema, 2002; Polizeli et al., 2005). On the basis of amino acid sequence similarities of their catalytic domain in hydrophobic cluster analyses, most endoxylanases can be classified into glycosyl hydrolase families (GH) 10 and 11, whereas a minority is categorised into GH5, 7, 8 and 43 (Collins et al., 2005).

GH11 members adopt a β-jelly-roll fold, which has been likened to a partly closed right hand due to the existence of a unique two β-strand ‘thumb’ structure embracing the active site located within the two β-pleated sheet ‘palm’ and ‘fingers’ (Torronen and Rouvinen, 1997). In nature, this type of endoxylanases is produced by a bewildering array of micro-organisms, including phytopathogens for which it is generally assumed that these enzymes are indispensable for plant infection because of their crucial role in cell wall degradation (Beliën et al., 2006).

In recent years, a growing number of GH11 endoxylanases has been isolated from, in particular, extremophilic microorganisms to meet increasing industrial demands for enzymes that can cope with the often harsh conditions of biotechnological processes (Collins et al., 2005). In addition, numerous protein engineering endeavours have been directed towards improvement of their stability and/or optimal activity at elevated temperatures and/or extreme pHs. By far most of these research efforts aimed at the production of thermoalkalophilic endoxylanases, which are optimally suited for the pulp and paper industries, given the high temperature and alkaline pH of the pulp substrate during biobleaching (Subramaniyan and Prema, 2002). From technical and economical point of view, however, the development of endoxylanases with increased stability under acid conditions is of great interest, in view of their increasing use as feed additive working in the gastrointestinal tract, as processing aid for clarification of juices and wines, or as bread improver ingredient for the manufacture of sourdough breads (Collins et al., 2005). However, only a few studies have dealt with the acidophilic adaptation of GH11 endoxylanases (Fushinobu et al., 1998; de Lemos Esteves et al., 2004), all of them focusing on the complex hydrogen bond network occurring between the two catalytic glutamic acids and surrounding residues within the active site region (Joshi et al., 2000). Although several interesting mutants with optimal activity at decreased pH levels have been described, none of the protein engineering efforts made so far has been directed at specific improvement of the acid stability of these enzymes. This, together with the fact that up till now less than a handful acid-stable GH11 endoxylanases have been discovered in nature (Fushinobu et al., 1998; Kimura et al., 2000; Tanaka et al., 2004), makes that our knowledge regarding molecular features conferring acid stability to this class of proteins is very limited to date.
We here report on the protein engineering of the GH11 Bacillus subtilis endoxylanase (accession number M36648) towards enhanced acid stability. Since the pH dependence of protein stability is governed by the ionisation states of the side chains of its titrable amino acid residues, we explored the strategy of changing pH-stability profiles by altering pK\textsubscript{a} values of key residues through \textit{in silico} designed mutations. To this end, computational predictions and molecular modeling were carried out using the recently developed pKD software package (Tyan-Connolly and Nielsen, 2006, 2007). In contrast to previous studies aiming at an acidophilic or alkali-lphilic adaptation of the pH-dependent enzymatic activity of endoxylanases, ionisable residues outside the active cleft were avoided, avoiding modification of residues in the close proximity of the two catalytic glutamic acids.

Materials and methods

Computational design procedure

A detailed description of the pKD design algorithm for re-engineering pK\textsubscript{a} values in a protein has recently been published (Tyan-Connolly and Nielsen, 2006, 2007). The program integrates the functionality of a number of Python-based software packages, i.e. the WHAT IF pK\textsubscript{a} calculation package, the WHAT IF construction of point mutations and the pK\textsubscript{a}Design algorithm. The PDB file of \textit{B. subtilis} XynA endoxylanase (1XXN) was prepared for a pKD design by removing all crystal water molecules and non-protein atoms. Missing side-chain atoms were rebuilt using the position-specific rotamer libraries in WHAT IF. Following calculation of the pK\textsubscript{a} values of the wild-type (wt) XynA structure, the algorithm selects mutations that possibly contribute to a design solution, calculates pK\textsubscript{a} values for each of these mutations and finally combines them to optimise the structure. The algorithm selects mutations that possibly contribute to a design solution, calculates pK\textsubscript{a} values for each of these mutations and finally combines them to optimise the structure. During the process, pK\textsubscript{a} values and interaction energies between a site-directed mutation and all other residues are calculated using the WHAT IF pK\textsubscript{a} calculation package. Point mutations are modelled using position-specific rotamer libraries as implemented in WHAT IF, targeting only residues that are at least 30% solvent exposed in order to minimise the effect on the protein structure. The program was run two times with separate design criteria. The first objective comprised a reduction of the pK\textsubscript{a} value of Asp4 as well as Asp11. The second design goal involved a decrease of the pK\textsubscript{a} value of His149. For both specific primers. Sequencing products were analysed on an ABI 3100-Avant Genetic Analyzer (Applied Biosystems).

Table I. Mutants with adjusted pK\textsubscript{a} values corresponding to design solutions as calculated by the pKD algorithm

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Target residue(s), wild-type pK\textsubscript{a}</th>
<th>Mutated residues</th>
<th>Target residue(s), mutant pK\textsubscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>XynA-pKaD4D11-1</td>
<td>D4, wt pK\textsubscript{a}: 4.4</td>
<td>W\textsuperscript{9}→H\textsuperscript{9}, T\textsuperscript{10}→H\textsuperscript{10}, G\textsuperscript{23}→K\textsuperscript{23}, P\textsuperscript{116}→H\textsuperscript{116}, Q\textsuperscript{175}→H\textsuperscript{175}</td>
<td>D4, mut pK\textsubscript{a}: 4.1</td>
</tr>
<tr>
<td>XynA-pKaD4D11-2</td>
<td>D4, wt pK\textsubscript{a}: 4.4</td>
<td>S\textsuperscript{3}→K\textsuperscript{2}, W\textsuperscript{9}→H\textsuperscript{9}, T\textsuperscript{10}→H\textsuperscript{10}, G\textsuperscript{23}→R\textsuperscript{23}, Q\textsuperscript{175}→K\textsuperscript{175}</td>
<td>D4, mut pK\textsubscript{a}: 1.4</td>
</tr>
<tr>
<td>XynA-pKaH149-1</td>
<td>D4, wt pK\textsubscript{a}: 4.0</td>
<td>D\textsuperscript{83}→N\textsuperscript{83}, S\textsuperscript{134}→K\textsuperscript{134}, R\textsuperscript{136}→Q\textsuperscript{136}</td>
<td>D4, mut pK\textsubscript{a}: 3.8</td>
</tr>
<tr>
<td>XynA-pKaH149-2</td>
<td>H149, wt pK\textsubscript{a}: 4.0</td>
<td>D\textsuperscript{83}→N\textsuperscript{83}, R\textsuperscript{136}→Q\textsuperscript{136}, S\textsuperscript{155}→R\textsuperscript{155}</td>
<td>H149, mut pK\textsubscript{a}: 1.9</td>
</tr>
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Construction of XynA mutants

On the basis of the outcomes of the computational designs, XynA variants with multiple mutations (Table I) were created using the Quick Change Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. Briefly, thermal cycling was performed using 50 ng of pEXP5-CT-xynA (Belien \textit{et al}., 2008) and 100 ng of each mutagenic primer (Table II) under the conditions of denaturation at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min) and extension at 68°C for 7 min. Primers were designed according to the desired mutations, taking into account a minimum melting temperature of 75°C, wherever possible. Reaction mixtures were treated with DpnI and used to transform XL10-Gold (\textit{supE44 recA1 endA1 gyrA96 thi-1 relA1 lac out1 Tn10 (Tet\textsuperscript{R}) Amy Cam\textsuperscript{R}]) Ultracompetent cells (Stratagene) by heat shock according to the manufacturer’s instructions. All of the designed mutations were obtained in one mutagenesis round, with exception of XynA-pKaD4D11-2, for which first the pEXP5-CT-xynA-W9H-T10H-G23R-Q175K mutant was created. Subsequently, in a second mutagenesis round, the S2K substitution was introduced using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) with pEXP5-CT-xynA-W9H-T10H-G23R-Q175K as template DNA and a pair of appropriate complementary mutagenic primers (Table II) according to the manufacturer’s recommendations.

DNA sequence analysis

Expression plasmid xynA mutant inserts were sequenced by the chain-termination dideoxynucleoside triphosphate method with the BigDye\textsuperscript{®} Terminator V3.1 CycleSequencing Kit (Applied Biosystems, Foster City, CA, USA) and vector-specific primers. Sequencing products were analysed on an ABI 3100-Avant Genetic Analyzer (Applied Biosystems). VectorNTI Advance\textsuperscript{TM} 10 ContigExpress software (Invitrogen, Groningen, The Netherlands) was used to edit and align obtained DNA sequences. For all constructs, it was
confirmed that the intended mutations were introduced and that no additional mutations had occurred.

Heterologous expression and purification
Recombinant XynA and mutant enzymes were produced in Escherichia coli BL21 (DE3)pLysS (F' lac dcm ompT hsdS (r59 m5) gal λ (DE3) [pLysS Cam']) cells (Stratagene) transformed with pEXP5-CT-xynA or mutant constructs, respectively. Protein expression was performed according to instructions of the pEXP5-CT/TOPO TA Expression Kit (Invitrogen). Cultures were grown in Luria–Bertani medium supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol at 37°C with shaking at 250 rpm until the optical density at 600 nm reached ~0.5. Expression was induced with isopropyl-β-D-thiogalactopyranoside (0.2 mM) and cells were further grown at 18°C. After ~18 h incubation, cells were harvested by centrifugation at 2800g for 20 min. Pellets were resuspended in an appropriate amount of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 30 mM imidazole, pH 7.4) for a 50-fold concentration of cell density. Protease inhibitor Pefablock® SC (VWR International, Leuven, Belgium) was added to a final concentration of 1 mM. Lysis was performed by incubation with 1 mg/ml lysozyme (Sigma-Aldrich, St Louis, MO, USA) for 30 min on ice followed by four cycles of freeze-thawing (~80°C to 25°C) and sonication on ice [three bursts of 15 s with 1 min pause in between for each 2 ml of lysate by using the microtip of a Vibra Cell™ model VCX 13 PB sonicator (Sonic & Materials, Newtown, CT, USA) set at an amplitude of 40%]. Ni-NTA Spin Columns (Qiagen) were used to purify the His6-tagged proteins from the lysate under native conditions according to the manufacturer’s instructions. Up to 6 ml of cleared lysate was loaded onto one column, and the concentration of imidazole during washing steps was increased to 60 mM. Protein expression and purification were assessed by standard sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining. The low molecular mass SDS–PAGE markers of GE Healthcare (Diegem, Belgium) were used to compare band sizes. Protein concentrations were determined spectrophotometrically measuring the absorbance at 280 nm using the specific extinction factor corresponding to each variant.

Endoxylanase activity assay
Endoxylanase activity of B. subtilis wt XynA and the mutants described in this work was determined with the colorimetric Xylazine-AX method (Megazyme, Bray, Ireland). Ni-NTA purified endoxylanase fractions were diluted in 0.50 ml sodium phosphate buffer (250 mM, pH 7.0). The suspension was incubated for 10 min at 40.0°C, prior to addition of a Xylazine AX tablet. Incubation was prolonged for 60 min at 40.0°C and stopped by adding 2.0% (w/v) Trizma base solution (5.0 ml) and vortex stirring. After filtering through a 90 nm filter (VWR International, Leuven, Belgium), the absorbance at 590 nm (A590) of filtrates was measured. The Xylazine AX substrate consists of water-insoluble azurine-crosslinked wheat arabinoxylan, which, upon hydrolysis by endoxylanases, produces water-soluble dyed fragments. Thus, the increase of absorbance at 590 nm (A590) is directly related to the rate of release of these fragments and, hence, to enzyme activity. One enzyme unit (EU) corresponds to an increase in A590 of 1.00 under the conditions of the assay.

Effect of pH, ionic strength and temperature on optimal activity
The pH dependency of endoxylanase activity was determined by measuring activities at varying pH (pH 3–11) using the Xylazine-AX method as described above. Ni-NTA purified endoxylanase solutions were appropriately diluted in universal buffer at different pHs. Universal buffer was prepared from a stock solution of 31.2 mM citric acid, 28.7 mM potassium dihydrogen phosphate, 29.0 mM trihydrogen borate, 18.0 mM diethyl barbituric acid. pHs were adjusted with
sodium hydroxide. The effect of ionic strength on enzymatic activity was studied by performing the endoxylanase activity assay using sodium phosphate buffer pH 7.0 with molarities ranging from 25 to 1000 mM. The temperature dependency of endoxylanase activity of XynA and mutants was determined by measuring enzymatic activity as described above at temperatures ranging from 30.0°C to 60.0°C. To this end, enzyme solutions were prepared in 250 mM sodium phosphate buffer (with appropriate pH for optimal activity). All assays were performed in triplicate.

**Effect of pH, ionic strength and temperature on functional stability**

To test the functional pH stability of XynA and mutants, residual endoxylanase activity was measured after 120 min of incubation (40.0°C) of the enzymes in buffers with varying pH (pH 3–5). Following incubation, pH values were adjusted to pH 7.0 by diluting the enzyme solutions 10 times in sodium phosphate buffer (250 mM) and endoxylanase activities were determined using the Xylazyme-AX method as described above. For testing the influence of ionic strength on functional stability, residual endoxylanase activity was measured with the Xylazyme-AX method after 2.0 h of incubation (45°C) in sodium phosphate buffers (pH 7.0) with molarities ranging from 0.025 to 1.000 M. Functional temperature stability was assessed by pre-incubating appropriately diluted endoxylanases at different temperatures (40.0–60.0°C) in sodium phosphate buffer (250 mM, pH 7.0) for 10.0 min. After cooling the samples in an ice water bath, residual activities were determined at 40.0°C using the Xylazyme-AX method. All tests were performed in triplicate and the statistical significance of differences was tested at a significance level (P) of <0.05 using a two-tailed Student’s t-test. The rate constants for inactivation were estimated from a plot of Ln per cent residual activity versus time. Non-linear and linear regression fits were generated using GraphPad Prism software, obtaining fairly good correlations for all fits \( R^2 > 0.98 \) and >0.80, respectively.

**Results**

**Computational design and generation of XynA mutants**

In order to create novel mutants of the *B. subtilis* XynA GH11 endoxylanase with an improved stability under acidic conditions, a novel approach based on computational design was followed. Our strategy involved the redesigning of pK\(_a\) values of particular residues, i.e. an adaptation of their degree of protonation at a certain pH, by changing the way one or more of the protonation states interact with the rest of the amino acid residues of the protein. To this end, *in silico* predictions were generated by the automated computer algorithm pKD (Tynan-Connolly and Nielsen, 2006, 2007), as described in the Materials and methods section. Briefly, the program predicts which set of point mutations will change the pK\(_a\) values of targeted titratable groups in a given direction. In addition, for each calculated solution, a modelled 3D structure containing the proposed mutations is presented.

On the basis of the observation that acid-mediated inactivation of the wt enzyme starts in the region of pH 4, residues that are expected to change their protonation state around pH 4 or lower were selected for redesign calculations, aiming at a lowering of their pK\(_a\) values. Asp4, Asp11 and His149 were chosen as target residues. After two runs with separate design goals (as described Materials and methods), a set of design solutions fulfilling the intended criteria was obtained for both design stipulations. Table 1 lists four sets of multiple point mutations corresponding to four generated design solutions, indicating for each of them the wt pK\(_a\) value(s) of the target residue(s) as well as the calculated pK\(_a\) shift, and Fig. 1 shows their molecular representation.

All four described mutants were expressed as soluble proteins in the cytoplasm of *E. coli*. The recombinant endoxylanase variants were purified to near homogeneity on a Ni-NTA affinity column, and migrated as single protein bands on SDS–PAGE (Fig. 2), similar to the wt enzyme (Belien et al., 2008). Yields varying from 0.5 (for XynA-pKaH149 variants) to 10 μg (for XynA-pKaD4D11 variants) of purified protein per millilitre of cell culture were obtained. Measuring the specific enzyme activity (U/mg) of the endoxylanase variants using chromophoric AX substrate revealed markedly decreased catalytic activities for both XynA-pKaD4D11 mutants (~710 and ~540 U/mg for XynA-pKaD4D11-1 and XynA-pKaD4D11-2, respectively) compared with the wt enzyme (~7200 U/mg). Specific enzymatic activities of XynA-pKaH149-1 and XynA-pKaH149-2 were also reduced in comparison with wt XynA, but to a far lesser extent (~6600 and ~6800 U/mg).

**pH dependence of enzymatic activity**

The pH dependence of the enzymatic activity of all four generated XynA variants as well as the wt enzyme is shown in Fig. 3. The pH-activity dependencies of the mutants were comparable to those of wt XynA, following a bell-shaped profile with an optimum near neutral pH. In the case of the XynA-pKaD4D11-2, XynA-pKaH149-1 and XynA-pKaH149-2 mutants, a slightly increase in the pH optimum for maximal activity in comparison with the wt and the XynA-pKaD4D11-1 variant can be noticed. Upon acidification and alkalinisation, the catalytic activity of all tested enzymes decreased gradually, leaving only ~10% activity or less above pH 10 and below pH 4. Hence, optimal activity of the endoxylanases variants as well as the wt enzyme showed a clear dependency on the pH of the buffer. The degree of hydrolysis was also affected by the ionic strength of the buffer system. Although the wt enzyme displays reasonable catalytic activity at low molarity (25 mM) (retaining half of its activity at 175 mM after 1 h of incubation), the activity of the mutants is significantly lower at low molarities (retaining only ~20% of its optimal activity) (data not shown). The temperature at which a maximum amount of substrate was hydrolysed within 1 h of incubation was considerably lower in the case of the mutants compared with the wt enzyme (40°C, 45°C and 50°C for both XynA-pKaD4D11 mutants, both XynA-pKaH149 mutants and wt XynA, respectively (data not shown).

**pH, ionic strength and temperature dependence of functional stability**

The pH sensitivity of the engineered pKa mutants with respect to their functional stability was assessed after pre-incubation of the enzymes for 2 h at decreased pH values. As shown in Fig. 4, acid-induced inactivation of wt XynA was initiated once the pH dropped below pH 5, retaining
57% residual activity after pre-incubation at pH 4 under the experimental conditions, and only 24% residual activity after pre-incubation at pH 3. The generated mutants, however, clearly showed more tolerance towards acid-mediated

![Fig. 1. Molecular view of the modelled structures of pKaD4D11-1 and -2 (A and B), and pKaH149-1 and -2 (C and D), superimposed on the wt XynA protein (PDB code 1XNN). The side chains of the mutated residues are shown as sticks (red) as well as those of the corresponding wt residues (blue). The residues whose pK_a is targeted for redesign are indicated by atom coloring (C white, O red, N blue). All figures were generated using Pymol (http://pymol.sourceforge.net/).](image)

![Fig. 2. SDS–PAGE analysis of purified pKaD4D11 and pKaH149 mutants. Left low molecular mass protein marker, lane 1: XynA-pKaD4D11-1, lane 2: XynA-pKaD4D11-2, lane 3: XynA-pKaH149-1, lane 4: XynA-pKaH149-2.](image)

![Fig. 3. pH dependence of enzymatic activity of the pKa mutants and wt XynA. Displayed activities are relative to the activities measured at optimal pH, which were expressed as 100%. Means and standard deviations of triplicate experiments are shown.](image)

~57% residual activity after pre-incubation at pH 4 under the experimental conditions, and only ~24% residual activity after pre-incubation at pH 3. The generated mutants, however, clearly showed more tolerance towards acid-mediated
denaturation. XynA-pKaD4D11-1 remained almost fully active (≈95% residual activity) after pre-incubation at pH 4, in significant (P = 0.0013) contrast with the wt enzyme (Fig. 4). Also for inactivation at pH 3, higher residual endoxylanase activities than in the case of wt XynA could be observed, although differences were not significant (P = 0.14). XynA-pKaH149-2 displayed quite similar pH-stability characteristics, as drastically less enzymatic activity was lost after incubation at pH 4 (only ≈10%). Like for the XynA-pKaD4D11-1 mutant, the improved acid-stability was less explicit at pH 3 (no significant difference with wt XynA, P = 0.097). XynA-pKaD4D11-2 and XynA-pKaH149-1, on the other hand, also showed significantly increased stability at pH 4 (P = 0.0064 and 0.025, respectively), but to a lesser extent than the former mutants (retaining ≈83% and ≈77% residual activity, respectively). In the case of pre-incubation at pH 3, however, substantial residual activities were measured, which differed significantly from those obtained for the wt enzyme (P = 0.012 and 0.0012, respectively). Especially, the pKaH149-1 mutant displayed relative high stability under these low pH conditions. Kinetic parameters for denaturation of enzymes at low pH (pH 3) were determined by extensive analyses of residual activities at subsequent time points (Fig. 5). In line with the end-point stability measurements, the unimolecular rate constants for inactivation of the mutant enzymes were found to be substantially lower (~0.009, ~0.006, ~0.005 and ~0.008 min\(^{-1}\) for XynA-pKaD4D11-1, XynA-pKaD4D11-2, XynA-pKaH149-1 and XynA-pKaH149-2, respectively) than that of the wt enzyme (~0.012 min\(^{-1}\)). As a consequence, all mutants showed prolonged half-lives (t\(1/2\) ≈ 77, ~116, ~154 and ~87 min, for XynA-pKaD4D11-1, XynA-pKaD4D11-2, XynA-pKaH149-1 and XynA-pKaH149-2, respectively) in comparison with wt XynA (t\(1/2\) ≈ 58 min) under the experimental conditions.

In addition, influence of the ionic strength and temperature on functional stability was evaluated. The wt enzyme showed optimal stability at 175.0 mM (Fig. 6). Molarity increases above 250.0 mM resulted in a significant fall in residual activity, which progressively worsened as the ionic strength increased. In contrast, both XynA-pKaD4D11 mutants displayed gradually improving stability at increasing molarities. pKaH149-1 and -2 were optimally stable at 500 and 375 mM, respectively, under the experimental conditions. Further increase in the ionic strength led to a decrease in residual activity. Finally, testing the stability of the pKa mutants at increasing temperatures revealed a significant drop of thermostability for all four generated mutants compared with the wt enzyme (Fig. 7). The temperatures at which half of the enzymes lost their activity under the
conditions of the assay (midpoints of the inactivation curves) decreased with ~4-5°C for the mutants.

**Discussion**

Structural adaptations that confer enhanced acidostability to enzymes are of great interest, not only with regard to our general understanding and insights into protein-centred science, but also for the advantage such improved stability could provide in an increasing number of industrial applications. However, in comparison with thermostable proteins, there are only few well characterised acid-stable proteins to date. The reason for this is simply that far less natural occurring proteins have acquired acid resistance, since only a minority of the proteome from acidophilic organisms need to be acid-stable (in fact, only the proteins that are actually exposed to the acidic environment) as in general a circum-neutral intracellular pH is maintained (Baker-Austin and Dopson, 2007). This is quite different from the situation in thermophilic organisms, for which all proteins have to be functional at high temperature. A number of acidophilic proteins isolated thus far, such as endoglucanase from *Sulfolobus solfataricus* (Huang et al., 2005), Rieske iron–sulphur protein from *S. acidocaldarius* (Bonisch et al., 2002) and, noticeably, GH11 endoxylanase from *Aspergillus kawachii* (Fushinobu et al., 1998), are typified by a low overall pI predominantly caused by an overabundance of acidic residues at their surface. These molecular features are regarded as the main reason for their acid stability, arguing that the negative charges neutralise the positive electrostatic repulsion resulting from protonated residues at low pH. However, as a consequence of this adaptation strategy, negative electrostatic charges are inevitably accumulating as the pH rises, leading to poor stability at neutral and alkaline pH due to repulsion forces.

In this study, we explored an alternative strategy to change the enzyme pH-stability profile of GH11 endoxylanases, which involves the specific redesigning of pKₐ values of particular amino acid residues. Since the pH dependence of protein stability is determined by the difference in titrational behaviour of protein residues between the denatured and the folded form, we anticipated that the acid stability might be improved by mutations that significantly lower pKₐ values of residues becoming protonated at critical pH transitions. Therefore, the recently developed pKD server (Tynan-Connolly and Nielsen, 2006, 2007) was utilised to design novel mutations that alter the pKₐ value of one or more of such key ionisable groups. As we focused on engineering the pH-stability properties of the enzyme, rather than changing the pH dependence of optimal enzymic activity, residues located inside the active cleft were excluded from targeted redesign. Taking these considerations into account, ionisable residues Asp4, Asp11 and His149 were selected for pKₐ redesign, all three of them at a minimum distance of 8.5 Å from both catalytic glutamic acids. The pKₐ values of both aspartate side chains and the histidine side chain in the wt enzyme are less than their reference values in unfolded polypeptide (Plesniak et al., 1996; Joshi et al., 1997), indicating that the environment of each in the folded protein favours its deprotonation. In other words, the ionisation state of these amino acid groups contributes to the stability of the native enzyme relative to its denaturated form. However, as these residues are considered to become protonated at decreasing pH levels, pKD design computations were carried out in order to introduce structural adaptations further shifting down their pKₐ values, and hence, postponing their protonation at acidification. In analysing the output solutions, it was ascertained that all four XynA variants which were further evaluated experimentally (Table I), did not contain any mutation within hydrogen-bonding distance (2.8 Å) of the catalytic residues, again in order to avoid disturbing the pH profile of catalytic efficiency.

For all recombinantly expressed mutant enzymes, significantly increased functional stability at acid pH levels was observed, whereas no acidophilic adaptation of the catalytic activity was detected. The improved acid stability of both pKaD4D11 mutants is likely to be due to stabilisation of the ionisation states of both aspartic acids. In the wt protein, neither Asp4 nor Asp11 is located in the immediate proximity of a charged side chain (Joshi et al., 1997). In pKaD4D11-1 and -2, the pKₐ value of Asp4 is lowered through the replacement of Gly23 by Lys23 and Arg23, respectively (Fig. 1A and B). The insertion of these positively charged side chains stabilises the negatively charged ionisation state of Asp4. The significant effect of long-range charge–charge interactions on the pKₐ of ionisable groups and their influence on protein stability has previously been reported by several other authors (Pace et al., 2002; Laurents et al., 2003; Kaushik et al., 2006). In the case of pKaD4D11-2, the deprotonated carboxylate group of Asp4 is additionally stabilised by the nearby presence of the basic epsilon-amino group of the lysine residue introduced at position 2 (Fig. 1B), leading to a further reduction of its pKₐ. The drastically decreased pKₐ of Asp11 in pKaD4D11-1 is caused by the encircling of the target’s carboxylate group by four positively charged imidazole rings originating from replacement of Trp9, Thr10, Pro116 and Gln175 by histidine residues (Fig. 1A). For pKaD4D11-2, on the other hand, the shift down of Asp11 sidechain’s pKₐ is somewhat less pronounced, which can easily be declared by the absence of the His substitution at position 116. However, this is partly compensated by the nearness of the inflammatory cationic Lys residue inserted at position 175, begetting a calculated overall reduction of the target’s pKₐ by 1.1 unit.

His149, whose pKₐ is designed to decrease in the other two mutants, pKaH149-1 and pKaH149-2, is completely buried within the hydrophobic core and fully conserved in all known GH11 endoxylanases (Sapag et al., 2002). Moreover, the imidazole side chain of the corresponding residue of the *Bacillus circulans* GH11 endoxylanase was shown to be never protonated significantly while buried within the interior of the folded enzyme, indicating its structural importance, which was further proved by the destabilising effect of amino acid substitutions at this position (Plesniak et al., 1996). However, as a negatively charged aspartate (Asp83) is located directly adjacent to this His, its pKₐ is not minimal and, hence, could be additionally lowered by optimising the electrostatic environment of the imidazole ring. Therefore, two mutants were designed by the pKD program, both exhibiting a neutral asparagine instead of the acidic aspartate at position 83 (Fig. 1C and D). The proper insertion of an Asn side chain in the structural context of position 83, however, required the additional replacement of Arg136 by the neutral polar Gln136. The latter residue is modelled to stabilise the asparagine by two hydrogen bonds,
one between $O^\text{e}_{1}$ of Gln136 and $N^\text{e}_{2}$ of Asn83, and one between Gln136 main chain N and Asn83 main chain O. Furthermore, for both endoxylanase variants, an extra positively charged amino acid residue was incorporated in the vicinity of His149 (Lys134 and Arg155 in the case of pKaH149-1 and -2, respectively), further stabilising its deprotonated form. The decreased $pK_a$ of His149 is most likely responsible for the relative good acid-stability observed for both pKaH149 mutants, keeping the protonation grade of this key His residue at a minimum in acidic solutions. The phenomenon that acid-induced unfolding is largely influenced by the protonation behaviour of one or a couple of His residues has previously been observed for several other proteins (Barrick et al., 1994; Geierstanger et al., 1998; Kay and Baldwin, 1998). However, besides the stabilisation of the ionic form of the target residue, subtle influences of one or

![Fig. 8. Electrostatic surface representation of the modelled structures of pKaD4D11-1 and -2 (A and B) and pKaH149-1 and -2 (C and D), compared with the wt XynA protein (E and F, PDB code 1XNN). The surface is coloured blue for positively charged residues and red for negatively charged residues. The saturation of the colour is proportional to the degree of electrostatic charge from $-50 \text{kT/e}$ to $+50 \text{kT/e}$. All figures were generated using Pymol (http://pymol.sourceforge.net/).]
more additional residues, whose (electrostatic) interaction characteristics are adjusted, cannot be excluded. For instance, putative subtle supplementary molecular adaptations might explain the observed differences in stability at various acidic pH values between the XynA-pKaH149-1 and the XynA-pKaH149-2 mutants for which only a small distinction in pK_a value was predicted (2.5 versus 2.4, respectively).

Rather than avoiding positive charge on the surface, the endoxylanase variants generated during this study are decorated with some extra basic side chains (Fig. 8). Hence, in comparison with the highly acid-stable A. kawachii GH11 endoxylanase (Fushinobu et al., 1998), the XynA pKa mutants have many fewer uncompensated acidic residues on their surface. However, the slight overabundance of basic surface residues might still contribute to the improved pH stability by preventing the surface from destabilising charge fluctuations or even whole charge inversions as the pH varies from neutral to acidic. This alternative mechanism to defend proteins against pH-induced denaturation has also been observed for the acidophilic maltose binding protein (Schafer et al., 2004) and N²-carboxyaminoimidazole ribonucleotide mutase (Settembre et al., 2004) from Alicyclobacillus acidocaldarius, as well as a hexameric type II citrate synthase from Acetobacter acetii (Francois et al., 2006). The altered electrostatic properties of the pKa mutants are further reflected by their distinct activity and stability with respect to varying buffer molarities. Although it is hard to elucidate the precise mechanism responsible for the relative better activity and stability of the mutants at higher molarities, it is clear that ion binding plays an important role, similar to what was observed for thoroughly characterised halophilic proteins (Madern et al., 2000).

Despite their increased stability under acid conditions, none of the generated XynA variants showed an acidophilic adaptation of the catalytic activity. In the case of the XynA-pKaD4D11-2, XynA-pKaH149-1 and XynA-pKaH149-2 mutant, even a slight increase in the pH optimum for maximal activity in comparison with the wt enzyme could be detected. This small shift of the pH optimum towards alkaline pH is possibly caused by the incorporation of basic amino acid residues at the surface of the molecule, similarly to previous reported results for engineering of multiple arginines into the surface of the Trichoderma reesei GH11 XynII endoxylanase (Turunen et al., 2002). The fact that pKaD4D11-1 has only one basic residue engineered into its surface (Lys23), whereas pKaD4D11-1 exposes—besides Arg23—two supplementary basic amino acid residues (Lys2 and Lys175) might explain the slight difference in pH optimum between both endoxylanase variants. Interestingly, the XynII variants decorated with several Arg residues on their Ser/Thr surface also showed decreased thermal stability in the absence of substrate (Turunen et al., 2002), similarly as was observed for the here described mutants. The reduced thermostability of the pKa mutants is probably due to the lost of certain favourable interactions, such as hydrogen bonds associated with the replaced polar Ser, Thr and Gly residues or salt bridges like the one occurring between Asp83 and Arg136. In this context, it should also be emphasised that both pKaD4D11 mutants are considerably redesigned within their N-terminal region. After all, this part of the protein structure has been shown to have a large impact on the thermostability of multiple GH11 endoxylanases (Fenel et al., 2004; Janis et al., 2004; Sun et al., 2005). Moreover, recently it was shown that unfolding of this type of enzymes is initiated at the N-terminus, identifying this region as one of the weak spots of the structure, in addition to the α-helix region from which denaturation alternatively sets out (Purmonen et al., 2007). The latter observation might be related to the poor thermostability of both pKaH149 mutants, as His149 is located in this α-helix and was indeed shown to be of significant structural importance (Plesniak et al., 1996).

The generated mutant enzymes can be used as a template in additional, more straightforward, engineering strategies in order to enhance their catalytic efficacy at altering pH. Since the optimum pH for enzymatic activity is related to the pK_a of catalytic residues, the optimum pH can be moved up or down by altering the electrostatic potential (Joshi et al., 2000). In a rational protein engineering approach, the electrostatic potential can be calculated by the finite difference Poisson–Boltzmann (FPDB) continuum method. Several computational procedures for solving FPDB equation in proteins have been developed, for example, Delphi, UHBD, CHARMM, etc. (Warwicker, 1999; Nielsen and Vriend, 2001). Alternatively, structural comparisons of the target endoxylanase and known acidophilic endoxylanases might allow to identify differences in residues surrounding the two glutamic acid side chains involved in the catalysis that could be responsible for the acidophilic adaptation, as illustrated by de Lemos Esteves et al. (2004). Additional engineering towards improved enzymatic activity at low pH can also be achieved by directed evolution strategies (for an overview of strategies see Turner, 2003; Rubin-Pitel and Zhao, 2006). Libraries of endoxylanase variants created by random/combinatorial/saturation mutagenesis and/or DNA shuffling can be screened for elevated catalytic activity at altered pH values (as exemplified by Wang and Xia, 2008). For instance, one could take advantage of the phage display selection system developed for GH11 endoxylanases (Belien et al., 2008) to optimise the (arabino)xylan binding performance at acidic pH values, similarly as has been done for α-amylase (Verhaert et al., 2002).

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