Crystallographic and mutational analyses of an extremely acidophilic and acid-stable xylanase: biased distribution of acidic residues and importance of Asp37 for catalysis at low pH

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Xylanase C from \textit{Aspergillus kawachii} has an optimum pH of 2.0 and is stable at pH 1.0. The crystal structure of xylanase C was determined at 2.0 Å resolution (R-factor = 19.4%). The overall structure was similar to those of other family 11 xylanases. Asp37 and an acid–base catalyst, Glu170, are located at a hydrogen-bonding distance (2.8 Å), as in other xylanases with low pH optima. Asp37 of xylanase C was replaced with asparagine and other residues by site-directed mutagenesis. Analyses of the wild-type and mutant enzymes showed that Asp37 is important for high enzyme activity at low pH. In the case of the asparagine mutant, the optimum pH shifted to 5.0 and the maximum specific activity decreased to about 15% of that of the wild-type enzyme. On structural comparison with xylanases with higher pH optima, another striking feature of the xylanase C structure was found; the enzyme has numerous acidic residues and importance of Asp37 for catalysis at low pH.

Keywords: acid stability/\textit{Aspergillus kawachii}/low pH optimum/X-ray crystallography/xylanase

Introduction

endo-1,4-β-Xylanase (EC 3.2.1.8) is one of the hemicellulases and microbial xylanases are of considerable interest for biotechnological applications such as in the food industry and biobleaching (Coughlan and Hazlewood, 1993; Gilbert and Hazlewood, 1993; Poutanen, 1997). Of the two xylanase families, the family 11(G) xylanases are β-sheet proteins each composed of a single domain of about 20 kDa (Davies and Henrissat, 1995). The catalytic mechanisms have mainly been studied for xylanases from \textit{Bacillus} species, which have relatively high pH optima (Miao et al., 1994; Lawson et al., 1996, 1997). The catalytic residues of xylanases are two conserved glutamic acid residues (McCarter and Withers, 1994; Davies and Henrissat, 1995). According to the retaining mechanism (Biely et al., 1994), these residues act as a nucleophile and an acid–base catalyst, respectively (Miao et al., 1994; Wakarchuk et al., 1994; Havukainen et al., 1996). The crystal structures of a number of xylanases have been reported (\textit{B.pumilus}, Matsube et al., 1990; Arase et al., 1993; \textit{B.circulans}, Wakarchuk et al., 1994; \textit{Trichoderma harzianum}, Campbell et al., 1993; \textit{T.reesei}, Törnönen et al., 1994; Törnönen and Rouvinen, 1995; \textit{Aspergillus niger}, Krengel and Dijkstra, 1996; and \textit{Bacillus} strain D3, Harris et al., 1997). In addition, the NMR assignment of \textit{B.circulans} xylanase (BCX) has also been reported (Plesniak et al., 1996b).

\textit{Aspergillus kawachii} is used for making ‘shochu’ (a Japanese traditional spirit) and produces cellulolytic enzymes that contribute to the digestion of barley (Ogasawara et al., 1991). In the saccharification of starch in crops such as barley, both hemicellulases and amylases are important (Iwano et al., 1988). The pH of shochu mash is very low (about 3.0) because of the citric acid production by \textit{A.kawachii}; nevertheless, the enzymes act efficiently without inactivation. Hence the enzyme systems involved are very interesting as acid-stable systems (Mikami et al., 1987; Kaneko et al., 1996). Xylanase C (XynC) is one of the xylanases of \textit{A.kawachii} and it is an interesting acid xylanase, because its optimum pH is 2.0 and it is stable at pH 1.0 (Ito et al., 1992a,b). Because of its extremely low pH optimum and acid stability, XynC is also useful for the bleaching of Kraft pulp under acidic conditions (Tenkanen et al., 1997). In order to determine the structural basis of its extreme acid stability and low pH optimum, we conducted X-ray crystallography of the enzyme and mutational analyses.

Two crystal structures of xylanases with low pH optima, \textit{T.reesei} XYN1 (Törnönen and Rouvinen, 1995) and \textit{A.niger} xylanase I (Krengel and Dijkstra, 1996), have been reported and an aspartic acid residue is suggested to be a critical residue for their low pH optima. In this study, we observed similar structural features to those of the two xylanases with low pH optima and then examined the hypothesis by means of mutational analyses. We also discuss additional structural features of XynC which may be responsible for its low pH optimum and extreme acid stability.

Materials and methods

\textit{Crystallization and X-ray data collection}

XynC was purified according to the procedure of Ito et al. (1992b) from \textit{A.kawachii} carrying the multi-copy gene of XynC on its chromosome (Ito, 1993). Crystallization was performed by the hanging drop vapor diffusion method at 25°C. A 20 µl drop was made by mixing 10 µl of a reservoir solution and 10 µl of a protein solution (5–8 mg/ml XynC in 5 mM acetic acid, pH 4.5). The drop was equilibrated against a 1 ml reservoir solution comprising 100 mM HEPES–NaOH buffer (pH 7.5) and 1.5 M sodium sulfate. Large pillar-shaped crystals (>1.0×0.4×0.4 mm) grew in 1–2 months. Before data collection, the crystals were harvested in a solution comprising 60 mM MES–NaOH buffer (pH 6.5) and 1.75 M sodium sulfate. Data were collected using the Weissenberg method with synchrotron radiation at the BL6A station of the Photon Factory, High Energy Accelerator Research Organization (KEK), Tsukuba, Japan (Sakabe et al., 1995). Two crystals were mounted with their crystallographic c* and a* axes almost parallel to the spindle axis. A Weissenberg camera and Fuji imaging plates were used to record the reflections. A total

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of 35 images were digitized using a Fuji BAS-2000 analyzer and then processed with the HKL program suite (Otwinowski and Minor, 1997). Reflections up to 2.0 Å resolution were merged into a data set, as summarized in Table I. Structure factors were obtained from intensities using F2MTZ and TRUNCATE from the CCP4 program suite.

Structure determination and refinement

The crystal structure was determined by the molecular replacement method using program X-PLOR, version 3.1 (Brunger, 1993). Modified coordinates of XYNI from T.reesei (Törnøen and Rouvinen, 1995) were used as a search model (sequence identity, 51%; PDB code 1xyn). On the basis of sequence alignment between XynC and XYNI, all non-conserved residues of the XYNI structure other than glycine residues were replaced with alanine and loop regions having an insertion or deletion were omitted. After calculation of the rotation function, a translation search with space group P4_2_2 gave a proper solution (R value 45.8%, after rigid body refinement), but not with P4_2_2_2. Structural refinement was performed by repeatedly applying a simulated annealing or positional refinement procedure using X-PLOR (Brunger, 1993) and computer graphics-aided model manipulation using program O (Jones et al., 1991). After the refinement range had been extended gradually to 2.0 Å, individual B-factor refinement cycles were included. On the basis of 2|F_o|-|F_c| and |F_o|-|F_c| maps and the existence of hydrogen bonds, water molecules were included in the model. The quality of the model was assessed by using the programs X-PLOR, PROCHECK (Laskowski et al., 1993) and What-check (Hooft et al., 1996). The final refinement statistics are given in Table I. The coordinates have been deposited at the Brookhaven Protein Data Bank (ID code 1BK1).

Mutagenesis and vector construction

Site-directed mutagenesis was performed with a QuickChange Site-Directed Mutagenesis Kit (Stratagene) and the DNA fragments were sequenced to check the mutations. For the cDNA clone subcloned into pUC118 (Ito et al., 1992a), the codon of the XynC gene for Asp37 (GAC) was replaced with AAT (D37N), TCT (D37S), GAA (D37E) and AAA (D37K), respectively. Each of the mutated DNAs could be confirmed by DNA gel electrophoresis of their plasmids after treatment with restriction enzymes, because the AspI site (GACTTGTC) in the wild-type gene includes the codon for Asp37 and is absent in each mutant gene. Each mutant gene as well as the wild-type gene was ligated under the GPD promoter of yeast expression vector pG3 (Schena et al., 1991). The N-terminal signal sequence was shortened to facilitate secretion of the XynC into the medium (Ito, 1993). The wild-type and mutant proteins were produced as described by Ito (1993) and purified from the medium as described by Ito et al. (1992b). The purities of the proteins were checked by SDS–PAGE (Laemmli, 1970) stained with Coomassie Brilliant Blue. Protein concentrations were determined by measuring the absorbance at 280 nm.

Measurement of enzymatic activity

A 2% beechwood xylan (Sigma) suspension was solubilized by heating for 10 min in a boiling water-bath. A 400 µl volume of a 1% xylan solution in Walpole or McIlvaine buffer of various pHs was incubated for 30 min at 40°C. Depending on its specific activity, an appropriate concentration of each enzyme was added to start the reaction. Released reducing sugar was measured by Somogyi and Nelson’s method (Somogyi, 1952) using reagents from Wako Chemicals (Tokyo). Walpole sodium acetate–hydrochloric acid buffer was prepared by changing the concentration of hydrochloric acid in 200 mM sodium acetate. McIlvaine NaHPO_4–citric acid buffer was prepared by changing the ratio of NaHPO_4 to citric acid. The pH of the buffers was checked with a pH meter. One unit of enzyme was defined as the activity producing 1 µmol of reducing sugar as a xylose equivalent per minute.

Results

Crystallography

On initial screening for the crystallization conditions using Crystal Screen kits (Hampton Research), some conditions with ammonium sulfate as a precipitant were found to give thin needle-shaped crystals. With further optimization, large pillar-shaped crystals were obtained with sodium sulfate as a precipitant at 25°C. Since the XynC crystals were grown at pH 7.5, which was not within the active pH range of the enzyme (Ito et al., 1992b), they were soaked in a solution of pH 6.5 before data collection (see Materials and methods). The three-dimensional structure of XynC was solved by molecular replacement using the coordinates of XynC from T.reesei (Törnøen and Rouvinen, 1995) as a search model. Crystallographic parameters, data collections and refinement statistics are shown in Table I. During the refinement steps, the electron density around the two residues, reported to be Ala15 and Ser53 by Ito et al. (1992a), clearly showed the shapes of glycine and threonine residues, respectively. On the basis of careful inspection of omit maps and the result of re-sequencing the cloned cDNA, these residues were confirmed to be Gly15 and Thr53.
and Thr53, respectively. The XynC structure consisted of a single domain comprising two β-sheets (A and B) and a single α-helix (Figure 1), which is unique for family 11 xylanases. The overall structure of the molecule resembled a right hand (Törnönen et al., 1994). In Figure 1, the XynC structure is represented as ‘fingers’ at the bottom, a ‘thumb’ at the top and a long ‘cord’ (or ‘chord’) loop at the front of the molecule.

**Difference from the model structure**

The backbone structure of XynC was very similar to the XYNI structure. The overall structure of the molecule resembled a right hand (Törnönen et al., 1994). In Figure 1, the XynC structure is represented as ‘fingers’ at the bottom, a ‘thumb’ at the top and a long ‘cord’ (or ‘chord’) loop at the front of the molecule.

**Difference between A.kawachii XynC and A.niger xylanase I**

During the course of this study, the crystal structure of xylanase I from A.niger was reported (Krengel and Dijkstra, 1996). The amino acid sequences of XynC and xylanase I differ in only three residues: Ser64, Glu154 and Met167. However, the characteristics of the crystals were clearly different. As for xylanase I, several crystal forms belonging to different space groups, $P_2_1$, $P_2_12_12_1$ and $P_4_3_2$, have been obtained and can be converted into one another through flash freezing or macroseeding (Krengel et al., 1996). A refined structure has only been reported for the orthorhombic $P_2_12_12_1$ form (Krengel and Dijkstra, 1996). Among these crystal forms, the arrangements of the molecules in the crystal lattice are very similar and a pseudo-4-fold screw axis is present in the $P_2_1$ and $P_2_12_12_1$ crystal forms.

In the case of XynC, a crystal form of a space group with higher symmetry ($P_4_32_12_1$) and diffractions to higher resolution was grown under similar but simpler conditions in comparison with those for A.niger xylanase I (Krengel et al., 1996). The crystal packing of XynC was also similar to that of xylanase I, but a crystallographic 4-fold screw axis was present because of slight differences in the molecular orientations. The structures of XynC and xylanase I were almost identical (r.m.s. deviation = 0.5–0.6 Å), differences being several side-chains sticking out into the solvent. The three residues different between the two xylanases are located far from the packing site. It is not clear why these residues caused such differences in crystal formation.

The optimum pH of A.niger xylanase I has been reported to be 3.0 (Krengel and Dijkstra, 1996), which is different from that of XynC (2.0). Because the assay conditions for A.niger xylanase I were not reported in detail, it is not clear whether the pH optima of the two xylanases are different or not.

**A.niger xylanase I** was crystallized at high pH (8.0), at which the enzyme is completely inactive, and its structure was solved at the same pH. XynC could also only be crystallized at high pH (above 7.5), but crystals soaked in a lower pH (6.5) buffer, in which XynC is slightly active, were used to solve the structure. In spite of the pH difference, the two structures exhibited no significant structural differences between their molecules.

**The ‘cord’ region and a disulfide bond**

The central part (Pro91–Ser94) of the ‘cord’ region (Gly87–Ala95) was found to comprise a short helix-like turn structure (Figure 1). In this region, Cys92 formed a disulfide bond with Cys111 on the β-strand B8. The crystal structures of xylanases other than A.niger xylanase I contain no disulfide bonds. In these structures, the ‘cord’ region has a well defined conformation, although it does not have any secondary structure or any hydrogen bonds with the rest of the molecule. A conserved proline residue (Pro91 in XynC) is thought to define the conformation of the ‘cord.’ In the XynC structure, this disulfide bond seems additionally to stabilize the ‘cord’ structure, but its contribution to the thermostability is probably small, because its thermostability is moderate (stable about up to 40°C; Ito et al., 1992b). At present, it is not known how the disulfide bond contributes to the acid stability of XynC.

**The active site cleft**

The active site cleft of XynC (Figure 2a, front view; Figure 2b, side view) is composed of aromatic residues (represented in yellow and orange) lining the inner wall of the large extended open cleft. The subsites are defined as –2, –1, +1, +2 and +3 (Figure 2a), where positive numbers represent the reducing end direction of the substrate (Bray and Clarke, 1992; Törnönen and Rouvinen, 1995). In Figure 2, the two xylose rings found in the reducing end direction and negative numbers the non-reducing end direction of the substrate (Wakarchuk et al., 1994) are superimposed, being represented in cyan. On the basis of the results of structural comparison between the group of xylanases with acidic pH optima (XynC, A.niger xylanase I and T.reesei XYNI) and the other with relatively high pH optima (T.reesei XYNII and BCX), the aromatic residues along the active site cleft were classified into two types. The three residues represented in orange (Tyr10, Phe131 and Trp172) in XynC showed significant

![Fig. 1. Ribbon diagram of the overall crystal structure of A.kawachii XynC.](Image 60x539 to 266x760)
features depending on the pH optima. These residues correspond to Tyr, Phe/Ile and Trp in acidic xylanases and Thr, Trp and Tyr in alkaline xylanases. In particular, the Thr residue in acidic xylanases (Thr172 in XynC) bends into the cleft, but the corresponding Tyr residue in alkaline xylanases sticks out into the solvent. Other residues, represented in yellow, are fully conserved or are not significantly different between the two groups.

The two catalytic residues, nucleophile Glu79 and acid–base catalyst Glu170, are shown in red in Figure 2; Glu79 is shown on the upper side and Glu170 on the lower side. Both residues extend their side-chains to the bottom of the cleft from opposite sides. The two residues near nucleophile Glu79, Arg115 and Gln129 are shown in blue and green. These residues are fully conserved, but their functions in catalysis are unknown. However, on mutational analysis of BCX (Wakarchuk et al., 1994), the conserved arginine residue was indicated not to be essential for the activity. Glu118, which contributes to the negative charge around the active site cleft (described below), is shown in magenta.

As in other xylanases with low pH optima (T.reesei XYNI and A.niger xylanase I), Asp37 (shown in pink in Figure 2) is located at hydrogen-bonding distance (2.8 Å between the nearest oxygen atoms) from the acid–base catalyst Glu170. On the other hand, in the structures of xylanases with relatively high pH optima (T.reesei XYNII and BCX), this residue is...
replaced with asparagine and is located relatively far away (3.1–3.5 Å) from the acid–base catalyst, glutamic acid. Hence Asp37 was expected to influence critically the pH dependence of xylanase activity, as described previously (Törroenen and Rouvinen, 1995; Krengel and Dijkstra, 1996).

**Mutagenic analysis of Asp37**

Therefore, Asp37 of XynC was replaced with Asn (D37N), Ser (D37S), Glu (D37E) or Lys (D37K) by site-directed mutagenesis. These mutants and the wild-type enzyme were produced in a *Saccharomyces cerevisiae* expression system that can secrete the XynC enzyme into the medium (see Materials and methods). On the plate assay involving the detection of halo formation on Remazol Brilliant Blue–xylan plates, the wild-type XynC and all mutants except the D37K mutant showed significant xylanase activity (data not shown) and were purified as described previously (Ito et al., 1992b). The purified enzymes were assayed as described under Materials and methods. The stabilities of the purified enzymes were the same as that of XynC purified from *A. kawachii*, at various pHs (data not shown).

The relative activities of the wild-type and mutant enzymes at various pH values are shown in Figure 3. The highest activity (204 U/mg protein) of the wild-type XynC at pH 2.0 is indicated as 100% (Figure 3a). Although the maximum specific activity of the D37N mutant was decreased to about 15% of that of the wild-type XynC, its optimum pH was shifted to 5.0, as expected (Figure 3b). The D37S mutant also showed decreased activity (about 10%) and a higher pH optimum (4–5) (Figure 3c). Although a glutamic acid residue has a carboxyl moiety, the D37E mutant exhibited very low activity (about 1.5%) and a pH optimum of 3–4 (Figure 3d). It is likely that the longer side-chain of the glutamic acid residue cannot interact with the acid–base catalyst and/or causes steric hindrance at the edge of the cleft that interferes with the substrate binding.

**Electrostatic features on the surface**

Electrostatic potentials on the surface of XynC, *T. reesei* XYNII, XYNI and BCX are illustrated in Figure 4, obtained using the program GRASP (Nicholls et al., 1991). Figure 4a–d show the active site clefts in the same orientation as in Figure 2a. The active sites of all xylanases were clearly acidic (red) because there are two catalytic glutamic acid residues at the bottom of the cleft. However, the xylanases with low pH optima (XynC and XYNI in Figure 4a and b, respectively) were more negatively charged than the xylanases with higher pH optima (XYNII and BCX in Figure 4c and d, respectively). The presence of acidic residues at the edge of the cleft resulted in the stronger negative charge of the former xylanases. There was an aspartic acid residue (Asp37 in XynC and Asp33 in XYNI) at the lower edge of the cleft and a glutamic acid residue (Glu118 in XynC and Glu112 in XYNI) at the other side of the edge.

The convex flat face of β-sheet A, which faces the bottom in Figure 4a–d and the front in Figure 4e–h, corresponds to the ‘Ser/Thr surface’ (Törroenen et al., 1994), which consists of conserved serine and threonine residues in all xylanase structures known at the present time. As shown in Figure 4f–h, all xylanases other than XynC have this conserved ‘Ser/Thr surface’. Interestingly, XynC had a very negatively charged surface (Figure 4e) instead of the ‘Ser/Thr surface’. The acidic residues on the surface were Asp16, Asp20, Glu21, Glu31, Asp32 and Glu57 and most of these residues were present in the three short helical link regions (Asn11–Gly15, Asp20–Gly24 and Glu31–Gly33) between the two β-sheets. These electrostatic features well corresponded to the low $pI$ (3.5) of XynC (Ito et al., 1992b).

In spite of the enormous number of negatively charged residues on the surface of XynC, crystal contacts with neighboring molecules were formed with no severe charge repulsion. Most negatively charged regions were exposed to the solution and most contacts were mediated by solvent moieties. More...
over, a few acidic residues at the contact regions were accompanied by adjacent arginine and histidine residues.

Discussion

Implications for the pH dependence of xylanase activity

Mutational analysis of Asp37 of XynC revealed that the amino acid residue at position 37 has to be aspartic acid for the expression of high activity at low pH. The upward shift of the optimum pH of the D37N mutant was predictable from the results of structural and amino acid sequence comparison. The similar results for the D37S mutant to those for the D37N mutant are interesting because no natural xylanases with serine at this position have yet been found. Why an aspartic acid residue near an acid–base catalyst glutamic acid increases the activity at low pH and why an asparagine residue does not remain unknown. The abnormally high pKₐ (6.7) of the acid–base catalyst glutamic acid of BCX is thought to be caused by the electrostatic interaction with a nucleophile glutamic acid located at a distance of 5.3 Å (Davoodi et al., 1995). On the basis of the results of a ¹³C-NMR study of BCX, the pKₐ's of the nucleophile and acid–base catalyst are suggested to determine the lower and upper limits of the active pH range, respectively (McIntosh et al., 1996). However, the knowledge of the catalysis of BCX is not applicable to xylanases with low pH optima. The replacement of Asp37, which interacts with the acid–base catalyst, resulted in a large decrease in the activity at low pH, apparently affecting the lower limit of the active pH range.

In the initial step of the enzymatic reaction, Glu170 acts as a general acid by donating a proton to the substrate oxygen. The protonated state of Glu170 is stabilized through a hydrogen bond with Asp37, which involves the hydrogen of Glu170. A hydrogen bond between two carboxylic acids was shown, on quantitative measurement, to be less stable when both carboxylic acids are protonated than when one carboxylic acid is deprotonated (Schwartz and Drueckhammer, 1995). Hence Asp37 is protonated and the proton of Glu170 can easily dissociate and contribute to the catalysis only at low pH, as also described previously (Krengel and Dijkstra, 1996). This pH effect is not seen for xylanases with asparagine instead of aspartic acid.

In the crystal structures of xylanases with asparagine at the position corresponding to Asp37, this residue does not form a strong hydrogen bond with the acid–base catalyst glutamic acid. Because the activities of the D37N and D37S mutants were decreased, the asparagine and serine residues of these mutants may maintain the hydrogen bond with the acid–base catalyst and prevent the proton of Glu170 from dissociating. Alternatively, some other differences in the environment around the Asn/Ser37 residue in the case of natural Asn-containing xylanases may cause the decrease in the activity. Crystallographic analysis of XynC mutants is now in progress.

Because the mutations of Asp37 did not increase the activity at higher pH, other factors which control the pH dependence of the activity may exist. On the basis of a structural comparison between xylanases exhibiting different pH dependences, the acidic residues at the edge of the cleft and some aromatic residues forming subsites were suggested to be characteristic structural differences. It has been stated that the negative charge at the active site may be important for molecular recognition and substrate specificity, because xylan exhibits a certain degree of substitution, especially of negatively charged glucuronic acid substituents (Törnönen and Rouvinen, 1997). We assume that the acidic residues at the edge of the cleft (Glu118 and Asp37) also play a role in determining the pH dependence of the activity. Electrostatic repulsion between the cleft and xylan should disappear at low pH, at which the acidic residues are protonated. Additionally, a decrease in the water binding capacity of acidic residues in the protonated state at low pH (Kuntz, 1971) may facilitate the substrate binding.

The aromatic residues forming subsites are also worth examining. In particular, the residues corresponding to Tyr10, Phe131 and Trp172 of XynC are Tyr, Phe/Ile and Trp in acidic xylanases, but Trp, Trp and Tyr in alkaline xylanases, respectively. In the complex structures of both BCX-substrate (Wakarchuk et al., 1994) and XYNII-epoxylkyl xylosides (Havukenen et al., 1996), the Trp residue corresponding to Tyr10 in XynC forms the stacking interaction and hydrogen bonds with the xylose ring at subsite -2. Moreover, the Tyr10/Trp and Trp172/Tyr residues are located near the Asp37/Asn residue. The large aromatic ring of the Trp residue may sterically affect a possible conformational change of the Asp/Asn residue during the catalytic cycle. Phe131 and Trp172 are located at subsite +1. The proximity of Phe131 to the fully conserved Gln129 near nucleophile Glu79 and the structural difference between Trp172 and the corresponding Tyr residue in XYNII and BCX highlight the importance of these residues.

Implications for the acid stability of XynC

The negatively charged nature of the ‘Ser/Thr surface’ of the XynC molecule was difficult to predict from the results of sequence analysis, because most of the acidic residues on the surface are present in the coiled regions exhibiting relatively low conservation, with short insertions or deletions in the N-terminal half of the xylanase molecule. The negatively charged surface is certainly a distinct structural feature of XynC and also of A.niger xylanase I. However, there has been no such description for A.niger xylanase I or T.reesei XYNI. We postulate that this striking feature is the major mechanism underlying the acid stabilization of XynC.

Porcine pepsin is a well-studied acidophilic and acid-stable enzyme, which is active and stable at pH <2.0 (Sielecki et al., 1990; Andreeva and James, 1991). Pepsin has an extraordinarily high ratio of potentially negatively charged groups to positively charged groups such as XynC and has an extremely low pI (<1.0), which is much lower than that of XynC (3.5). Several aspartyl carboxylate groups of pepsin are thought to have low pKₐ values because of their unusual hydrogen-bonding environments and retain the net negative charge even at very low pH. This extremely low pI is regarded as the cause of the acid stability of pepsin. Even at low pH, the electrostatic repulsion of the positively charged residue is not severe, unlike in the case of usual proteins with higher pI values. As for XynC, there are only three acidic residues (Glu79, Glu84 and Asp104) that seem to have low pKₐ values as judged from their hydrogen-bonding environments. While the buried His148 residue of XynC seems to have a very low pKₐ value, as observed for BCX (Plesniak et al., 1996a), XynC has at least five positively charged groups at acidic pH, i.e. N-terminal NH²⁺, the imidazole ring of a solvent-exposed histidine residue (His155) and the guanidium groups of three arginine residues (Arg115, Arg134 and Arg138). In total, XynC is positively charged at pH <3.5, but the positive charge repulsion may not be severe enough to cause denaturation. Pepsin also has excessive acidic residues on its surface like
XynC. These acidic residues on the surface and the low pI seem to be important for the acid stabilization of the protein.

In the context of the relationship between the charge repulsion and the protein stability, proteins with a large excess of acidic residues over basic residues must be unstable at neutral pH because of the charge repulsion of negative charges. This is true for pepsin, which is rapidly denatured above pH 6.5, but is not applicable for XynC, as it is stable up to pH 9.0. The difference in stability at neutral pH may be caused by different spatial distributions of the acidic residues on the surface. Some acidic residues of XynC concentrated in the active site cleft and the so-called ‘Ser/Thr surface’ may have relatively high pK_a values because of the electrostatic interaction between them and the negative charge repulsion at neutral pH may not be so severe. In contrast to the case of XynC, the acidic residues on the surface of pepsin are not concentrated, hence they probably have typical pK_a values of 3.0–5.0.

Interestingly, two halophilic proteins were reported to have many acidic residues on their surfaces and this structural feature is suggested to stabilize these proteins at high salt concentrations (Dym et al., 1995; Frolow et al., 1996). In particular, the crystal structure of ferredoxin from Haloarcula marismortui has an extra domain made up of two helices carrying highly negative charges (Frolow et al., 1996). In the halophilic ferredoxin structure solved at cryogenic temperature (100 K), bound surface water molecules have more hydrogen bonds than in a typical non-halophilic protein. Carboxylates on the surface are the most hydrated side-chain moieties in the protein. Frolow et al. (1996) suggested that the numerous negatively charged carboxylates can keep the protein extensively hydrated even with the maximum number of inorganic cations in a supersaturated salt solution and thus would prevent it from undergoing self-aggregation. Acidic residues exhibit a strong water binding capacity in the deprotonated state, but not in the protonated state at low pH (Kuntz, 1971). Hence the explanation that the strongly bound waters shield XynC at low pH seems not to be applicable. Since the XynC structure was solved with data collected at room temperature, the side-chains of acidic residues on the surface were often disordered and solvent peaks were merely observed around these acidic residues. Another similar example of a protein exhibiting stability under extreme pH conditions has been reported. A subtilisin-family serine protease exhibiting optimal enzymatic activity at pH 12.3 has many arginine residues localized to a hemisphere of the globular protein (Shirai et al., 1997). Phylogenic analysis revealed that a decrease in the number of negatively charged amino acids and lysine residues and an increase in arginine and neutral hydrophilic amino acids (His, Asn and Gln) are correlated. A spatially biased distribution of charged residues on proteins which are stable against extreme pH conditions or high salt concentrations seems to widely exist, but the mechanism underlying this stabilization of the proteins remains to be elucidated.

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