Efficient constitutive expression of *Bacillus subtilis* xylanase A in *Escherichia coli* DH5α under the control of the *Bacillus* BsXA promoter

Roberto Ruller*, José César Rosa*†, Victor M. Faça*†, Lewis J. Greene*† and Richard J. Ward‡[1]

**Departamento de Biologia Molecular e Celular e Bioagentes Patogênicos, FMRP-USP (Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo), Ribeirão Preto, SP, Brazil, †Centro de Química de Proteínas, FMRP-USP, Ribeirão Preto, SP, Brazil, and ‡Departamento de Química, FFCLRP-USP (Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo), Ribeirão Preto, SP, Brazil*

Xylanase A (XynA) is a class G/11 xylanase secreted by *Bacillus subtilis*. XynA was purified to homogeneity from *B. subtilis* strain 168 culture supernatants by ethanol precipitation and cation-exchange chromatography. The DNA fragment encoding the XynA together with the BsXA promoter region was amplified by PCR from *B. subtilis* 168 genomic DNA, and cloned into the plasmid pT7T3 to give the plasmid pT7BsXA. After transformation of *Escherichia coli* DH5α with pT7BsXA, a 19-fold increase in the levels of the secreted XynA was detected in the supernatant as compared with the *B. subtilis* culture. Correct post-translation modification of the recombinant protein was confirmed by N-terminal amino acid sequencing and MS analyses. The pH- and temperature-dependences of the native and recombinant proteins were identical, indicating that the pT7BsXA may be useful for the constitutive expression of heterologous protein in *E. coli*.

Introduction

Previous studies have shown that the Gram-positive *Bacillus* species are a rich source of industrially important enzymes [1], including xylanases [2,3]. Xylanases (1,4-β-D-xylan xylanhydrolase, EC 3.2.1.8) are endo-enzymes that randomly hydrolyse the β-1,4-xylose linkages of xylans, the principal polysaccharide of hemicelluloses, to release xylo-oligosaccharides (for a review, see [4]). Owing to their industrial potential, xylanases are the focus of many research efforts to harness this catalytic function. On the basis of amino-acid-sequence similarity, xylanases are currently classified into large distinct families referred to either as 10 and 11 or, alternatively, as F and G [5,6]. Members of the G/11 family of xylanases with molecular masses below 30 kDa have been reported from several *Bacillus* sp. (e.g. [7,8]), and these enzymes show no cellulase activity [9].

Xylanase production by diverse native bacterial strains has been analysed (e.g. [10,11]); however a frequent drawback in the use of these native enzymes is the low level of production by the natural strains [4]. In addition, co-production of endogenous enzymes with undesirable catalytic properties may compromise the applicability of the enzyme of interest [12]. Heterologous expression of these enzymes is therefore an interesting option, and the development of efficient and economic protein expression systems is of paramount importance. In the present study, we report that BsXA, the promoter of the XynA (xylanase A) from *Bacillus subtilis*, can drive the efficient expression and secretion of the enzyme in the *Escherichia coli* DH5α host. We have further demonstrated that the *B. subtilis* peptide secretion signal in the secreted protein product is efficiently cleaved by the *E. coli* secretory apparatus. This construct has the potential for an efficient and economic expression system for the constitutive production and secretion of heterologous proteins in *E. coli*.

Materials and methods

Bacterial strains and plasmids

The *B. subtilis* strain 168 [13] was obtained from the Bacillus Genetic Stock Center, Columbus, OH, U.S.A. (BGSC code 168/168). *E. coli* DH5α [*supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 relA1 gyrA96 thi-1 recA1*] (Invitrogen, Carlsbad, CA, U.S.A.) was used both as the cloning host for all plasmid manipulations and as the host cell for heterologous protein expression. Plasmid pT7T318U (Amersham Biosciences, Piscataway, NJ, U.S.A.) was used for cloning of the fragment derived from PCR. The plasmid pT7T318U containing the *B. subtilis* 168 genomic fragment encoding the G/11 xylanase will be referred to as pT7BsXA.

Key words: *Bacillus subtilis* xylanase A, BsXA, *Escherichia coli* DH5α, expression system, industrial enzyme, recombinant protein.

Abbreviations used: DNSA, 3,5-dinitrosalicylic acid; LB, Luria–Bertani; LS, leader sequence; ORF, open reading frame; XynA, xylanase A.

[1] To whom correspondence should be addressed (email rjward@fmrp.usp.br).

Chemicals

DNSA (3,5-dinitrosalicylic acid) and birchwood (*Betula*) xylan were obtained from Sigma–Aldrich (São Paulo, Brazil) and used without further purification. SOURCE 15S cation exchange support was obtained from Amersham Biosciences (São Paulo, Brazil) and used according to the manufacturer's instructions. Reagents and solvents used in the present study were of the highest quality [HPLC or sequencing grade, from Fisher Scientific (São Paulo, Brazil), Applied Biosystems (Foster City, CA, U.S.A.) and Sigma].

Growth of *B. subtilis* strain 168

Spores of *B. subtilis* 168 were cultivated on solid LB (Luria–Bertani) agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5%, w/v, bacteriological agar) for 12 h at 37 °C. An individual colony was used to inoculate 30 ml of liquid LB medium, which was cultured for a further 16 h at 37 °C in an orbital shaker at 180 rev./min. After centrifugation at 6000 ***g*** for 5 min, the xylanase activity of the native protein in the supernatant was estimated (see the next subsection), and the cell pellet was processed to extract the genomic DNA (see the subsection 'Preparation of *B. subtilis* 168 genomic DNA').

Enzyme assays

The effect of pH on the catalytic activity of xylanase was evaluated by incubating 100 μl of culture supernatant for 10 min in a buffered solution of 1% birchwood xylan over a range of pH between 3.6 and 10.4 at 55 °C (0.1 M acetate buffer, pH 3.6–6.0; 0.1 M Tris/HCl, pH 6.4–7.6; 0.1 M glycine/NaOH buffer, pH 8.0–10.4). The effect of temperature on the catalytic activity was evaluated by incubating 100 μl of culture supernatant with 1% xylan suspended in 0.1 M phosphate/citrate buffer (pH 6.5) for a period of 10 min over a temperature range 30–80 °C. In all experiments, the reactions were halted by the addition of 200 μl of a 10 mM DNSA solution and the mixture was heated to 100 °C for 5 min, followed by incubation for 5 min on ice. After centrifugation at 10000 ***g*** for 2 min, the reaction volume was adjusted to 2 ml with distilled water, and the absorption was measured at 540 nm. The final concentration of reducing sugar was quantified using a standard curve [14]. One unit of enzyme was defined as the quantity of xylanase required to liberate 1 μmol of xylose per min under the assay conditions described.

Purification of XynA

Ice-cold ethanol was added to a final concentration of 80% (v/v) to 1 litre of a cell-free culture supernatant of *B. subtilis* 168 while stirring. The mixture was incubated for 1 h at −20 °C, during which time a dense flocculent precipitate was formed. After centrifugation at 20000 ***g*** for 10 min at 4 °C, the pellet was partially dried and resuspended in 50 ml of buffer A (20 mM acetate buffer, pH 4.8), and dialysed for 48 h at 4 °C against the same buffer. Xylanase was purified by cation-exchange chromatography using a 100 mm × 7 mm column packed with SOURCE 15S resin (Amersham Biosciences) previously equilibrated in buffer A. The column was eluted with buffer A supplemented with 1 M NaCl, and the A_{280} of the eluate was monitored continuously. The eluted protein was dialysed against 1 mM Hepes (pH 7.0) for 36 h with buffer changes every 12 h. The protein was quantified by the Lowry method [15], using a standard curve constructed using BSA over the range 0–50 μg/ml, and stored at 4 °C before further use.

SDS/PAGE and N-terminal amino acid sequence determination

Samples of protein were mixed with an equal volume of sample buffer (6%, v/v, 2-mercaptoethanol, 6%, w/v, SDS, 0.1% Bromophenol Blue and 20%, v/v, glycerol) and applied to a denaturing 16% (w/v) polyacrylamide gel. After electrophoresis at 20 mA for a period of 90 min, gels were stained with 0.25% Coomassie Brilliant Blue in a 50:20 (v/v) methanol/acetic acid solution. Proteins were electroblotted on to PVDF membranes as described previously [16]. After staining with Coomassie Blue R250, the 21 kDa protein band was excised from the membrane and submitted to N-terminal amino acid sequencing by automatic Edman degradation using a Sequenator Procise 491 (Applied Biosystems).

MS

Purified xylanase (5 μg) was desalted in a microtip containing POROS 50 R2 (PerSeptive Biosystems, Foster City, CA, U.S.A.) previously equilibrated with 0.2% formic acid and the sample was eluted in 50% (v/v) acetonitrile and 0.2% formic acid. The MS analysis of recombinant xylanase was performed in electrospray triple-quadrupole mass spectrometer Quatro II (Micromass, Wythenshaw, Manchester, U.K.). The sample was injected at a flow rate of 300 nl/min with a capillary voltage of 2.8 kV, a cone voltage of 40 V and a cone temperature of 100 °C. Spectra were collected as the average of 20–50 scans (2–5 s/scan) over the range 1500–3000 a.m.u. (atomic mass units), and the ion envelope was deconvoluted using the MaxEnt1 algorithm (MassLynx software v.3.3, Micromass).

Preparation of *B. subtilis* 168 genomic DNA

The cell pellet from the *B. subtilis* 168 culture was resuspended in 600 μl of STET buffer (8%, w/v, sucrose, 5%, v/v, Triton X-100, 50 mM Tris/HCl, pH 8.0 and 50 mM EDTA), to which was added 60 μl of RNase A/lysozyme (10 mg/ml lysozyme and 1 mg/ml RNase A in 50 mM Tris/HCl, pH 8.0) and the mixture was heated for 1 min 45 s at 100 °C. After cooling, the mixture was centrifuged at 12000 ***g*** for 10 min at 4 °C, and the supernatant was extracted with

Table 1 Purification of the XynA from *B. subtilis* 168 culture supernatants

Step	Volume (ml)	Total protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (-fold)
Culture supernatant	1000	6700	73 300	11.0	100	1.0
Precipitation	250	900	2560	2.80	3.5	0.26
Dialysis	260	668	1190	1.80	1.6	0.16
Resource 15S	1	1.47	890	604	1.2	56

STET equilibrated phenol. LiCl was added to the upper phase to a final concentration of 0.4 M and the mixture incubated on ice for 10 min. After centrifugation (12 000 ***g*** for 10 min at 4 °C), an equal volume of propan-2-ol was added to the supernatant and the mixture was incubated at room temperature (26 °C) for 5 min. After centrifugation at 12 000 ***g*** for 10 min at 4 °C, the pellet was washed with ice-cold 80 % (v/v) ethanol, dried and resuspended in TE buffer (10 mM Tris/HCl and 2 mM EDTA, pH 8.0).

Amplification of the *xynA* gene and cloning in pT7T318U

B. subtilis genomic DNA (5 μg) was digested with 10 units of EcoRI and, after a 100-fold dilution in distilled water, it was used as the template for a PCR with oligonucleotides complementary to the *B. subtilis* genomic DNA sequence [17] regions 2053689–2053717 (XYL5: 5′-ATGAGAATTCGTGGTATTATACTGAAGGG-3′), 2053799–2053814 (XYL5Δ103: 5′-*AAAAA*GAATTCTAGGAGGTAACATATG-3′, non-complementary bases are shown in italics) and 2054472–2054494 (XYL3: 5′-GATCAGGATCCCGTTAGCTACCC-3′). A recognition site (underlined in the oligonucleotide sequences) for the restriction enzyme EcoRI was incorporated into the XYL5 and XYL5Δ103 oligonucleotides and a recognition site for BamHI was included in the XYL3 oligonucleotide. Amplification by PCR (96 °C for 2 min, 50 °C for 90 s and 72 °C for 2 min) using 2.5 units of *Taq*DNA *Platinum* High Fidelity (Invitrogen) with XYL5 and XYL3 produced an 805 bp fragment, which not only includes the coding region of the *xynA* gene, but also the putative upstream transcription regulation sequences. Amplification with XYL5Δ103 and XYL3 produced a 714 bp fragment in which the putative upstream transcription regulation sequences are excluded. After sequential digestion with EcoRI and BamHI, the resulting 791 and 688 bp fragments were ligated into plasmid pT7T318U previously digested with the same two restriction enzymes, and used to transform *E. coli* DH5α. The plasmid DNA extracted from the transformants was fully sequenced using oligonucleotide primers immediately upstream and downstream to the pT7T318U plasmid multiple cloning site. The plasmid pT7T318U containing the 791 bp *B. subtilis* genomic fragment encoding the XynA was referred to as pT7BsXA, and the same plasmid containing the 688 bp fragment without the putative 5′ control regions was named pT7BsXA-Δ103.

Heterologous expression of the XynA in *E. coli* DH5α

A 1 litre portion of LB culture medium (1 % tryptone, 0.5 % yeast extract, 1 % NaCl and 100 mg/l ampicillin) was inoculated with a single colony of *E. coli* DH5α transformed with pT7BsXA. After a 48 h incubation at 37 °C in an orbital shaker at 180 rev./min, the culture was centrifuged at 5000 ***g***, and the recombinant protein was purified as described earlier for the native xylanase.

CD spectroscopy

Far-UV CD spectra of native and recombinant XynA were measured between 185 and 250 nm in 10 mM Hepes (pH 7.0) at 25 °C with a Jasco (Hachioji City, Tokyo, Japan) J-810 spectropolarimeter using 0.1-mm-path-length cuvettes and a protein concentration of 1.5 mg/ml. For each measurement, a total of nine spectra were collected, averaged and corrected by subtraction of a buffer blank.

Results and discussion

Purification of native XynA from *B. subtilis* 168 culture supernatants

As shown in Table 1, the total xylanase activity in the culture supernatant of the *B. subtilis* 168 strain suggests an efficient secretion of the enzyme. However, after precipitation with ethanol, the yield, total activity and specific activity all decreased sharply. This may be due to the production of several distinct xylanases by the *B. subtilis* strain 168, only one of which is precipitated efficiently. Indeed, a search of the complete *B. subtilis* genomic DNA sequence [17] available at KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.ad.jp/kegg/) reveals that, in addition to the gene encoding the XynA, four other genes that encode proteins containing glycohydrolase motifs and that are orthologues of endo-1,4-β-xylanases are also present. The ORFs (open reading frames) of three of these genes (*xynD*, *yheN* and *ynfF*) encode proteins with predicted secretion signal amino acid sequences and which have predicted molecular masses for the mature proteins of 52 (XynD), 27 (YheN) and 44 (YnfF) kDa. These values are significantly different from the predicted molecular mass of 20 kDa for the mature XynA.

Figure 1(A) presents the chromatographic elution profile of the XynA from the cation-exchange column in

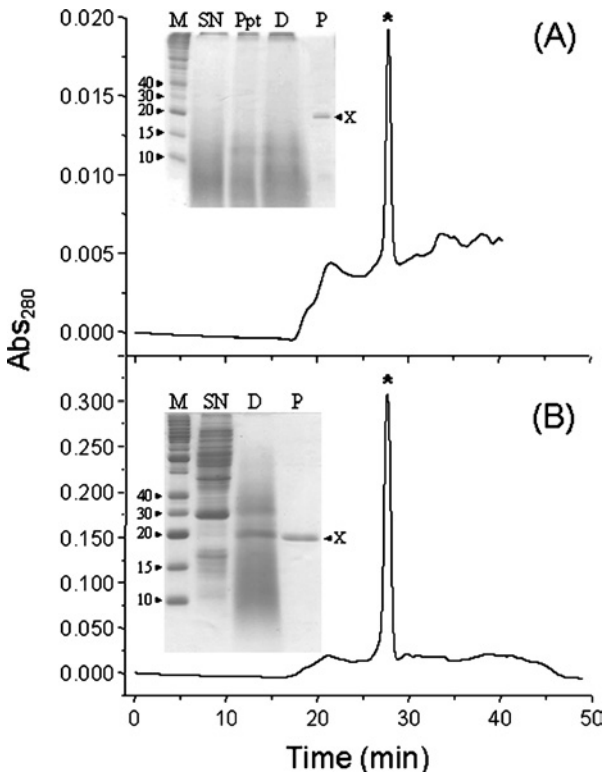

Figure 1 Purification of native and recombinant XynA

(**A**) Elution profile from a SOURCE 15S cation-exchange column of the native XynA isolated from the culture supernatants of *B. subtilis* 168. The absorption of the eluate was monitored at 280 nm ('Abs_{280}'), and the protein peak containing xylanase activity is marked with an asterisk. Inset: an SDS/16% polyacrylamide gel with samples. SN, *B. subtilis* 168 culture supernatant; Ppt, pellet after ethanol precipitation; D, sample after dialysis; and P, sample after cation-exchange chromatography; M, protein standards with their molecular masses indicated in kDa. (**B**) Elution profile from a SOURCE 15S cation-exchange column of recombinant XynA from culture supernatants of *E. coli* DH5α transformed with pT7BsXA. Details were as described for the native protein. The inset shows an SDS/16% polyacrylamide gel with samples. SN, *E. coli* culture supernatants; D, sample after ethanol precipitation and dialysis; and P, sample after purification using cation-exchange chromatography; M, standards with molecular mass in kDa as indicated. The band marked 'X' corresponds to the purified XynA.

which the major peak was eluted after approx. 28 min. As shown in the inset to Figure 1(A), SDS/PAGE demonstrated a single protein band with an apparent molecular mass of 20 kDa. Although xylanase activity was detected at all stages during purification, the low levels of expression of the XynA by the *B. subtilis* 168 were below the detection limit of SDS/PAGE, and the total yield of native protein was approx. 1.4 mg/l culture.

Cloning of the *B. subtilis* XynA gene

The complete nucleotide sequencing of the 791 bp insert resulting from the PCR using *B. subtilis* genomic DNA confirmed the correct amplification and successful cloning in the pT7T318U vector to yield the construct referred to as pT7BsXA. As shown in Figure 2, the amplified fragment contains a 639 bp ORF that encodes the complete 213-amino-acid XynA. The XynA is secreted into culture supernatants, and the predicted amino acid sequence includes a 28-amino-acid leader peptide (shown in Figure 2 as the hatched region denoted LS). The amplified genomic DNA fragment also includes *B. subtilis* regulatory elements and, as indicated in Figure 2, a Shine–Dalgarno sequence (5′-AGGAGGT-3′) is located between the -6 and -12 positions located upstream from the ATG initiation codon (where the $+1$ position is defined as the first A in the ATG codon).

Comparison of the 5′ region of the cloned fragment with a database of *B. subtilis* transcription factor recognition sites {Database of Transcriptional Regulation in *Bacillus subtilis* (DBTBS; http://dbtbs.hgc.jp/), [19]} identified a putative binding site for the Mta protein [20] between nt -40 and -27. The *B. subtilis* Mta is a MerR-type transcription regulator [21], which is a member of a highly conserved family of transcription factors found in many prokaryotes, including *E. coli* [22]. We suggest that the presence of a conserved regulatory recognition site in the promoter region might explain the efficient expression of the heterologous *B. subtilis* XynA that could be observed in the *E. coli* host. This hypothesis was tested by deleting the 5′ region of the *xynA* gene by amplification with the XYL5Δ103/XYL3 oligonucleotide pair to give the pT7BsXA-Δ103 plasmid, as described in the Materials and methods section. As shown in Figure 2, in this construct, the putative 5′ promoter region which includes the Mta recognition site is deleted, yet the preserved region between the -13 and -1 positions maintains the Shine–Dalgarno sequence. The absence of detectable xylanase activity in the supernatants of cultures of *E. coli* DH5α transformed with this construct (results not shown) demonstrates that the native promoter from *B. subtilis* can drive efficient expression of XynA in the *E. coli* host, and we name this upstream region of the *xynA* gene as the BsXA promoter.

Heterologous expression and purification of the recombinant XynA

As presented in Table 2, culture supernatants of *E. coli* DH5α transformed with pT7BsXA contained high levels of xylanase activity. Supernatants of the *E. coli* DH5α transformed with the same plasmid without the insert showed no detectable xylanase activity, demonstrating that the host cell has no endogenous xylanase production. This high level of constitutive heterologous protein expression was achieved in standard LB culture medium and demonstrates that the upstream *B. subtilis* regulatory elements amplified along with the *xynA* coding region support efficient transcription and translation in the *E. coli* host. Figure 1(B) presents the chromatographic profile of the XynA elution from the cation-exchange column, and shows that the retention time of the recombinant xylanase is 28 min, which is in excellent

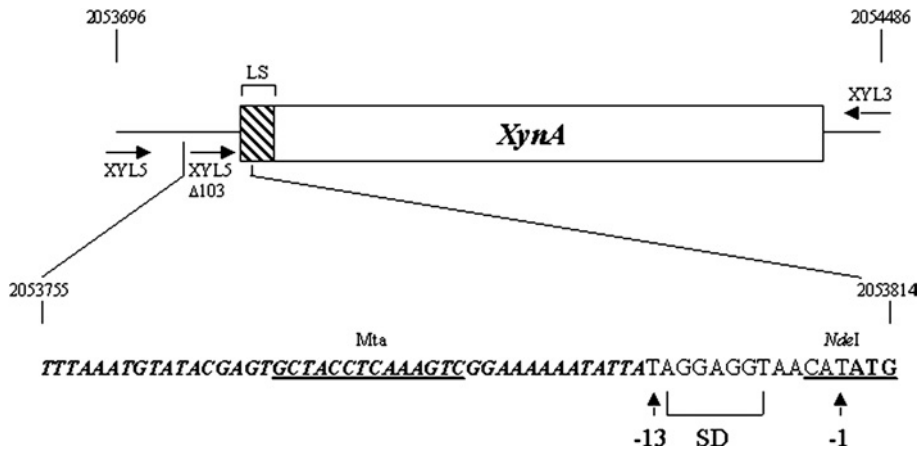

Figure 2 Details of the *xynA* locus in the *B. subtilis* strain 168 genome

Genomic positions between 2053 696 and 2054 486 are shown, which includes the coding region for XynA together with an 84-nucleotide sequence that encodes a 28-amino-acid leader sequence (LS – shown as the hatched region). The annealing regions for the oligonucleotides XYL3, XYL5 and XYL5Δ103 are indicated (see the Materials and methods section for details). The upstream region between nt 2053 755 and 2053 814 is also shown and includes a Shine–Dalgarno sequence (SD) between positions −12 and −6 (where the +1 position is defined as the first 'A' in the initiation codon ATG, shown in boldface and underlined). Recognition sites for the restriction enzyme NdeI and the transcription regulator Mta are underlined. A part of the 5′-region of the gene coding the Mta site, which is excluded by amplification with the XYL5Δ103/XYL3 oligonucleotide pair, is shown in italics.

Table 2 Purification of XynA from *B. subtilis* 168 expressed as a heterologous protein in *E. coli* DH5α

Step	Volume (ml)	Total protein (mg)	Activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (-fold)
Culture supernatant	1000	27000	216000	8.00	100	1.0
Precipitation	250	2880	74400	25.9	35	3.2
Dialysis	270	1810	57000	31.5	26	3.9
Resource 15S	6	26.4	16600	628	7.7	79

agreement with that of the native protein. The inset to Figure 1(B) presents a Coomassie Blue-stained SDS/16% polyacrylamide gel showing that the major peak contains a single protein of approx. 20 kDa, in good agreement with the size of the native protein shown in the inset to Figure 1(A). Table 2 reveals that the yield from 1 litre of culture was approx. 26 mg of recombinant protein, which has a specific activity that is equivalent to the native XynA purified from *B. subtilis* 168 culture supernatants. This significantly increased level of XynA production represents a 19-fold improvement as compared with the native protein yield from the *B. subtilis* 168 strain and suggests that the BsXA promoter and signal sequence may be useful for the constitutive expression of other heterologous proteins in *E. coli* hosts. Indeed, we have found that the use of this construct reduces the cost per litre of recombinant protein produced in the laboratory, as compared with alternative expression systems that rely on the induction of protein expression by expensive synthetic compounds.

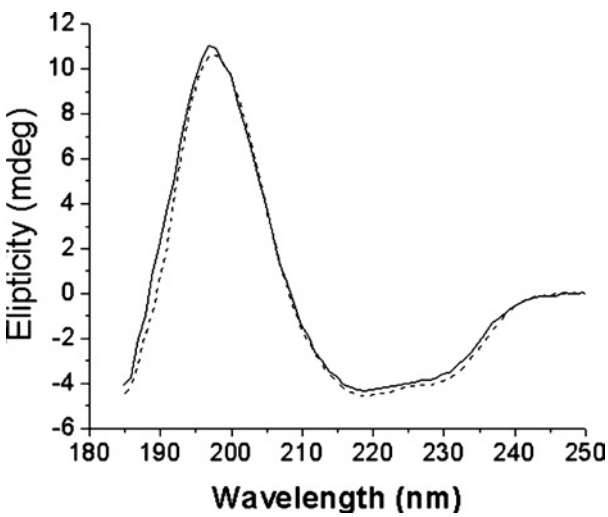

Figure 3 Far-UV CD spectra of purified native (continuous line) and recombinant (broken line) XynA

Spectra were measured as described in the Materials and methods section. Abbreviation: mdeg, millidegrees.

Correct post-translational modification of recombinant XynA

The sequence of the first 15 N-terminal amino acids of the purified protein was ASTDTWQNWTDGGGI, which is identical with the native protein, and demonstrates that the predicted leader polypeptide of the recombinant protein has been correctly cleaved. Furthermore, characterization

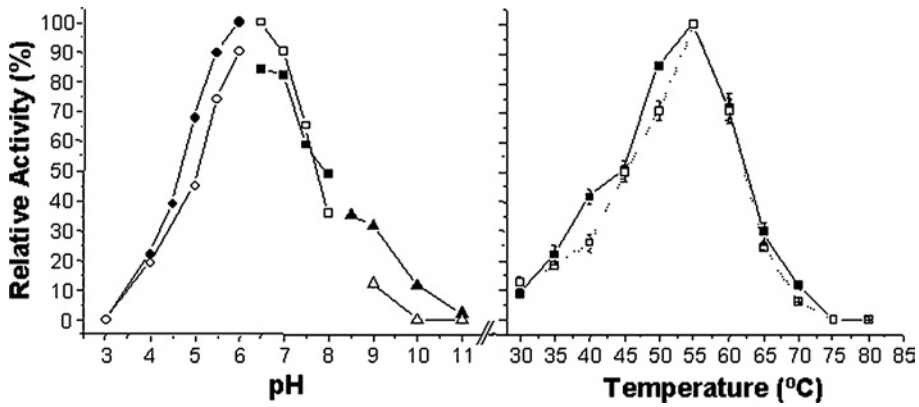

Figure 4 Effects of pH and temperature on the catalytic activity of native (open symbols) and recombinant (closed symbols) XynA

(**A**) The pH was varied in the range 3.6–6.0 with acetate buffer (○), 6.4–7.6 with Tris/HCl (□) and 8.0–10.4 with glycine/NaOH buffer (Δ). Details of the enzyme assay conditions are provided in the 'Enzyme assays' subsection of the Materials and methods section. All data are normalized with respect to the activity observed at pH 6 or 6.5 for the native and recombinant enzymes respectively. (**B**) Temperature-dependences of native (□) and recombinant (■) XynA were measured as described in the Materials and methods section. All data are normalized with respect to the activity observed at 55 °C for the native and recombinant enzymes. The error bars represent the S.D. for three independent activity measurements.

of the mature recombinant xylanase by MS reveals that the protein has a molecular mass of 20 383 Da, which is in good agreement with the molecular mass of 20 381 Da deduced from the DNA sequence after cleavage of the 28-amino-acid LS. This demonstrates that the protein-export pathway in the *E. coli* DH5α strain recognizes and efficiently processes the *B. subtilis* preprotein sequence.

Structural and functional conservation of native and recombinant XynA

Figure 3 demonstrates that the far-UV CD spectra of the native and recombinant xylanases are essentially identical and present defined minima at 185 and 217 nm and a maximum at 196 nm, which are typical values for proteins rich in β-sheet structures. This observation is in agreement with the high β-sheet content observed in the crystal structure of the G11 xylanase from *B. circulans* [23], a protein that is highly homologous with the *B. subtilis* XynA. In addition, the effects of pH and temperature on the catalytic activity of native and recombinant proteins were compared, and Figure 4(A) shows that the pH-dependences on activity are similar for both enzymes and that the native and recombinant proteins present optimum pH values of 6–6.5. Furthermore, Figure 4(B) demonstrates that both proteins show a similar optimum temperature of 55 °C, confirming that the functional properties of the native and recombinant proteins have similar functional properties.

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