

Señores
DIVISIÓN DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y CC
Bogotá, D.C.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 02-074872- -00010-0000
Fecha: 2007-05-17 10:13:30 Dep 2020 NUECREACIONI
Tra. 11 PATENTEIYII Evs. 378 FASENACIONALI
Act. 444 RESPUESTAREQUE Fallos: 42

Re.: Código 011-379-444
Expediente No. 02-74.872
Fase Nacional de Solicitud
de Patente PCT
DANISCO A/S

En relación con el expediente de la referencia y dando cumplimiento a la última providencia recaída sobre este asunto por medio del presente escrito me permito manifestarles lo siguiente:

1. Entendemos que el Examinador considera que las reivindicaciones deben definirse por el resultado deseado (sensibilidad alterada al inhibidor de xilanas) el cual puede lograrse utilizando técnicas conocidas (mutagenesis).

Sin embargo, estas reivindicaciones no reivindican simplemente el resultado deseado (es decir, la sensibilidad alterada al inhibidor de xilanas). La presente invención describe exactamente qué estructuras tendrían este resultado deseado.

2. El Ejemplo 4 demuestra que una modificación en todas y cada una de las posiciones específicas mencionadas en la reivindicación 1 ha sido probada en *Bacillus subtilis* y ha demostrado alterar la sensibilidad de una enzima

de xilanasa *B. subtilis* a un inhibidor de xilanasa. Por ello, el ejemplo 4 provee soporte experimental para modificaciones a las posiciones específicas mencionadas en la reivindicación 1.

Además, el Ejemplo 4 (y en particular, la Tabla 10) provee soporte para cualquier combinación de estas mutaciones. Si una modificación en cada una de las posiciones afecta la sensibilidad de un polipéptido de xilanasa a un inhibidor, puede observarse que si se altera más de una posición se afectará la sensibilidad de un polipéptido de xilanasa a un inhibidor.

Por ello, el Ejemplo 4 provee soporte explícito en el sentido de que las modificaciones en las posiciones específicas mencionadas en la reivindicación 1 resultarán en un polipéptido de xilanasa que tenga una sensibilidad alterada a un inhibidor de xilanasa si se compara con un polipéptido de xilanasa que no haya sido modificado de este modo.

3. Además, se ha modificado la reivindicación 1 para referirse a un método de alterar la sensibilidad de un polipéptido de xilanasa de la Familia 11. Se sabe que las xilanasas de la Familia 11 comparten una estructura similar. De hecho, la presente solicitud de patente indica expresamente esto (ver página 12 líneas 15.17). Con esta carta también adjuntamos dos documentos que respaldan este hecho.

Por ello, si se altera un amino ácido en una posición equivalente en una familia 11 diferente, la xilanasa tendría el mismo efecto que modificar un polipéptido de xilanasa *Bacillus subtilis* en esa posición.

4. El Ejemplo 7 respalda esto. El Ejemplo 7 muestra que la modificación de la posición 11 (D11F) aumentó el nivel de inhibición de un polipéptido de xilanasa *Trichoderma reesei* tal como lo hizo la modificación de D11F para el polipéptido de xilanasa *B. subtilis* que se muestra en el Ejemplo 4.

De este modo puede observarse que la solicitud de patente ofrece soporte adecuado para que una persona versada en la técnica modifique la sensibilidad de un polipéptido de xilanasa modificando una o más de las posiciones que se mencionan en la reivindicación 1.

5. Por lo tanto, consideramos que las reivindicaciones de método tal como están presentadas actualmente no se definen por un resultado deseado sino por las características técnicas (las posiciones específicas que vayan a ser modificadas) que dan lugar a este resultado.
6. Creemos que las Reivindicaciones de polipéptido variante están definidas por medio de su estructura o secuencia química.

Estas reivindicaciones están definidas por su estructura o secuencia química porque una persona versada en la técnica conoce fácilmente las secuencias de amino ácido de las xilanasas de la familia 11 (por ejemplo, la Figura 2 de la solicitud de patente provee las secuencias de amino ácido para un número de xilanasas de la familia 11).

Por lo tanto, si se describe la modificación específica que se vaya a hacer, una persona versada en la técnica puede hacer fácilmente las sustituciones en el polipéptido de xilanasa de la familia 11 (como los que se muestran en la Figura 2) y determinar la secuencia de amino ácidos que resulta de hacer dicha sustitución.

Por ello, el lenguaje que se utiliza en las reivindicaciones del polipéptido variante pueden verse como un resumen para describir todas y cada una de estas secuencias específicas de amino ácidos.

Por las razones anteriormente expuestas, todas y cada una de estas secuencias de amino ácidos tendrá una sensibilidad alterada a un inhibidor de xilanasa.

7. Como pueden observar, se han eliminado las Reivindicaciones 23 y 24
8. Teniendo en cuenta lo dispuesto por el Consejo de Estado en sentencia proferida dentro del Expediente No. 7684 el 22 de Octubre de 2004 y acogida por esa Superintendencia mediante memorando interno de fecha 22 de Diciembre de 2004 dirigido a los funcionarios de la Delegatura de Propiedad Industrial, no se hace necesario acompañar comprobante de pago por concepto de modificación de la solicitud por cuanto el nuevo pliego reivindicatorio se presenta a instancia de la Superintendencia en cumplimiento de lo ordenado por el artículo 45 de la Decisión 486.

Renuncio al término faltante de traslado, y les solicito ordenar la publicación del extracto.

De Ustedes Atentamente,

GERMAN CASTILLO GRAU
T.P. No. 2.978 del C.S.J.

Reivindicaciones :

1. Un método para alterar la sensibilidad de un polipéptido de xilanasa de la Familia 11 a un inhibidor; dicho método comprende:

modificar uno o más residuos amino ácidos de dicho polipéptido; y medir la sensibilidad de dicho polipéptido de xilanasa a un inhibidor de xilanasa;

de modo que el polipéptido o fragmento del mismo tenga sensibilidad alterada a un inhibidor de xilanasa si se compara con la enzima de xilanasa precursora, en donde dicha xilanasa tiene al menos una de dichas modificaciones de amino ácidos en cualquiera de las posiciones, 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 ó 175 de la secuencia amino ácida *B. subtilis* mostrada como la SEQ. ID No.1, o en posiciones equivalentes en otras xilanasas homólogas, en donde al menos una de dichas modificaciones de amino ácidos está al menos en la posición 11, 12 o 13 de la secuencia de amino ácidos *B subtilis* mostrada como la SEQ ID. No. 1 o en una posición equivalente a otras xilanasas homólogas.

2. Un método para alterar la sensibilidad de un polipéptido de xilanasa a un inhibidor; dicho método comprende:

modificar uno o más residuos amino ácidos de dicho polipéptido; y opcionalmente, medir la sensibilidad de dicho polipéptido de xilanasa a un inhibidor de xilanasa;

de modo que el polipéptido o fragmento del mismo tenga sensibilidad alterada a un inhibidor de xilanasa si se compara con la enzima de xilanasa precursora, en donde dichas modificaciones son a dos o más de las posiciones 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 y 175 de la secuencia de amino ácido *B subtilis* mostrada como la SEQ ID. No. 1, o en una posición equivalente en otras xilanasas homólogas, en donde una de dichas modificaciones de amino ácidos esta en la posición 11.

3. Un método según cualquiera de las reivindicaciones anteriores, en donde dicha modificación de amino ácidos se hace por mutagenesis dirigida en sitio.

4. Un método para alterar la sensibilidad de un polipéptido de xilanasa a un inhibidor, dicho método comprende:

modificar dos o más residuos amino ácidos de dicho polipéptido; y
opcionalmente, medir la sensibilidad de dicho polipéptido de xilanasa a un inhibidor de xilanasa;

de modo que el polipéptido o fragmento del mismo tenga sensibilidad alterada a un inhibidor de xilanasa si se compara con la enzima de xilanasa precursora,

en donde dichas modificaciones son a dos o más posiciones 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 y 175 de la secuencia de amino ácidos *B. subtilis* mostrada como la SEQ ID. No. 1, o a una posición equivalente en otras xilanasas homólogas,

en donde dichas modificaciones de amino ácidos se hace por mutagenesis dirigida en el sitio,

y

en donde una de dichas modificaciones de amino ácido esta en la posición 11, 12 o 13.

5. Un método según cualquiera de las reivindicaciones anteriores, en donde una o más modificaciones de amino ácidos es cualquiera de: D11Y, D11N, D11F, D11K, D11S, D11W, G12F, G13F, I15K, N17K, N17Y, N17D, N29K, N29Y, N29D, S31K, S31Y, S31D, N32K, G34D, G34F, G34T, Y113A, Y113D, Y113K, N114A, N114D, N114F, N114K, D119K, D119Y, D119N, G120K, G120D, G120F, G120Y, G120N, D121N, D121K, D121F, D121A, R122D, R122F, R122A, T123K, T123Y, T123D, T124K, T124Y, T124D, I175E, I175S y Q175L.

6. Un método según cualquiera de las reivindicaciones anteriores, en donde una o más modificaciones de amino ácidos en cualquiera de: D11Y, D11N, D11F, D11K, D11S o D11W.

7. Un método según cualquiera de las reivindicaciones anteriores, en donde una de dichas modificaciones de amino ácidos es D11F.

8. Un método según cualquiera de las reivindicaciones anteriores, en donde dos de dichas modificaciones de amino ácidos son D11F y R122D.

9. Un polipéptido variante o un fragmento del mismo que tiene actividad de xilanasa, que comprende dos o más modificaciones de amino ácido, en donde dichas modificaciones de amino ácido están en dos o más de las posiciones 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124, y 175 de la secuencia de amino ácidos *B subtilis* mostrada como SEQ ID. No.1, o en posiciones equivalentes en otras xilanasa homólogas, de modo que el polipéptido o fragmento del mismo tenga una sensibilidad alterada si se compara con la xilanasa precursora, en donde una de dichas modificaciones de amino ácidos está en la posición 11, y en donde dichas modificaciones de amino ácidos son cualquiera de: D11Y, D11N, D11F, D11K, D11S, D11W, G12F, G13F, I15K, N17K, N17Y, N17D, N29K, N29Y, N29D, S31K, S31Y, S31D, N32K, G34D, G34F, G34T, Y113A, Y113D, Y113K, N114A, N114D, N114F, N114K, D119K, D119Y, D119N, G120K, G120D, G120F, G120Y, G120N, D121K, D121F, D121A, R122D, R122F, R122A, T123K, T123Y, T123D, T124K, T124Y, T124D, 1175E, 175S y Q175L.

10. Un polipéptido variante o fragmento del mismo que tiene actividad de xilanasa, que comprende dos o más modificaciones de amino ácido, en donde dichas modificaciones de amino ácido están en dos o más de las posiciones 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 y 175 de la secuencia de amino ácidos *B subtilis* mostrada como SEQ ID. No.1, o en posiciones equivalentes en otras xilanasa homólogas, de modo que el polipéptido o fragmento del mismo tenga una sensibilidad alterada si se compara con la xilanasa precursora, en donde dichas modificaciones de amino ácidos son introducidas por mutagenesis dirigida y en donde al menos una de dichas modificaciones de amino ácidos está en la posición 11, 12 ó 13, y en donde dichas modificaciones de amino ácidos son cualquiera de : D11Y, D11N, D11F, D11K, D11S, D11W, G12F, G13F, 115K, N17K, N17Y, N17D, N29K, N29Y, N29D, S31K, S31Y, S31D, N32K, G34D, G34F, G34T, Y113A, Y113D, Y113K, N114A, N114D, N114F, N114K, D119K, D119Y, D119N, G120K, G120D, G120F, G120Y, G120N, G121N, D121K, D121F, D121A, R122D, R122A, R122F, T123K, T123Y, T123D, T124K, T124Y, T124D, 1175E, 175S y Q175L.

11. Un polipéptido variante según cualquiera de las reivindicaciones 9 ó 10, en donde una de dichas modificaciones de amino ácidos es D11Y, D11N, D11F, D11K, D11S o D11W.

12. Un polipéptido variante según cualquiera de las reivindicaciones 9 a 11, en donde una de dichas modificaciones de amino ácidos es D11F.

13. Un polipéptido variante según cualquiera de las reivindicaciones 9 a 12, en donde una de dichas modificaciones de amino ácidos es D11F y R122D.

14. Un polipéptido variante según cualquiera de las reivindicaciones 9 a 13, en donde dicho Inhibidor es un inhibidor que se encuentra naturalmente en los tejidos de las plantas.

15. Un polipéptido variante según cualquiera de las reivindicaciones 9 a 14, en donde dicha sensibilidad a un inhibidor es reducida.

16. Una composición que comprende un polipéptido variante según cualquiera de las reivindicaciones 9 a 15.

17. Un método para degradar o modificar una pared de célula vegetal el cual comprende contactar dicha pared vegetal con un polipéptido según cualquiera de las reivindicaciones 9 a 15, o una composición según la reivindicación 16.

18. Un método para procesar un material vegetal, el cual comprende contactar dicha pared vegetal con un polipéptido según cualquiera de las reivindicaciones 9 a 15, o una composición según la reivindicación 16.

19. Una secuencia nucleótida que codifica un polipéptido variante según cualquiera de las reivindicaciones 9 a 15.

20. Una construcción que comprende la secuencia nucleótica de la reivindicación 19.



The endoxylanases from family 11: computer analysis of protein sequences reveals important structural and phylogenetic relationships

Amalia Sapag ^{a,1}, Johan Wouters ^b, Christophe Lambert ^b, Pablo de Ioannes ^a,
Jaime Eyzaguirre ^{a,a*}, Eric Depiereux ^b

^a Laboratorio de Bioquímica, Departamento de Genética Molecular y Microbiología, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile

^b Department of Biology, Faculté Universitaire Notre-Dame de la Paix, Namur, Belgium

^a Escuela de Artes Liberales, Universidad Nacional Andrés Bello, Santiago, Chile

Received 5 June 2001; received in revised form 5 November 2001; accepted 20 December 2001

Abstract

Eighty-two amino acid sequences of the catalytic domains of mature endoxylanases belonging to family 11 have been aligned using the programs MATCHBOX and CLUSTAL. The sequences range in length from 175 to 233 residues. The two glutamates acting as catalytic residues are conserved in all sequences. A very good correlation is found between the presence (at position 100) of an asparagine in the so-called 'alkaline' xylanases, or an aspartic acid in those with a more acidic pH optimum. Four boxes defining segments of highest similarity were detected; they correspond to regions of defined secondary structure: B5, B6, B8 and the carboxyl end of the alpha helix, respectively. Cysteine residues are not common in these sequences (0.7% of all residues), and disulfide bridges are not important in explaining the stability of several thermophilic xylanases. The alignment allows the classification of the enzymes to groups according to sequence similarity. Fungal and bacterial enzymes were found to form mostly separate clusters of higher similarity. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Family 11 endoxylanases; Factor analysis classification; Sequence alignment; Structural relationships

1. Introduction

Xylan, a heteroglycan, is the main constituent of plant hemicelluloses. It is composed of a linear chain of xylose residues linked by $\beta(1 \rightarrow 4)$ glycosidic bonds, with a variety of substituents, depending on its source (Joclean et al., 1992). Biodegradation of xylan is accomplished by a complex set of enzymes generically called 'xy-

* Corresponding author. Tel.: +562-686-2664; fax: +562-222-4515.

E-mail address: jeyzag@genes.bio.puc.cl (J. Eyzaguirre).

¹ Present address: Laboratorio de Farmacoterapia Génica, Facultad de Ciencias Químicas y Farmacia, Universidad de Chile, Santiago, Chile.

170 -15

lanases', which are produced by fungi and bacteria (Biely, 1985).

The hydrolysis of the xylan backbone is performed mainly by the action of the endoxylanases (E.C. 3.2.1.8), which liberate xylooligosaccharides of different length (Biely, 1985). A large number of endoxylanases have been described, purified, characterized and sequenced from different microorganisms (Sunna and Antranikian, 1997), and based on sequence similarities and hydrophobic cluster analysis, they have been grouped in families 10 (or F) and 11 (or G) of the glycosyl hydrolases (Gilkes et al., 1991; Henrissat and Davies, 1997). Family 10 xylanases have a catalytic domain with molecular weights in the range of 32–39 K. The structure of the catalytic domain of several family 10 xylanases has been determined by X-ray crystallography and it consists of an eightfold β/α barrel (Harris et al., 1996). These enzymes show a greater catalytic versatility than family 11 endoxylanases (Biely et al., 1997). Family 11 xylanases, on the other hand, have a much smaller catalytic domain (around 20 kDa), with an all β -strand sandwich fold structure (Himmel et al., 1997). Some enzymes of family 10 and family 11 possess, in addition, cellulose or xylan binding domains (Törrönen and Rouvinen, 1997).

The endoxylanases have been found to be useful for several different biotechnological applications (Section 3.4). Therefore, for the design and protein engineering of endoxylanases, a good knowledge of their sequence and structure is of great importance.

For this purpose, a comparative analysis of the sequences of 82 catalytic domains of family 11 glycanases has been performed in this work. The large number of sequences available allows a fine analysis of sequence similarities, with the purpose of establishing structural relationships, determine location of conserved (and possibly essential) sequences and establish phylogenetic relationships.

2. Materials and methods

Sequences were obtained from the literature and from the public databases (SwissProt, GenBank and CAZY ([pedro/CAZY/ghf.html\)\). The 82 sequences were aligned by the method described below.](http://afmb.cnr-mrs.fr/~</p>
</div>
<div data-bbox=)

To define conserved residues in a reliable alignment of numerous sequences of very different length and of poor similarity is not trivial (de Fays et al., 1999). Although alignments are widely used, the rate of false positive functional similarities deduced from skewed aligned positions is not easily overcome (Briffeuil et al., 1998). In this study, an original methodology has been applied to obtain reliable alignments allowing a precise delineation of the corresponding relevant residues in the different sequences, despite the fact that some sequences share a very low percentage of identity (10%).

The first step of this methodology is based on the local alignment method MATCHBOX (Depiereux and Feytmans, 1992). Default parameters were used to delineate conserved boxes (defining Predicted Structural Conserved Regions, PSCR) with an estimated level of confidence (from 1 to 9) at each position of the alignment (Depiereux et al., 1997). In this work, PSCR's are defined for reliability scores below 5 on more than 2 non-redundant sequences, which corresponds to a rate of confidence of over 90%. This ensures less than 10% of 'false positive' aligned positions, a remarkable rate at this level of low similarity.

The second step of the procedure is based on the global alignment method CLUSTAL (Higgins et al., 1992). It is used to align the regions inserted between the anchor points defined above. Thus, the final alignment obtained by this original procedure takes advantage of both approaches: MATCHBOX for definition of the boxes (PSCR's), and CLUSTAL for the multiple alignment of the segments outside the boxes.

The proposed classification of the family 11 endoxylanases is based on a principal coordinates analysis computed from a similarity matrix between the sequences (Depiereux and Feytmans, 1991). The method takes advantage of a grouping of the sequences independent of any alignment steps and thus avoids a misclassification due to misalignments. Briefly, the similarity coefficient is based on matches, for a given threshold, between short unaligned segments of the sequences compared; signvectors and eigenvalues are computed

171

12

by matrix diagonalization, and sequences are plotted for factors 2 and 3, factor 1 being poorly discriminant. Eigenvector coordinates have been submitted to a cluster analysis, Ward's method, run on STATISTICA 4.1 (Statsoft, Inc) and plotted in an EXCEL spreadsheet.

PSCR delineation and factor analysis have been performed by the programs Align and Explore on the MATCHBOX server http://www.fundp.ac.be/sciences/biologie/bma/matchbox_submit.shtml and global alignment on the Clustal server <http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html>.

3. Results and discussion

Table 1 lists the 82 enzymes used in this study. It includes 36 sequences from bacteria, 43 from fungi, 2 from protozoa and 1 from insects. Twenty-two bacterial and 8 fungal enzymes are multidomain proteins; they correspond mainly to anaerobic microorganisms. Only the sequence of the catalytic domain of the mature enzymes is considered in this analysis. The amino terminus of the mature enzymes was taken to be either the experimentally determined terminus, when reported in the literature, or the predicted terminus, determined by means of the program SIGNALP (Nielsen et al., 1997). The enzymes from *Fibrobacter succinogenes*, *Neocallimastix frontalis* and *Neocallimastix patriciarum* have two family 11 catalytic domains each; these domains are considered separately in the analysis. The size of the catalytic domain of the multidomain enzymes is somewhat arbitrary; in Fig. 1 the sequences belonging to these enzymes are marked with a star at the beginning and/or end to indicate this fact.

The enzymes analyzed (not considering the multidomain proteins) range in molecular mass from 19035 (*Trichoderma reesei*) to 25908 (*Clostridium acetobutylicum*), and in sequence length from 175 (*Polypastron multivesiculatum* Xyn A) to 233 amino acid residues (*C. acetobutylicum*). A shorter sequence (Xyn 4 from *Aspergillus niger*; 153 residues, accession number U39785) was not included in this study because it aligned poorly to the other sequences due to its

shorter length, although it is considered part of family 11 by CAZy.

Table 2 shows the pI's, pH and T° optima of those enzymes from the above list which have been purified and characterized. The pI values given are values measured in the laboratory; no theoretical estimations have been included. A wide range of pI values has been found: from a low of 3.5 for *Aspergillus kawachii* and *Aspergillus nidulans* to a high of > 10.25 for *Streptomyces lividans* xylanases. Bacterial enzymes show pH optima ranging from 5.5 to 7, while those from fungi show a much wider range (from pH 2.0 to 8.0), the majority having acidic pH optima.

The three-dimensional structure of family 11 endoxylanases has been determined for several enzymes, from both bacteria and fungi (Table 3). The catalytic domain folds into two β sheets (A and B) constituted mostly by antiparallel β strands and one short alpha helix and resembles a partially closed right hand (Törrönen and Rouvinen, 1997). The loop between strands B7 and B8 forms the 'thumb' and the loop linking strands B6 and B9 is the 'cord'. A great similarity is found in all these structures. Fig. 2 presents a topology diagram showing the sequence of the secondary structure elements found in these enzymes. Two glutamic acid residues have been found to be catalytically essential and are located in strands B4 and B6, respectively (Törrönen and Rouvinen, 1997).

3.1. Multiple sequence alignment

The original alignment methodology used in this work is aimed at delineating structural features shared by all the xylanase sequences included in the analysis. First, boxes obtained from the MATCHBOX alignment program have been shown to accurately outline conserved structural motifs (De Bolle et al., 1995; Vinals et al., 1995; Bertrand et al., 1997, 1998; Depierreux et al., 1997). The fact that these predicted structurally conserved regions actually reflect robust structural similarities among the endoxylanases has been checked by comparison of the three-dimensional structures of the 11 xylanases of known crystallographic structure (Table 3) using the HOMOLGY

15
172

Table 1
Family 11 xylanases used in this study

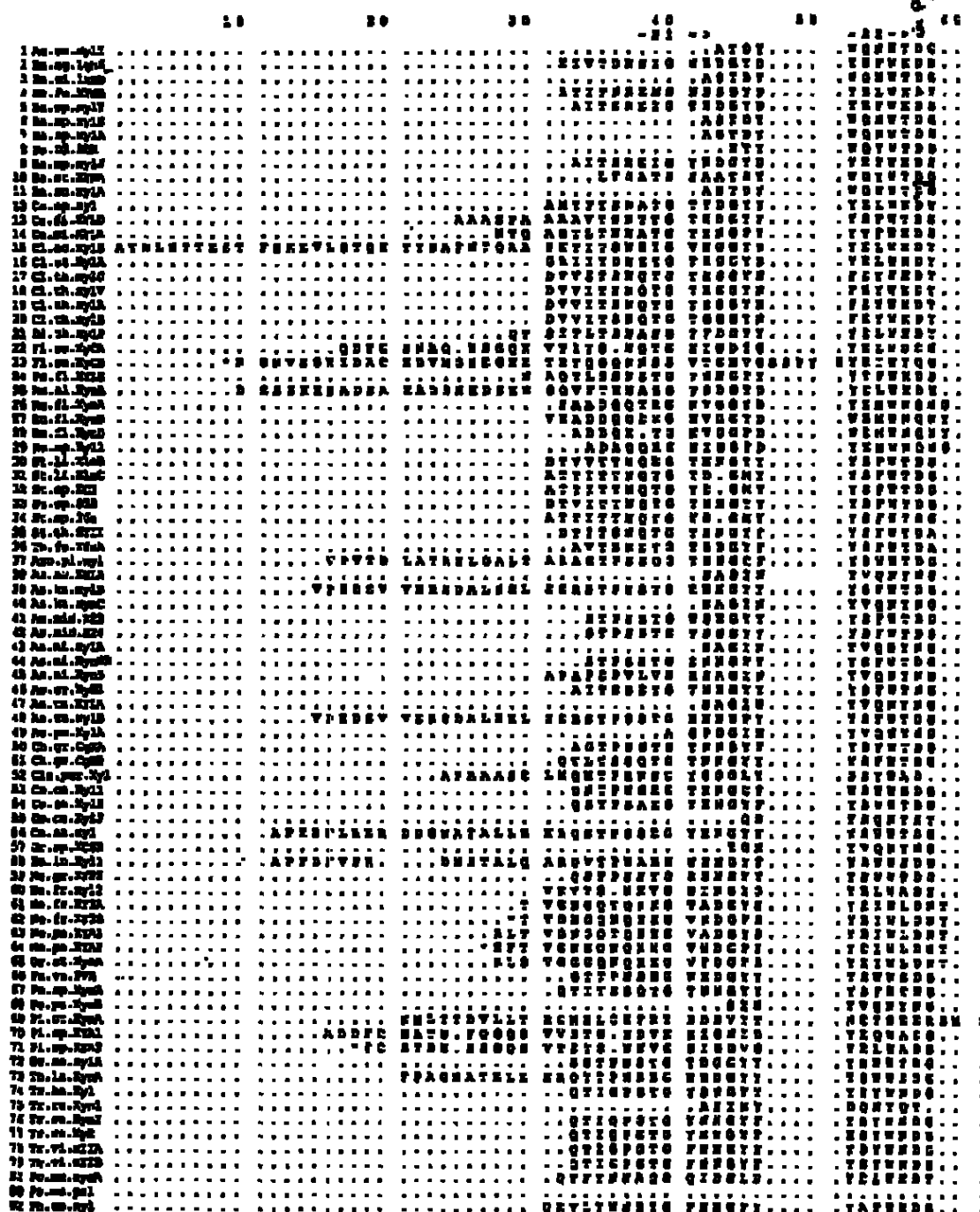
Organism	Protein	Gene	Accession No. (GenBank)	Catalytic domain		References
				Length ^a	MW	
Bacteria						
<i>Aeromonas caviae</i>	Xylanase I	<i>xynA</i>	D32065	183	20 212	Kubota et al. (1997)
<i>Bacillus agaradhaerens</i>	Xylanase		A48223	221	24 681	Sabini et al. (1999)
<i>Bacillus clausii</i>	XLNA	<i>xlnA</i>	X07723	185	20 382	Yang et al. (1988)
<i>Bacillus pumilus</i>	XYNA	<i>xynA</i>	X00660	201	22 515	Fukumaki et al. (1994)
<i>Bacillus</i> sp.	Xylanase Y ^a	<i>xynY</i>	B51779	200	22 201	Yu et al. (1993)
<i>Bacillus</i> sp.	Xylanase S	<i>xynS</i>	X59058	185	20 164	Yu et al. (1993)
<i>Bacillus</i> sp.	Xylanase	<i>xynA</i>	U51675	185	20 220	Jeong et al. (1998)
<i>Bacillus</i> sp. D3	Xylanase	<i>xyn</i>		182	20 683	Harris et al. (1997)
<i>Bacillus</i> sp. 41 M-1	Xylanase J ^b	<i>xynJ</i>	AB028319	199	22 098	Nakai et al. (1994)
<i>Bacillus</i> <i>thermophilus</i>	XYNA	<i>xynA</i>	U15985	191	21 083	Cho and Choi (1995)
<i>Bacillus subtilis</i>	Xylanase A	<i>xynA</i>	M36648	185	21 451	Palen et al. (1984)
<i>Caldicellulosiptychus</i> sp. R169B.1	Xylanase ^a	<i>xynD</i>	AF036925	199	22 158	Morris et al. (1999)
<i>Cellulomonas fimi</i>	XYLD ^b	<i>xynD</i>	X76729	198	21 451	Millward-Sadler et al. (1994)
<i>Cellulobio mixtus</i>	XYLA ^b	<i>xynA</i>	Z48925	207	22 550	Millward-Sadler et al. (1995)
<i>Clostridium</i> <i>neobutylicum</i>	Xylanase B	<i>xynB</i>	M31726	233	25 908	Zappe et al. (1990)
<i>Clostridium sporosporium</i>	XynA ^b	<i>xynA</i>	D13324	193	22 130	Sakka et al. (1993)
<i>Clostridium thermocellum</i>	Xylanase U ^b	<i>xynU</i>	AF047761	204	22 842	Unpublished
<i>Clostridium thermocellum</i>	Xylanase V ^b	<i>xynV</i>	AF047761	204	22 787	Unpublished
<i>Clostridium thermocellum</i>	XynA ^b	<i>xynA</i>	AB010958	200	22 445	Hayashi et al. (1999)
<i>Clostridium thermocellum</i>	XynB ^b	<i>xynB</i>	AB010958	200	22 365	Hayashi et al. (1999)
<i>Dictyoglomus</i> <i>thermophilum</i>	Xylanase B ^b	<i>xynB</i>	U76545	198	22 204	Morris et al. (1998)
<i>Fibrobacter succinogenes</i>	XynC (domA) ^b	<i>xynC</i>	U01037	234	25 330	Parade et al. (1993)
<i>Fibrobacter succinogenes</i>	XynC (domB) ^b	<i>xynC</i>	U01037	216	24 457	Parade et al. (1993)
<i>Pseudomonas fluorescens</i>	XYLB ^b	<i>xynE</i>	Z48927	202	22 130	Millward-Sadler et al. (1995)
<i>Ramibacterium albus</i>	XynA ^b	<i>xynA</i>	U43089	236	26 314	Unpublished
<i>Ramibacterium flaviglutans</i>	XYLA ^b	<i>xynA</i>	Z11127	221	24 346	Zhang and Flint (1992)
<i>Ramibacterium flaviglutans</i>	XynB ^b	<i>xynB</i>	Z35226	216	24 216	Zhang et al. (1994)
<i>Ramibacterium flaviglutans</i>	XYLD ^b	<i>xynD</i>	S61204	213	24 031	Flint et al. (1993)
<i>Ramibacterium</i> sp.	Xylanase I ^b	<i>xynI</i>	Z49970	213	23 904	Unpublished
<i>Streptomyces lividans</i>	XlnB ^b	<i>xlnB</i>	M64552	192	21 064	Shareck et al. (1991)
<i>Streptomyces lividans</i>	XlnC	<i>xlnC</i>	M64553	191	20 715	Shareck et al. (1991)
<i>Streptomyces</i> sp. BC3	Xylanase	<i>xln</i>	X81045	191	20 931	Mury-Servais et al. (1996)
<i>Streptomyces</i> sp. 838	Xylanase	<i>xylI</i>	X385316	190	20 585	Georia et al. (1999)
<i>Streptomyces</i> sp. 36a	Xylanase			192	20 973	Nagashima et al. (1989)
<i>Streptomyces</i> <i>thermophilus</i>	STX-II ^b	<i>stx-II</i>	D85897	190	20 738	Tajima et al. (1997)
<i>Thermomonospora fusa</i>	TxA ^b	<i>xynA</i>	U01242	190	20 900	Irwin et al. (1994)
Fungi						
<i>Ascochyta pisi</i>	Xylanase	<i>xylI</i>	Z68891	208	22 185	Lisbeck et al. (1997)
<i>Aspergillus awamori</i>	EXLA	<i>exlA</i>	X78113	184	19 876	Hosking et al. (1994)

14
173

Table 1 (Continued)

Organism	Protein	Gene	Accession No. (GenBank)	Catalytic domain		References
				Length ^a	MW	
<i>Aspergillus kawachi</i>	Xylanase B	<i>xynB</i>	F48824 ^c	207	22 259	Unpublished
<i>Aspergillus kawachi</i>	XynC	<i>xynC</i>	D14848	184	19 876	Ito et al. (1992a)
<i>Aspergillus nidulans</i>	X21	<i>xhA</i>	Z49892	188	20 235	Perez-Gonzalez et al. (1996)
<i>Aspergillus nidulans</i>	X24	<i>xhB</i>	Z49893	188	20 077	Perez-Gonzalez et al. (1996)
<i>Aspergillus niger</i>	Xylanase A	<i>xylA</i>	A19535	184	19 890	Misut et al. (1992)
<i>Aspergillus niger</i>	XynB	<i>xynB</i>	D38071	188	20 069	Kinoshita et al. (1995)
<i>Aspergillus niger</i>	Xyn5	<i>XYN5</i>	U39784	195	21 143	Unpublished
<i>Aspergillus oryzae</i>	XynG1	<i>xynG1</i>	AB003085	189	20 589	Kinoshita et al. (1998)
<i>Aspergillus subspensus</i>	XYLA	<i>xhA</i>	L26918	184	19 837	de Graaff et al. (1994)
<i>Aspergillus subspensus</i>	Xylanase B		A39368	207	22 240	Patent WO 9414965-A (1994)
<i>Aspergillus subspensus</i>	XynA	<i>xynA</i>	U10298	187	20 074	Li and Ljungdahl (1994)
<i>Chaetomium gracile</i>	CgXA	<i>cgaA</i>	D49850	189	20 149	Yoshino et al. (1995)
<i>Chaetomium gracile</i>	CgXB	<i>cgaB</i>	D49851	211	22 525	Yoshino et al. (1995)
<i>Chaetops purpurea</i>	Xylanase	<i>xylI</i>	Y16969	197	21 460	Gilbert et al. (1992)
<i>Cochliobolus carbonum</i>	Xyl1	<i>XYL1</i>	L13596	191	20 856	Apel et al. (1993)
<i>Cochliobolus carbonum</i>	Xyl2	<i>XYL2</i>	U38915	191	21 199	Apel-Birkhold and Walton (1996)
<i>Cochliobolus carbonum</i>	Xyl3	<i>XYL3</i>	U38916	183	19 858	Apel-Birkhold and Walton (1996)
<i>Cochliobolus sativus</i>	Xylanase	<i>xyl2</i>	AJ004802	212	23 658	Emund and Hack (2001)
<i>Cryptococcus</i> sp. S-2	Xyn-CE2	<i>xyn-CE2</i>	D63382	184	20 209	Iefuji et al. (1996)
<i>Humicola insolens</i>	Xyl1	<i>xylI</i>	X78047	208	23 814	Dalboge and Holch-Hansen (1994)
<i>Magnaporthe grisea</i>	XYN22	<i>xyn22</i>	L57529	194	21 427	Wu et al. (1995)
<i>Neocallimastix frontalis</i>	Xylanase 2	<i>XYN2</i>	B48865	212	23 933	Unpublished
<i>Neocallimastix frontalis</i>	XYN3 (domA) ^b	<i>xyn3</i>	X82266	223	24 594	Durand et al. (1996)
<i>Neocallimastix frontalis</i>	XYN3 (domB) ^b	<i>xyn3</i>	X82266	223	24 532	Durand et al. (1996)
<i>Neocallimastix patriciarum</i>	XYLA (dom1) ^b	<i>xynA</i>	X65526	226	24 941	Gilbert et al. (1992)
<i>Neocallimastix patriciarum</i>	XYLA (dom2) ^b	<i>xynA</i>	X65526	225	24 770	Gilbert et al. (1992)
<i>Orpinomyces stria</i> PC-2	XynA ^b	<i>xynA</i>	U57819	225	24 810	Li et al. (1997)
<i>Panicumum variol</i>	PVX		F81538 ^c	194	21 365	Kumar et al. (2000)
<i>Panicumum</i> sp. 40	XynA	<i>xynA</i>	AB033540	190	20 713	Kinoshita et al. (2000)
<i>Panicumum purpurascens</i>	XynB	<i>xynB</i>	Z50050	183	19 571	Diaz et al. (1997)
<i>Pichia stipitis</i>	Xylanase A ^b	<i>xynA</i>	AF151379	232	26 291	Unpublished
<i>Phanerochaete</i> sp. (inactive)	XYLA ^b	<i>xynA</i>	X91858	234	25 558	Fanucci et al. (1995)
<i>Phanerochaete</i> sp. (active)	XYLA ^b	<i>xynA</i>	X91858	222	24 808	Fanucci et al. (1995)
<i>Schizopyllium commune</i>	Xylanase A	<i>xynA</i>	F35809 ^c	197	20 963	Oka et al. (1993)
<i>Thermomyces lanuginosus</i>	XynA	<i>xynA</i>	U35436	206	22 614	Schlaeber et al. (1996)
<i>Trichoderma harzianum</i> ES420 kD Xylanase			F48793 ^c	190	20 690	Yaguchi et al. (1992b)
<i>Trichoderma reesei</i>	XYN1	<i>xyn1</i>	X69574	178	19 035	Töröcsen et al. (1992)
<i>Trichoderma reesei</i>	XYN2	<i>xyn2</i>	X69573	190	20 829	Töröcsen et al. (1992)
<i>Trichoderma reesei</i>	Xyn2	<i>XYN2</i>	U34191	190	20 731	La Grange et al. (1996)
<i>Trichoderma viride</i>	Xylanase IIA		A44594	190	20 759	Yaguchi et al. (1992a)
<i>Trichoderma viride</i>	Xylanase IIB		A44595	190	20 743	Unpublished
<i>Protococcus</i>						
<i>Polypastrum</i>	XYN A	<i>xynA</i>	AJ009828	219	25 192	Unpublished
<i>Polypastrum multiseptatum</i>						
<i>Polypastrum</i>	Xylanase	<i>polyX</i>	AB011274	175	19 394	Unpublished
<i>Polypastrum multiseptatum</i>						
<i>Insect</i>						
<i>Phaeon cochllearius</i>	Xylanase		Y17908	200	22 070	Unpublished

^a Number of amino acid residues.^b Multidomain protein.^c SWISS-PROT entry.



176

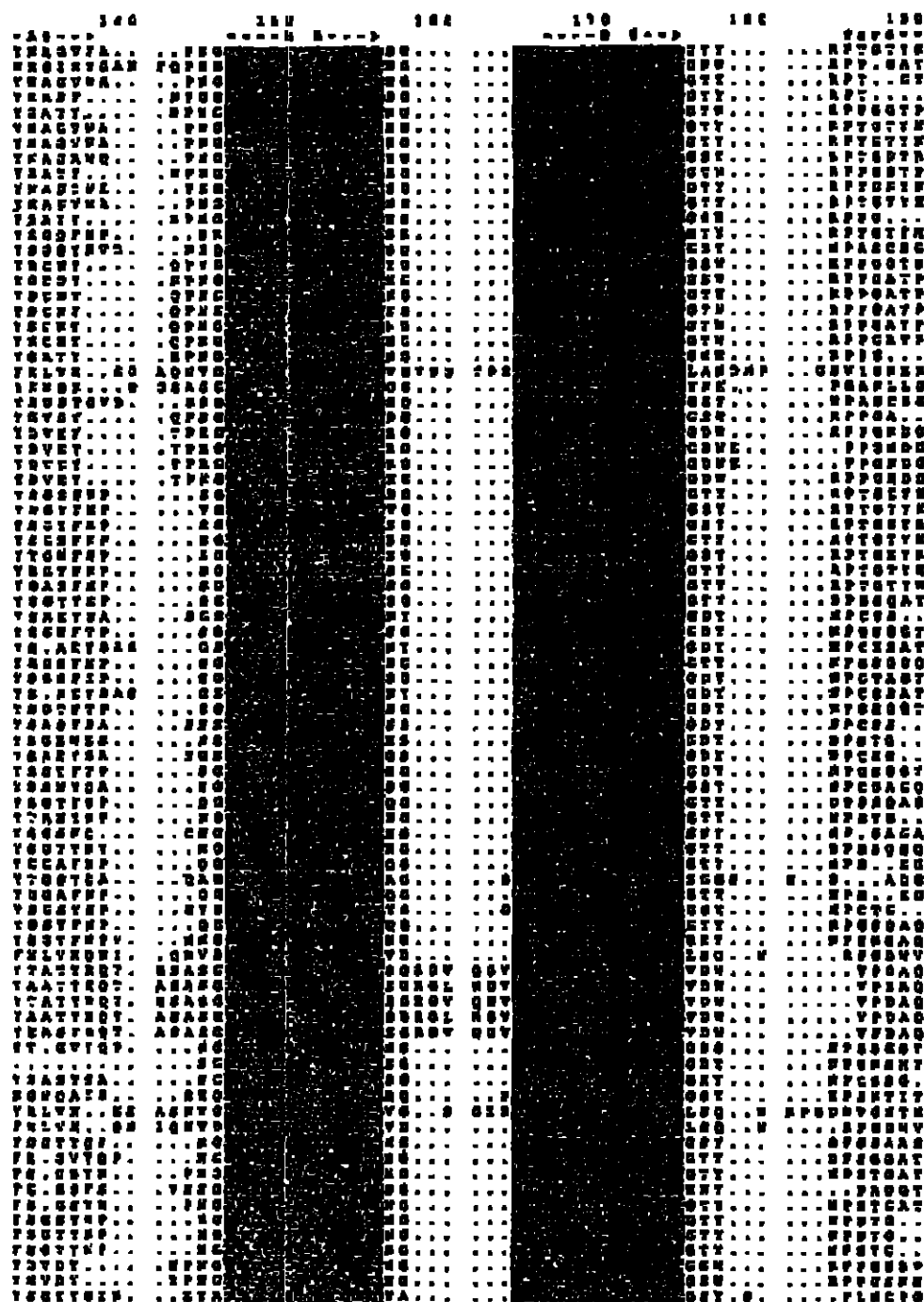


Fig. 1. (Continued)

177

	238	239	240	241	242	243	244	245	246	247	248	249	250
1 No. 01.01.01	U	U	U	U	U	U	U	U	U	U	U	U	U
2 No. 01.01.02	U	U	U	U	U	U	U	U	U	U	U	U	U
3 No. 01.01.03	U	U	U	U	U	U	U	U	U	U	U	U	U
4 No. 01.01.04	U	U	U	U	U	U	U	U	U	U	U	U	U
5 No. 01.01.05	U	U	U	U	U	U	U	U	U	U	U	U	U
6 No. 01.01.06	U	U	U	U	U	U	U	U	U	U	U	U	U
7 No. 01.01.07	U	U	U	U	U	U	U	U	U	U	U	U	U
8 No. 01.01.08	U	U	U	U	U	U	U	U	U	U	U	U	U
9 No. 01.01.09	U	U	U	U	U	U	U	U	U	U	U	U	U
10 No. 01.01.10	U	U	U	U	U	U	U	U	U	U	U	U	U
11 No. 01.01.11	U	U	U	U	U	U	U	U	U	U	U	U	U
12 No. 01.01.12	U	U	U	U	U	U	U	U	U	U	U	U	U
13 No. 01.01.13	U	U	U	U	U	U	U	U	U	U	U	U	U
14 No. 01.01.14	U	U	U	U	U	U	U	U	U	U	U	U	U
15 No. 01.01.15	U	U	U	U	U	U	U	U	U	U	U	U	U
16 No. 01.01.16	U	U	U	U	U	U	U	U	U	U	U	U	U
17 No. 01.01.17	U	U	U	U	U	U	U	U	U	U	U	U	U
18 No. 01.01.18	U	U	U	U	U	U	U	U	U	U	U	U	U
19 No. 01.01.19	U	U	U	U	U	U	U	U	U	U	U	U	U
20 No. 01.01.20	U	U	U	U	U	U	U	U	U	U	U	U	U
21 No. 01.01.21	U	U	U	U	U	U	U	U	U	U	U	U	U
22 No. 01.01.22	U	U	U	U	U	U	U	U	U	U	U	U	U
23 No. 01.01.23	U	U	U	U	U	U	U	U	U	U	U	U	U
24 No. 01.01.24	U	U	U	U	U	U	U	U	U	U	U	U	U
25 No. 01.01.25	U	U	U	U	U	U	U	U	U	U	U	U	U
26 No. 01.01.26	U	U	U	U	U	U	U	U	U	U	U	U	U
27 No. 01.01.27	U	U	U	U	U	U	U	U	U	U	U	U	U
28 No. 01.01.28	U	U	U	U	U	U	U	U	U	U	U	U	U
29 No. 01.01.29	U	U	U	U	U	U	U	U	U	U	U	U	U
30 No. 01.01.30	U	U	U	U	U	U	U	U	U	U	U	U	U
31 No. 01.01.31	U	U	U	U	U	U	U	U	U	U	U	U	U
32 No. 01.01.32	U	U	U	U	U	U	U	U	U	U	U	U	U
33 No. 01.01.33	U	U	U	U	U	U	U	U	U	U	U	U	U
34 No. 01.01.34	U	U	U	U	U	U	U	U	U	U	U	U	U
35 No. 01.01.35	U	U	U	U	U	U	U	U	U	U	U	U	U
36 No. 01.01.36	U	U	U	U	U	U	U	U	U	U	U	U	U
37 No. 01.01.37	U	U	U	U	U	U	U	U	U	U	U	U	U
38 No. 01.01.38	U	U	U	U	U	U	U	U	U	U	U	U	U
39 No. 01.01.39	U	U	U	U	U	U	U	U	U	U	U	U	U
40 No. 01.01.40	U	U	U	U	U	U	U	U	U	U	U	U	U
41 No. 01.01.41	U	U	U	U	U	U	U	U	U	U	U	U	U
42 No. 01.01.42	U	U	U	U	U	U	U	U	U	U	U	U	U
43 No. 01.01.43	U	U	U	U	U	U	U	U	U	U	U	U	U
44 No. 01.01.44	U	U	U	U	U	U	U	U	U	U	U	U	U
45 No. 01.01.45	U	U	U	U	U	U	U	U	U	U	U	U	U
46 No. 01.01.46	U	U	U	U	U	U	U	U	U	U	U	U	U
47 No. 01.01.47	U	U	U	U	U	U	U	U	U	U	U	U	U
48 No. 01.01.48	U	U	U	U	U	U	U	U	U	U	U	U	U
49 No. 01.01.49	U	U	U	U	U	U	U	U	U	U	U	U	U
50 No. 01.01.50	U	U	U	U	U	U	U	U	U	U	U	U	U
51 No. 01.01.51	U	U	U	U	U	U	U	U	U	U	U	U	U
52 No. 01.01.52	U	U	U	U	U	U	U	U	U	U	U	U	U
53 No. 01.01.53	U	U	U	U	U	U	U	U	U	U	U	U	U
54 No. 01.01.54	U	U	U	U	U	U	U	U	U	U	U	U	U
55 No. 01.01.55	U	U	U	U	U	U	U	U	U	U	U	U	U
56 No. 01.01.56	U	U	U	U	U	U	U	U	U	U	U	U	U
57 No. 01.01.57	U	U	U	U	U	U	U	U	U	U	U	U	U
58 No. 01.01.58	U	U	U	U	U	U	U	U	U	U	U	U	U
59 No. 01.01.59	U	U	U	U	U	U	U	U	U	U	U	U	U
60 No. 01.01.60	U	U	U	U	U	U	U	U	U	U	U	U	U
61 No. 01.01.61	U	U	U	U	U	U	U	U	U	U	U	U	U
62 No. 01.01.62	U	U	U	U	U	U	U	U	U	U	U	U	U
63 No. 01.01.63	U	U	U	U	U	U	U	U	U	U	U	U	U
64 No. 01.01.64	U	U	U	U	U	U	U	U	U	U	U	U	U
65 No. 01.01.65	U	U	U	U	U	U	U	U	U	U	U	U	U
66 No. 01.01.66	U	U	U	U	U	U	U	U	U	U	U	U	U
67 No. 01.01.67	U	U	U	U	U	U	U	U	U	U	U	U	U
68 No. 01.01.68	U	U	U	U	U	U	U	U	U	U	U	U	U
69 No. 01.01.69	U	U	U	U	U	U	U	U	U	U	U	U	U
70 No. 01.01.70	U	U	U	U	U	U	U	U	U	U	U	U	U
71 No. 01.01.71	U	U	U	U	U	U	U	U	U	U	U	U	U
72 No. 01.01.72	U	U	U	U	U	U	U	U	U	U	U	U	U
73 No. 01.01.73	U	U	U	U	U	U	U	U	U	U	U	U	U
74 No. 01.01.74	U	U	U	U	U	U	U	U	U	U	U	U	U
75 No. 01.01.75	U	U	U	U	U	U	U	U	U	U	U	U	U
76 No. 01.01.76	U	U	U	U	U	U	U	U	U	U	U	U	U
77 No. 01.01.77	U	U	U	U	U	U	U	U	U	U	U	U	U
78 No. 01.01.78	U	U	U	U	U	U	U	U	U	U	U	U	U
79 No. 01.01.79	U	U	U	U	U	U	U	U	U	U	U	U	U
80 No. 01.01.80	U	U	U	U	U	U	U	U	U	U	U	U	U
81 No. 01.01.81	U	U	U	U	U	U	U	U	U	U	U	U	U
82 No. 01.01.82	U	U	U	U	U	U	U	U	U	U	U	U	U

Fig. 1. (Continued)

Table 2

Isoelectric point, pI and T° optimum of family 11 xylanases of known sequence

Organism	Xylanase	pI	pH optimum	Optimum T°	Reference
Bacteria					
<i>Aeromonas caviae</i>	Xylanase I	7.1	7.0	55	Kubota et al. (1992)
<i>Bacillus agrachaeus</i>	xyl 11	8.8	n.d.	n.d.	Sabini et al. (1999)
<i>Bacillus pumilus</i>	XYNA	n.d.	6.5	45–60	Pambourand et al. (1983)
<i>Bacillus</i> sp. D3	Xylanase	7.7	6.0	75	Harris et al. (1997)
<i>Bacillus</i> sp. 41 M1	Xylanase J	5.5	9.0	50	Nakamura et al. (1992)
<i>Bacillus subtilis</i>	Xylanase A	8.9	n.d.	n.d.	Paice et al. (1986)
<i>Caldicellulosigraptus</i> sp. R169B.1	Xylanase	n.d.	5.5	70	Morris et al. (1999)
<i>Clostridium acetobutylicum</i>	Xylanase B	8.5	5.5–6	60	Lee et al. (1987)
<i>Clostridium sarcoarum</i>	XynA	4.5	7.0	75	Sakka et al. (1994)
<i>Clostridium thermocellum</i>	XynA	n.d.	6.5	65	Hayashi et al. (1999)
<i>Dicetoglomus thermophilum</i>	Xylanase B	n.d.	6.5	85	Morris et al. (1998)
<i>Fibrobacter succinogenes</i>	XynC	6.2	6.5	n.d.	Paradis et al. (1993)
<i>Ruminococcus flavigenicus</i>	XYLA	5.0	5.5	50	Flint et al. (1991)
<i>Streptomyces halidus</i>	XlnB	8.4	6.5	55	Knapfel et al. (1990)
<i>Streptomyces halidus</i>	XlnC	>10.25	6.0	57	Knapfel et al. (1992)
<i>Streptomyces</i> sp. EC3	Xylanase	9.1	n.d.	n.d.	Milly-Servais et al. (1996)
<i>Streptomyces</i> sp. E38	XylI	9.8	6.0–6.5	55–60	Geerts et al. (2000)
<i>Streptomyces thermotolerans</i>	STX-II	8.0	7.0	60	Tajima et al. (1992)
<i>Thermomonaspora favae</i>	ThA	10	7.0	n.d.	Irwin et al. (1994)
Fungi					
<i>Aspergillus awamori</i>	EXLA	3.7	n.d.	n.d.	Hanning et al. (1994)
<i>Aspergillus kawachi</i>	XynC	3.5	2.0	50	Ito et al. (1992)
<i>Aspergillus nidulans</i>	X23	6.4	5.5	62	Fernández-Espinar et al. (1993)
<i>Aspergillus nidulans</i>	X24	3.5	5.5	52	Fernández-Espinar et al. (1996)
<i>Aspergillus niger</i>	Xyl A (or I)	3.7	3.0	n.d.	Mant et al. (1992)
<i>Aspergillus niger</i>	XynB	n.d.	5.0	n.d.	Krengel and DeBoura (1996)
<i>Aspergillus subglauco</i>	XYLA	3.6	n.d.	n.d.	Kinoshita et al. (1993)
<i>Aureobasidium pullulans</i>	XynA (APX II)	9.4	4.8	54	de Graaff et al. (1994)
<i>Cochliobolus carbonum</i>	XylI	>9.3	4.0–8.0	45	Li et al. (1998)
<i>Cochliobolus carbonum</i>	Xyl2 and 3	>9.3	5.0	n.d.	Holden and Walton (1992)
<i>Cryptococcus</i> sp. S-2	Xyn-CS2	7.4	2.0	40	Holden and Walton (1992)
<i>Magnaporthe grisea</i>	XYN22	9.7	n.d.	n.d.	Irfuji et al. (1996)
<i>Panellomyces varioti</i>	PVX	3.9	5.5–7.0	65	Wu et al. (1993)
<i>Penicillium</i> sp. 40	XynA	4.7	2.0	50	Krishnamurthy and Vidyasathil (1989)
<i>Penicillium purpuroscentum</i>	XynB	5.9	3.5	50	Kimura et al. (2000)
<i>Schizophyllum commune</i>	Xylanase A	4.5	5.0	50	Behnke et al. (1993)
<i>Thermomyces lanuginosus</i>	XynA	4.1	6.5	65	Jurack and Paice (1988)
<i>Trichoderma harzianum</i> E58	20 kD xylanase	9.4	5.0	50	Gomez et al. (1993)
<i>Trichoderma reesei</i>	XYN1	5.2	3.5–4	n.d.	Schlechter et al. (1996)
<i>Trichoderma reesei</i>	XYN2	9.0	4.5–5.5	n.d.	Wong and Saddler (1992)
<i>Trichoderma viride</i>	Xylanase IIA	9.3	5.0	55	Törönen et al. (1992)

n.d.: not determined.

180

Table 3

Family 11 endoxylanases of known three-dimensional structure

Organism	Protein	PDB code	Reference
Bacteria			
<i>Bacillus agaradhaerens</i>	Xylase ^a	1qk6	Sabini et al. (1999)
<i>Bacillus circulans</i>	XLNA	1xmb	Walsby et al. (1994a)
<i>Bacillus</i> sp. D3	Xylase ^a		Harris et al. (1997)
<i>Dietzoglomer thermophilum</i>	XynB ^a	1daj	McCarthy et al. (2000)
Fungi			
<i>Aspergillus kawachi</i>	XynC	1bkl	Fushinobu et al. (1998)
<i>Aspergillus niger</i>	XyII or A	1ukr	Krangel and Dijkstra (1996)
<i>Penicillium variot</i>	Xylase ^a	1pvx	Kumar et al. (2000)
<i>Thermomyces lanuginosus</i>	XynA ^a	1yna	Gruber et al. (1998)
<i>Trichoderma harzianum</i> B58	Xylase ^a	1xad	Campbell et al. (1993)
<i>Trichoderma reesei</i>	XYN I	1xya	Törönen and Kouvola (1995)
<i>Trichoderma reesei</i>	XYN II	1xyp	Törönen et al. (1994)

^a Thermophilic protein.

package (MSI, San Diego) or the FSSP program (Holm and Sander, 1996). In addition, the use of GLUSTAL to globally align the regions inserted between these anchor points allows to highlight several key residues in less conserved regions. In short, the alignment obtained on the basis of sequence similarity also reflects structural similarities among the endoxylanases.

The alignment is shown in Fig. 1. The residues are numbered on top; the numbering exceeds the length of any individual sequence since it includes all gaps assigned by the alignment. The secondary structure elements are numbered as shown in Fig. 2.

Four boxes (shaded in Fig. 1) are found along the whole set of 82 sequences and correspond to the PSCRs. Interestingly, the sequence alignment provides a pattern of aligned segments and gaps consistent with the observed secondary structure elements. Indeed, the lowest sequence homology is found in the regions between beta strands (residues 94–103 between A3 and B3; residues 111–130 between B3 and A5; residues 238–254 between B7 and A6). The region corresponding to the thumb, (between strands B7 and B8) is, however, very well conserved along all the sequences, except for *Phromyces* sp. XYLA. This last coenzyme is reported to be the inactive form of the protein, in contrast to *Phromyces* sp. XYLB, the active

form, that also contains the conserved segment defining the thumb region. The cord region (between B6 and B9) is also conserved. The PSCR's, in particular, correspond to secondary structure elements: box 1 to B5, box 2 to B6, box 3 to B8 and box 4 to the helix.

The amino terminal regions of the aligned sequences show no similarities in the first 30 or so residues. One sequence is particularly long in this region (*C. acstobutyllicum*). One of the sequences (*P. multivesiculatum*, AB011274) starts only at residue 81, and lacks strands B1, B2 and part of A2, suggesting that B1 and B2 may not be necessary for enzymatic activity. Residue 40 is a G in 60 sequences, and is missing in 15. It belongs to B1 in the structure of *T. reesei* XYN II (Törönen et al., 1994). Its importance is unclear, since B1 is missing totally or in part in the indicated 15 sequences.

The segment corresponding to B2 shows good similarity among the sequences. Position 52 is in most cases an aromatic residue: 63 sequences have a Y, while 14 show a W or a F. At position 55, W predominates (70 sequences), while 62 sequences have a D in position 57. Between B2 and A2, two long insertions (probably forming a loop) are found in the endoxylanases from *Penicillium* sp. and *Pichia stipitis*. 50 G's are observed in position 76 and 50 in position 77. Strand A2 shows low similarity except at position 81, where 24 Y and

PC
181

35 M are found. In the loop linking A2 and A3, 61 G's are observed in position 86 and 67 in position 87, but not necessarily together in the same sequence. In A3, a highly conserved F or Y (79 sequences) is found in position 89 and 75 W in 93; in this last case, the few replacements are F or Y; only one non-conserved replacement (H) is observed in *P. stipitis*.

Residue 100 is in all cases a D (27) or N (65); as we will see below, this residue is related to the pH optimum of the enzyme. At the end of B3, G109 is present in 79 sequences (it is replaced by a K in *Bacillus agaradhaerens*) and allows a special twist to the chain (Törönen and Rouvinen, 1997).

A5 is not present in *P. stipitis*, which shows a big gap between 110 and 144; in this sequence, B3 and B5 are probably linked by a short loop. B5

corresponds to the first box detected by MATCHBOX, indicating a highly conserved stretch of the sequence, particularly YGW (152–154), present in all 82 enzymes. Y152 is strongly hydrogen bonded to E167; it seems to be of great functional importance since its mutation to F (in *Bacillus circulans* XLNA) leads to a totally inactive enzyme (Wakarchuk et al., 1994a). At position 150 21 Cys are present, the highest number found at a particular location in all the sequences (see below).

The next box corresponds to B6. It is preceded by a highly conserved Pro at 164 (present in 74 sequences) and Leu 165 (74 sequences). E167 is common to all sequences and corresponds to one of the catalytic residues (the nucleophile); it is followed by two highly conserved Y's (75 in position 168, replaced in the rest of the enzymes by F;

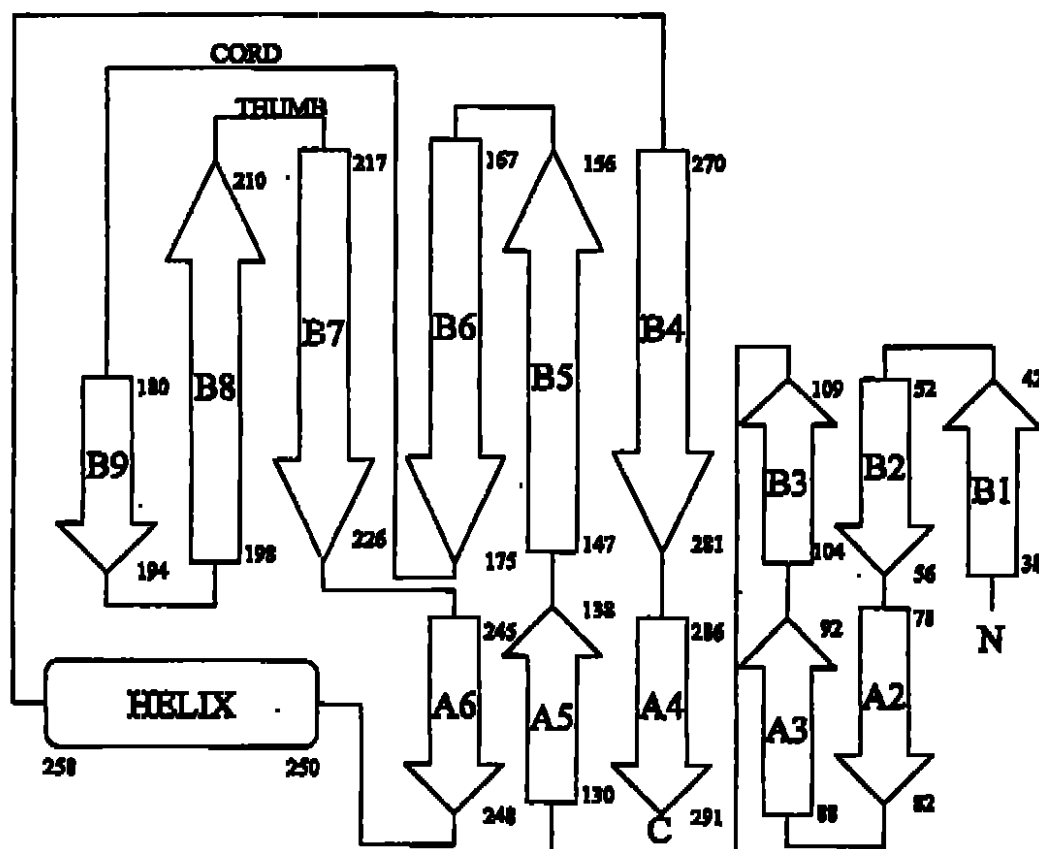


Fig. 2. Topology diagram of the family 11 α-D-glucosidases. The residue numbering refers to that of Fig. 1. The figure is adapted from Törönen et al. (1994).

23
182

and common to all in 169) and by two aliphatic hydrophobic residues in positions 170 and 171. Tyr 169 links both catalytic glutamates, acting as a charge stabilizing residue (Törrönen and Rouvinen, 1997), and its mutation to F in XLNA of *B. circulans* leads to a large decrease in activity (Wakarchuk et al., 1994a).

The 'cord' shows low similarity, except for a Pro (found in 70 sequences and missing in 7) in position 185. B9 is a stretch of very low similarity and varies considerably in length, but it is followed by the third high similarity box, corresponding mainly to B8. Between B9 and B8, two highly conserved residues are apparent: D204 (in 76 sequences) and G205 (in 80 sequences); this last residue is considered important in hairpin formation (Törrönen and Rouvinen, 1997). Y208 is present in all 82 sequences, R215 in 72 and N217 in 69 (this last residue is missing in 8 sequences).

The 'thumb' is well conserved; it has a consensus sequence PSIXG where X is almost any residue and the others show few and mainly homologous replacements. Pro 219 gives a twist to the strand at the beginning of the thumb. B7 is also well conserved; residue 227 is a T in 77 sequences and 228 is F in 81 cases; a consensus sequence QYWSVR can be proposed for residues 230–235 (R is replaced by K in one instance and the other residues show mainly conservative replacements).

The loop connecting B7 and A6 differs in length; the longest being in the enzyme from *P. stipitis*, which also shows the longest loop between B2 and A2. No significant similarities are apparent in A6, which is immediately followed by the helix.

The last box of high similarity comprises the carboxyl end of the α -helix and the following 6–7 residues. H262 is present in 79 sequences, W263 in 80 and G270 in 79.

B4 does not show high similarities, except for E287 (boldface in Fig. 1), a residue present in all sequences, which corresponds to the acid-base glutamate participating in catalysis, and which is followed by a highly conserved G (70 sequences). The last well defined secondary structure is A4. A highly conserved consensus sequence SSGS precedes and starts this strand.

The carboxyl terminus of the sequences is highly variable in length. The longest is found in *B.*

agaradhaerens and *P. multivacuatum*, stretching 25 and 22 residues, respectively, beyond the end of A4.

3.2. Acidophily and thermostability

A number of family 11 endoxylanases have been reported to be acidophilic. It has been postulated that for xylanases that function optimally under acidic conditions, residues spatially adjacent to the acid/base catalytic glutamate influence the pH optimum (Törrönen and Rouvinen, 1995). In particular, the substitution of N100 (underlined in Fig. 1) by D shifts the pH optimum from 5.7 to 4.6, as has been demonstrated by mutational, kinetic, and structural studies of N100D in *B. circulans* XYLA (Joshi et al., 2000). This agrees with the mutational analysis of xylanase C of *A. kawachii*, in which the single substitution of Asp 100 to Asn at this key position dramatically elevates its pH optimum from 2 to 5 (Fushinobu et al., 1998). Structural studies of xylanase A (or I) from *A. niger* led to a similar conclusion. In the crystal structure of this enzyme of low pH optimum (Krengel and Dijkstra, 1996), Asp 100 is assigned a critical role.

An examination of the enzymes of known sequence for which the pH optimum has been determined (Table 2), shows that this correlation holds true with only one exception. The enzymes showing a pH optimum below 5 have D at position 100, while N is present in those with pH optima of 5 or more. The exception is XynC from *F. succinogenes*, an anaerobic bacterium from the rumen. This enzyme shows a pH optimum of 6.5 but has a D at position 100. This enzyme has two catalytic domains, and the pH optimum reported in Table 2 corresponds to the native enzyme; the separate domains have a pH optimum of 6.0 (Zhu et al., 1994).

In conclusion, there is a strong correlation in that the residue hydrogen bonded to the general acid/base catalyst at position 100 is asparagine in the so-called 'alkaline' xylanases, whereas it is aspartic acid in those with a more acidic pH optimum.

Thermostability is an important issue in the properties of endoxylanases, due to their biotechnological applications, particularly in cellulose biobleaching. Of the enzymes listed in Table 2,

seven of bacterial origin (*Bacillus* D3, *Caldicellulosiraptor* sp., *Clostridium stercorarium*, *Clostridium thermocellum*, *Dictyoglomus thermophilus*, *Streptomyces thermotolaceus* and *Thermomonospora fusa*) and two from fungi (*Paeclomyces varioti* and *Thermomyces lanuginosus*) are considered thermophilic, based on their optimal temperature and stability at high temperature. Table 3 shows that the three-dimensional structure of four of these enzymes has been determined.

Thermophilicity and thermostability may be explained by a variety of factors and structural parameters (Kumar et al., 2000). Of those, the importance of S–S bridges and aromatic 'sticky patches' can be analyzed by sequence alignment. Additional structural features potentially involved in thermal stability, such as salt bridges, aromatic interactions and entropic effects have been postulated in family 11 xylanases (Georis et al., 2000).

Cysteine residues are not very common in these enzymes. Of the total number of residues in the 82 sequences listed in Table 1, only 117 are Cys, a 0.7%. Twenty-seven of the sequences have no Cys and 14 have only one; therefore, at least half of the total possesses no disulfide bridges. The three-dimensional structure shows that an S–S bridge is found in xylanase A from *A. niger* connecting Cys 186 to Cys 211, thus attaching the cord to the large beta-sheet (B8) (Krengel and Dijkstra, 1996). These two half-cysteine residues are conserved in a few other fungal family 11 xylanases (*A. kawachi*, *Aspergillus awamori*, *A. niger* Xyn5, *Aspergillus tubingensis*, *Cryptococcus* sp. and *Penicillium purpurogenum*). The xylanases from the bacteria *Cellvibrio mixtus* and *Pseudomonas fluorescens* and of the insect *Phaedon cochleariae* have Cys at positions 188 and 213, which may be forming a similar bridge. The presence of this disulfide bridge may influence the stability of these proteins, although it does not give (at least to the enzymes analyzed so far) a thermophilic character.

Of the seven thermophilic xylanases listed, three have no cysteines, and the three-dimensional structure of the *Dictyoglomus thermophilus* enzyme shows no S–S bonds, although its sequence has 3 Cys. On the other hand, *T. lanuginosus* and

P. varioti xylanases, both thermophilic, do have an S–S bridge linking Cys 203 (located in B9) and Cys 261 (in the α helix). *Cochliobolus carbonum* Xyl3 and *Schizophyllum commune* XylA also possess only two Cys and in the same positions; the T_m optimum of the former has not been reported, while the latter has a value of only 50 °C (Table 2). All these results suggest that S–S bridges are unlikely to be of importance in the thermophilicity of family 11 xylanases.

As pointed out by Turunen et al. (2001), thermophilicity (activity at high temperature) and thermostability do not necessarily depend on the same structural factors. They introduced a disulfide bridge plus other minor mutations in *T. reesei* XynII (C203–C261) significantly increasing the thermostability without affecting the temperature optimum. Wakarchuk et al. (1994b) have introduced an S–S bridge at the same position in *B. circulans* xylanase, obtaining similar results. Thus, the introduction of S–S bridges to enzymes may affect both properties differently.

Harris et al. (1997) propose that the *Bacillus* D3 xylanase (lacking S–S bridges) is stabilized through 'sticky patches' between pairs of molecules through the interaction of surface aromatic residues. These residues are Tyr 53, 78, 120, 201, 290, 295 and Trp 207 and 214. When the alignment is analyzed, it shows that Tyr 78, 120 and Trp 207 are unique to this sequence (no aromatic residues are present in those positions in the remaining 81 sequences), while Trp 214 is shared by only 1 sequence (not thermophilic) and no other aromatic residues are found in this location. The remaining four residues show no clear alignment pattern. Therefore, it can be concluded that the 'sticky patch' pattern described for the *Bacillus* D3 xylanase is very unique, and it is unlikely to play an important role in stabilizing the other thermophilic family 11 xylanases of known sequence.

Shibuya et al. (2000) using random gene shuffling between a mesophilic (*S. lividans* XlnB) and a thermophilic (*T. fusa* TfxA) enzyme have shown the importance of the amino terminal segment of the protein in temperature stability. However, if the first 50 residues (our numbering) of the thermophilic enzymes in this study are aligned, no

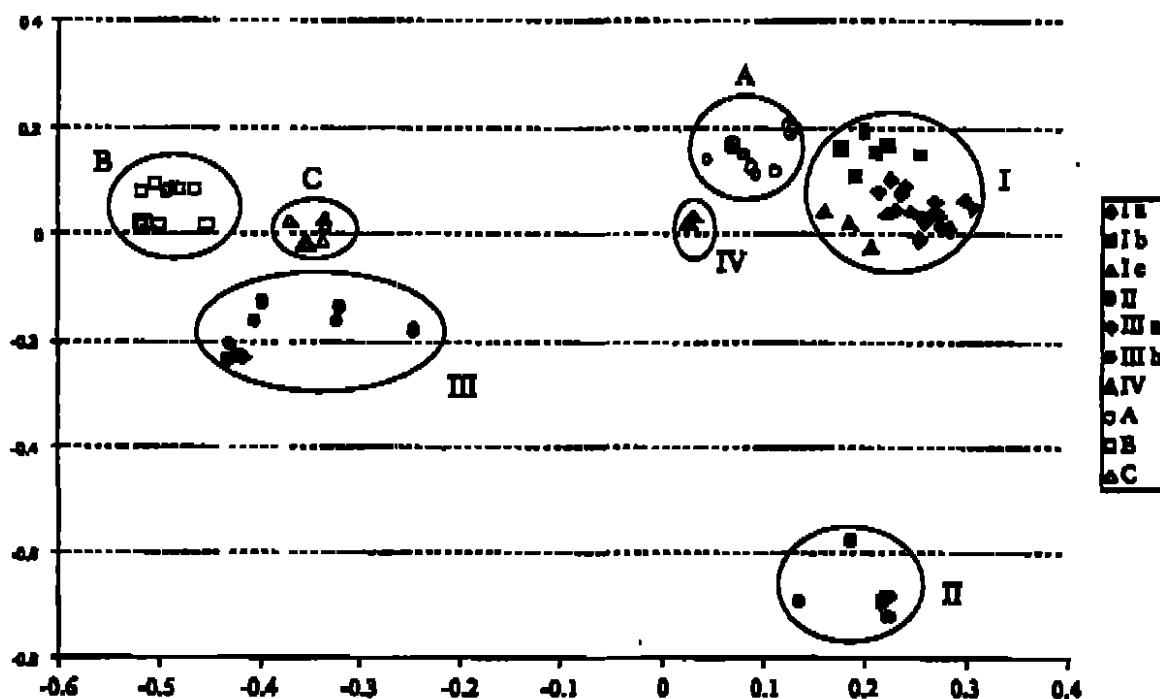
25
184

Fig. 3. Results from the factor analysis. Graphical representation of the sequences in the plane of factors 2 and 3. Each point corresponds to a sequence, the position on the plot being associated to a percentage of the total variability within the data set. Distances separating each position in the plot are directly related to the sequence homology. Groups containing mainly sequences of fungi (I, II, III, IV) are marked by filled symbols.

clear similarity pattern is observed, indicating that, again, the proposed thermostabilizing factor may be unique to the *T. fusca* coxyme.

3.3. Classification of the family 11 xylanases

A factor analysis (Explore subprogram of MATCHBOX) was performed in order to define groups and subgroups among the 32 sequences under study. Sequences have been represented in a three-dimensional space, each factor being associated to a percentage of the total variability between the sequences. The first factor is generally trivial, and the grouping of the sequences was performed in the plane of factors 2 and 3 (Deplereux and Feytmans, 1992). Graphical representation of the sequences in the plane of factors 2 and 3 (Fig. 3) was obtained directly from the program MATCHBOX and represented via Excel. The distances separating each position in the plot are directly related to the sequence homology, thus allowing a

classification. Sequences close in the plane of factors 2 and 3 were grouped/clustered, leading to the classification presented in Table 4. A first clustering, based on the distance between coordinates in the plane of factors 2 and 3, led to the definition of a total of seven groups. Those groups were labeled (A, B, C, I, II, III, IV) *a posteriori*, on the basis of the origin (fungal or bacterial) of the sequences belonging to them. The two larger groups (I and III), were further divided into subgroups on the basis of the distance between coordinates in the plane of factors 2 and 3 within the group. Group B, although containing many sequences (13) was not divided into subgroups as the distribution of points is narrow (factor 2 comprised between -0.453 and -0.518 ; factor 3 comprised between 0.015 and 0.087) in comparison to the distribution of, for example, group III ($-0.491 < \text{factor 2} < -0.240$; $-0.236 < \text{factor 3} < -0.161$).

This method avoids affecting the sequence classification by misalignments occurring in a previous

26
185

step, and represents the sequences on a plane taking into account Euclidian distance rather than hierarchical merging. The graphical representation of the eigenvectors (sequence coordinates) in the plane of factors 2 and 3 (Fig. 3) allows to classify and group the sequences by eye, since the distances separating each position in the plot are directly related to differences in the sequences. The classification obtained has been validated by a clustering (Ward method) performed on the sequence coordinates.

Interestingly, the groups obtained from the factor analysis are highly homogeneous, meaning that they mostly contain only sequences from either bacteria or fungi. It is also noteworthy that some bacterial sequences are more homologous to

sequences of fungal endoxylanases than to sequences of other bacteria (see for example group I).

In this work a robust classification methodology based on factor analysis, without previous sequence alignment and without reference to any phylogenetic inference is applied for the first time to classify a large set of sequences. Interestingly, the classification of xylanases presented in Table 4 compares favorably with previous classifications based on smaller sequence datasets. In particular, our results are in good agreement with the classification presented by Georis et al. (1999) deduced from a phylogenetic tree analysis on 63 sequences. According to their study, family 11 endoxylanases were subdivided into six main groups: three for

Table 4
Classification of family 11 xylanases based on a factor analysis

Ia	<i>Tr.re.XYNI</i>	II	<i>Aa.m.XyIA</i>	A	<i>Ca.f.XYLD</i>
Ia	<i>Tr.re.XYNII</i>	II	<i>Pe.pa.XynB</i>	A	<i>Th.f.XynA</i>
Ia	<i>Tr.vi.XIA</i>	II	<i>Aa.m.XYLA</i>	A	<i>St.p.SM</i>
Ia	<i>Tr.vi.XIB</i>	II	<i>Aa.m.Xyn5</i>	A	<i>St.h.STH</i>
Ia	<i>Th.h.XynA</i>	II	<i>Aa.h.XynC</i>	A	<i>St.B.XnC</i>
Ia	<i>Pa.sp.XynA</i>	II	<i>Aa.w.ECLA</i>	A	<i>Ba.c.l.m6</i>
Ia	<i>Pa.vi.lpx</i>	II	<i>An.pa.XyIA</i>	A	<i>St.p.EC3</i>
Ia	<i>Ch.gr.CgXB</i>			A	<i>St.h.XnB</i>
Ia	<i>Asp.p.xyl</i>	IIIa	<i>Na.f.XY3A</i>	A	<i>Ba.m.xylA</i>
Ia	<i>As.or.XyOI</i>	IIIa	<i>Na.f.XY3B</i>	A	<i>Ba.p.xylA</i>
Ia	<i>As.sil.XM</i>	IIIa	<i>Na.pa.XYA1</i>		
Ia	<i>Aa.h.xylB</i>	IIIa	<i>Na.pa.XYA2</i>	C	<i>D.h.xylB</i>
Ia	<i>Tr.ha.hmd</i>	IIIa	<i>Orgn.XynA</i>	C	<i>Ra.f.XynB</i>
Ia	<i>As.sil.XC2</i>			C	<i>Ra.f.XynD</i>
Ia	<i>Ma.gr.XY22</i>	IIIb	<i>Fl.m.XyCA</i>	C	<i>Ca.p.xyl</i>
Ia	<i>Co.m.XylI</i>	IIIb	<i>Fl.m.XyCB</i>	C	<i>Ra.p.XylI</i>
Ia	<i>Ch.gr.CgXA</i>	IIIb	<i>Fl.sp.XYA1</i>	C	<i>Ra.f.XynA</i>
Ia	<i>As.tu.xylB</i>	IIIb	<i>Fl.sp.XYA2</i>		
Ia	<i>Aa.m.XynB</i>	IIIb	<i>Na.f.xyl2</i>	B	<i>Ba.g.l.g6</i>
Ib	<i>Co.m.Xyl2</i>	IV	<i>Co.m.Xyl3</i>	B	<i>Ra.d.XynA</i>
Ib	<i>Cl.pa.Xyl</i>	IV	<i>Fl.at.XynA</i>	B	<i>Pa.m.POX</i>
Ib	<i>Co.m.xyl</i>			B	<i>Pa.m.xylA</i>
Ib	<i>Sc.co.xylA</i>			B	<i>Clac.xylB</i>
Ib	<i>Hu.h.XylI</i>			B	<i>Ba.p.xylU</i>
Ib	<i>Ba.D3.BDX</i>			B	<i>Ba.p.xylY</i>
Ic	<i>Ca.m.XYLA</i>			B	<i>Cl.h.xylV</i>
Ic	<i>Pa.f.XYLE</i>			B	<i>Cl.h.xylA</i>
Ic	<i>Ph.co.xyl</i>			B	<i>Cl.h.xylA</i>
				B	<i>Cl.h.xylB</i>
				B	<i>Cl.h.xylU</i>
				B	<i>Ba.pa.XYNA</i>

Sequence of bacterial xylanases are in *italics* and xylanases produced by protozoa or insects are underlined. For abbreviations see Table 1 and Fig. 1.

fungi, two for Gram positive bacteria and one for Gram negative bacteria.

A similar subdivision is found here: groups I, II and III contain mainly fungal enzymes. The enzymes in groups I and II are mostly the 20 kDa enzymes from *Ascomycota* and *Basidiomycota*. Most enzymes of group I exhibit a basic pI. Those in group II show an acidic pI. Enzymes of group III are mainly produced by anaerobic fungi. Like in the classification proposed by Georis et al. (1999) two enzymes produced by *F. saccharigenes* (XyCA and XyCB), an anaerobic Gram-negative bacterium, also fall in this group.

In addition to those three groups associated mainly to fungal enzymes, a fourth group that was not present in previous classifications emerges from the present factor analysis. It only contains two enzymes. One is produced by *C. carboxum* (Xyl3) and is clearly distinct from Xyl1 and Xyl2 produced by the same fungus. In the classification proposed by Georis et al. (1999), this enzyme is isolated in the unrooted phylogenetic tree. The second enzyme in group IV is produced by *P. stipitis* (XynA); this enzyme has not been included in any previous classification.

Bacterial enzymes are mainly divided into three groups (A, B, C). Group A contains mainly enzymes produced by members of the *Actinomycetaceae* and the *Bacillaceae* families, strictly aerobic Gram-positive bacteria. Groups B and C are more closely related and contain mainly enzymes from anaerobic Gram-positive bacteria, such as those from *Clostridium* or *Ruminococcus*, which usually live in the rumen. Two bacterial enzymes, XylE and XylA xylanases from *P. fluorescens* and *C. mixtus*, respectively, strictly aerobic Gram-negative bacteria, are found in subgroup Ic. In terms of sequence similarities, those two Gram-negative bacterial enzymes more closely resemble xylanases produced by fungi (group I) than other Gram-positive bacterial enzymes (e.g. groups A, B, C). They were also classified in a distinct group by Georis et al. (1999).

3.4. Biotechnological significance

Xylanases are finding an increasing number of applications, both alone and in combination with other enzymes. Among them are cellulose pulp

biobleaching (Buchert et al., 1994), bread-making (Courtin et al., 1999) and saccharification of lignocellulosic biomass (Lee, 1997). These applications require enzymes capable of operating under specific and often unnatural conditions. Parameters of particular interest are thermostability and pH optimum. Biobleaching, for instance, requires thermostable and alkali-stable enzymes. Family 11 xylanases may be of particular interest in biobleaching due to their smaller size; a fact which may facilitate penetration in the cellulose fiber network.

Optimizing enzyme properties for a particular application can be achieved by random and site-directed mutagenesis. Aruse et al. (1993) have achieved a significant stabilization of *Bacillus pumilus* XynA by random mutagenesis. Four heat-resistant mutants were isolated, and the stabilizing mutations were found to be clustered in the N-terminal region. In three of the four mutants, a mutation of G92 to S or to D was observed. Residue 92 is located in the A3 strand, and it is always a polar or charged residue except in *B. pumilus* and *Penicillium* sp. where it is a G (Fig. 1); the introduction of an S or a D at that position may allow the formation of a stabilizing hydrogen bond.

An example of the use of site-directed mutagenesis is given by Turunen et al. (2001). They have introduced a disulfide bridge by means of the mutations S203C–N261C, thus increasing significantly the half-life of the enzyme at 65°. As shown in Fig. 1, the thermophilic xylanase from *T. lanuginosus* possesses this bridge.

It has proved difficult to manipulate the properties of an enzyme in a predictable manner (Törönen and Rouvinen, 1997). However, for the design and protein engineering of endoxylanases with properties suitable for their different applications, a good knowledge of its sequence and structure is a necessary condition. The information and analysis presented in this publication should be useful for this purpose.

Acknowledgements

This work was financed in part by grants from DIPUC (Dirección de Investigación y Postgrado,

28
187

Pontificia Universidad Católica de Chile) and FONDECYT grants 1960241, 3950015 and 8990004 (Líneas Complementarias).

References

- Apel, P.C., Passalunghi, D.G., Holden, F.R., Walton, J.D., 1993. Cloning and targeted gene disruption of *XYL1*, a β -1,4-xylosidase gene from the maize pathogen *Cochliobolus carbonum*. *Molec. Plant Microbe Interact.* 6, 467–473.
- Apel-Mitchell, P.C., Walton, J.D., 1996. Cloning, disruption and expression of two endo- β 1,4-xylosidase genes, *XYL2* and *XYL3* from *Cochliobolus carbonum*. *Appl. Environ. Microbiol.* 62, 4129–4135.
- Arai, A., Yomo, T., Urabe, I., Hata, Y., Katube, Y., Okada, H., 1993. Stabilization of xylanase by random mutagenesis. *FEMS Lett.* 116, 123–127.
- Belandier, A., Scurpa, J., Pékano, A., Dine, R., Stinner, J., Byzantine, J., 1995. *Penicillium purpurescens* produces several xylanases: purification and properties of two of the enzymes. *J. Biotechnol.* 41, 71–79.
- Bertrand, L., Vertommen, D., Depierreux, E., Hua, L., Rider, M.H., Feytmans, E., 1997. Modelling the 2-kinase domain of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase on adenylate kinase. *Biochem. J.* 321, 615–621. erratum appears in *Biochem. J.* 323 (1997) 864.
- Bertrand, L., Vertommen, D., Freeman, J.M., Wouters, J., Depierreux, E., Di Pietro, A., Hua, L., Rider, M.H., 1998. Mutagenesis of the fructose-6-phosphate-binding site in the 2-kinase domain of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. *Eur. J. Biochem.* 254, 490–496.
- Bisby, P., 1985. Microbial xylanolytic systems. *Trends Biotechnol.* 3, 286–290.
- Bisby, P., Vránská, M., Teekman, M., Klusfeld, D., 1997. Endo- β -1,4-xylosidase families: differences in catalytic properties. *J. Biotechnol.* 57, 151–166.
- Briffault, P., Bandoux, G., Lambert, C., De Bolle, X., Vinals, C., Feytmans, E., Depierreux, E., 1998. Comparative analysis of seven multiple protein sequence alignment servers: clues to enhance reliability of predictions. *Bioinformatics* 14, 357–366.
- Buchert, J., Teekman, M., Konttinen, A., Viikari, L., 1994. Application of xylanases in the pulp and paper industry. *Bioresour. Technol.* 50, 65–72.
- Campbell, R., Rose, D., Wakarchuk, W., To, R., Sung, W., Yaguchi, M., 1993. A comparison of the structures of the 20 kD xylanases from *Trichoderma harzianum* and *Bacillus circulans*. In: Snoutmeier, P., Reinikainen, T. (Eds.), *Proceedings of the Second TRICEL Symposium on Trichoderma reesei Cellulases and other Hydrolyses*. Espoo, Finland, pp. 68–72.
- Cho, S., Choi, Y., 1995. Nucleotide sequence analysis of an endo-xylanase gene (*xynA*) from *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* 5, 117–124.
- Conrín, C.M., Roelants, A., Delcor, J.A., 1999. Fractionation-reconstitution experiments provide insight into the role of endoxylanases in bread-making. *J. Agric. Food Chem.* 47, 1870–1877.
- Dalhög, H., Heldt-Hansen, H.P., 1994. A novel method for efficient expression cloning of fungal enzyme genes. *Mol. Gen. Genet.* 243, 253–260.
- De Bolle, X., Vinals, C., Prud'homme, D., Piquet, J.-Y., Leprieux, E., Vandenhaute, J., Feytmans, E., 1995. Identification of residues potentially involved in the interactions between subunits in yeast alcohol dehydrogenase. *Eur. J. Biochem.* 231, 214–219.
- de Fays, E., Tibor, A., Lambert, C., Vinals, C., Dussol, P., De Bolle, X., Wouters, J., Lecomte, J.J., Depierreux, E., 1999. Structure and function prediction of the *Brassica oleracea* P39 protein by comparative modeling with marginal sequence similarities. *Prot. Eng.* 12, 217–223.
- de Groot, L.H., van den Broek, H.C., van Ooijen, A.J.J., Vlaser, J., 1994. Regulation of the xylanase-encoding *xlnA* gene of *Aspergillus nidulans*. *Mol. Microbiol.* 12, 479–490.
- Depierreux, E., Feytmans, E., 1991. Simultaneous and multivariate alignment of protein sequences. Correspondence between physico-chemical profiles and structurally conserved regions (SCR's). *Prot. Eng.* 4, 605–613.
- Depierreux, E., Feytmans, E., 1992. MATCH-box: a fundamentally new algorithm for the simultaneous alignment of several protein sequences. *Comp. App. Biosci.* 8, 501–509.
- Depierreux, E., Bandoux, G., Briffault, P., Ragusa, I., De Bolle, X., Vinals, C., Feytmans, E., 1997. Match-Box server: a multiple sequence alignment tool placing emphasis on reliability. *Comp. App. Biosci.* 13, 249–256.
- Díaz, R., Sepag, A., Pékano, A., Stinner, J., Byzantine, J., 1997. Cloning, sequencing and expression of the cDNA of endoxylanase B from *Penicillium purpurescens*. *Gene* 187, 247–251.
- Durand, R., Rancie, C., Fèvre, M., 1996. Molecular characterization of *xyn 3*, a member of the endoxylanase multigene family of the rumen anaerobic fungus *Nasostomatia fructosa*. *Curr. Genet.* 30, 531–540.
- Enami, K., Hase, K., 2001. Characterization of a xylanase gene from *Cochliobolus carbonum* and its expression. *Mycol. Res.* 105, 352–359.
- Fanouti, C., Pong, T., Black, G.W., Huxleywood, G.P., Gilbert, H.J., 1995. The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *J. Biol. Chem.* 270, 29314–29322.
- Fernández-Espinar, M.T., Piñaga, F., Sainz, P., Ramón, D., Vallés, S., 1993. Purification and characterization of a neutral endoxylanase from *Aspergillus nidulans*. *FEMS Microbiol. Lett.* 113, 223–228.
- Fernández-Espinar, M.T., Vallés, S., Piñaga, F., Pérez-González, J.A., Ramón, D., 1996. Construction of an *Aspergillus nidulans* multicopy transformant for the *xlnB* gene and its use in purifying the minor *X₂₄* xylanase. *Appl. Microbiol. Biotechnol.* 45, 338–341.

76
189

- Flint, H.J., McPherson, C.A., Martin, J., 1991. Expression of two xylanase genes from the rumen cellulolytic bacterium *Ruminococcus flaviglutinis* cloned in pUC13. *J. Gen. Microbiol.* 137, 123–129.
- Flint, H.J., Martin, J., McPherson, C.A., Daniel, A.S., Zhang, J.-X., 1993. A bifunctional enzyme with separate xylanase and β -(1,3-1,4) glucanase domains, encoded by the *xynD* gene of *Ruminococcus flaviglutinis*. *J. Bacteriol.* 175, 2943–2951.
- Fukuzaki, R., Panbangred, W., Shimmyo, A., Okada, H., 1994. The complete nucleotide sequence of the xylanase gene (*xynA*) of *Bacillus pumilus*. *FEBS Lett.* 171, 197–201.
- Fukudaoka, S., Ito, K., Kameo, M., Wakagi, T., Matsuzawa, H., 1998. Crystallographic and mutational analysis of an extremely acidophilic and acid-stable xylanase: Biased distribution of acidic residues and importance of Asp 37 for catalysis at low pH. *Prot. Eng.* 11, 1121–1128.
- García-Campayo, V., McCrea, S., Zhang, J.-X., Flint, H.J., Wood, T.M., 1993. Mode of action, kinetic properties and physicochemical characterization of two different domains of a bifunctional (1 $\rightarrow$ 4)- β -D-xylanase from *Ruminococcus flaviglutinis* expressed separately in *Escherichia coli*. *Biochem. J.* 296, 233–243.
- García, J., Giamotita, F., Lamotte-Braesser, J., Devreux, B., van Beestum, J., Grunier, B., Frire, J.M., 1999. Sequence, overproduction and purification of the family 11 endo-beta-1,4-xylanase encoded by the *xyl1* gene of *Streptomyces* sp. 338. *Gene* 237, 123–133.
- García, J., de Lencastre, F., Lamotte-Braesser, J., Bougnat, V., Devreux, B., Giamotita, F., Grunier, B., Frire, J.M., 2000. An additional aromatic interaction improves the thermostability and thermophilicity of a mesophilic family 11 xylanase: structural basis and molecular study. *Prot. Sci.* 9, 466–475.
- Gleason, S., Lapping, H.B., Tenberge, K.B., Tudyanski, P., 1998. The xylanolytic system of *Clostridium perfringens*: cytological evidence for secretion of xylanase in infected rye tissue and molecular characterization of two xylanase genes. *Phytopathology* 88, 1020–1030.
- Gilbert, H.J., Haslewood, G.P., Lancia, J.I., Orpin, C.G., Xue, G.P., 1992. Homologous catalytic domains in a rumen fungal xylanase: evidence for gene duplication and prokaryotic origin. *Mol. Microbiol.* 6, 2065–2072.
- Gilks, N.R., Hemmick, B., Kilburn, D.G., Miller, R.C. Jr., Warren, R.A.J., 1991. Domains in microbial β -1,4-glucanase: sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55, 303–315.
- Gomes, I., Gomes, I., Kreiner, W., Esterbauer, H., Stiner, M., Steiner, W., 1993. Production of high level of cellulase-free and thermostable xylanase by a wild strain of *Thermomyces longimanus* using beechwood xylan. *J. Biotechnol.* 30, 283–297.
- Gruber, K., Khotimskiy, G., Hayn, M., Schleicher, A., Steiner, W., Krafky, C., 1998. Thermophilic xylanase from *Thermomyces longimanus*: high-resolution X-ray structure and modeling studies. *Biochemistry* 37, 13475–13485.
- Harris, G.W., Jenkins, J.A., Connerton, I., Pickering, R.W., 1996. Refined crystal structure of the catalytic domain of xylanase A from *Pseudomonas fluorescens* at 1.8 Å resolution. *Acta Crystallog. Sec. D* 52, 393–401.
- Harris, G.W., Pickering, R.W., Connerton, I., Deleire, P., Toward, J.-P., Bruton, C., Pérez, S., 1997. Structural basis of the properties of an industrially relevant thermophilic xylanase. *Protein* 29, 77–86.
- Hayashi, H., Takahara, M., Hattori, T., Kimura, T., Karita, S., Sakita, K., Ohmura, K., 1999. Nucleotide sequences of two contiguous and highly homologous xylanase genes *xynA* and *xynB* and characterization of XynA from *Clostridium thermocellum*. *Appl. Microbiol. Biotech.* 51, 348–357.
- Hemmick, B., Davies, G., 1997. Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.* 7, 637–644.
- Hending, J.O.M., van Rotterdam, C.O., Verbeek, J.M.A., Rozema, M., Mast, J., van Gorkom, R.F.M., van den Hondel, C.A.M.J.J., 1994. Isolation and characterization of a 1,4- β -endoxylanase gene of *Aspergillus niger*. *Curr. Genet.* 26, 228–232.
- Higgins, D.G., Blessey, A.J., Fuchs, R., 1992. CLUSTAL V: improved software for multiple sequence alignment. *CABIOS* 8, 189–191.
- Hummel, M.E., Karypis, P.A., Sakon, J., Adney, W.E., Baker, J.O., Thomas, E.R., 1997. Polysaccharide hydrolase folds: Diversity of structure and convergence of function. *Appl. Biochem. Biotechnol.* 63–65, 313–323.
- Holden, R., Walton, J.D., 1992. Xylanases from the fungal maize pathogen *Cochliobolus carbonum*. *Phycol. Mol. Plant Pathol.* 30, 39–47.
- Hohn, L., Sander, C., 1994. Mapping the protein universe. *Science* 273, 595–603.
- Ishij, H., Chino, M., Kato, M., Imura, Y., 1996. Acid xylanase from yeast *Cryptococcus* sp. 5-2: purification, characterization, cloning and sequencing. *Biochem. Biotechnol.* 60, 1331–1338.
- Irwin, D., Jung, E.D., Wilson, D.B., 1994. Characterization and sequence of a *Thermomonospora favae* xylanase. *Appl. Environ. Microbiol.* 60, 743–770.
- Ito, K., Ogasawara, H., Sugimoto, T., Ishikawa, T., 1992. Purification and properties of acid stable xylanases from *Aspergillus kawachi*. *Biochem. Biotechnol. Biochem.* 56, 347–350.
- Ito, K., Iwahata, K., Iwano, K., 1992a. Cloning and sequencing of the *xynC* genes encoding acid xylanase of *Aspergillus kawachi*. *Biochem. Biotechnol. Biochem.* 56, 1338–1340.
- Jaong, K.I., Lee, P.C., Park, I.Y., Kim, M.S., Kim, S.C., 1998. Molecular cloning and characterization of an endoxylanase gene of *Bacillus* sp. in *Escherichia coli*. *Braz. J. Microb. Technol.* 21, 599–605.
- Jondani, J.P., Cowpat, J., Ruel, K., 1992. Chemical structure of xylans and their interaction in the plant cell walls. In: Vlasar, J., Soldman, G., Knitzer-Van Someren, M.A., Voragen, A.G.J. (Eds.), *Xylans and Xylanases*. Elsevier, Amsterdam, pp. 1–15.

28
189

- Joshi, M.D., Sidim, G., Pot, L., Beayer, G.D., Withers, S.G., McIntosh, L.P., 2000. Hydrogen bonding and catalytic: a novel explanation for how a single amino acid substitution can change the pH optimum of a glycosidase. *J. Mol. Biol.* 299, 255–279.
- Juzsak, L., Palcs, M.G., 1988. Xylanase A of *Schizophyllum commune*. *Meth. Enzymol.* 160, 659–662.
- Kimura, T., Kitamoto, N., Kito, Y., Karita, S., Sakita, K., Ohmura, K., 1998. Molecular cloning of xylanase gene *xynG1* from *Aspergillus oryzae* KEN 616, a shoyu koji mold, and analysis of its expression. *J. Ferment. Bioeng.* 85, 10–16.
- Kimura, T., Ito, J., Kawano, A., Makino, T., Kondo, H., Kachi, S., Sakita, K., Ohmura, K., 2000. Purification, characterization and molecular cloning of acidophilic xylanase from *Pseudomonas* sp. 40. *Biochem. Biotechnol. Biochem.* 64, 1230–1237.
- Kinochita, K., Takano, M., Kozaki, T., Ito, K., Iwano, K., 1995. Cloning of the *xynB* gene encoding xylanase B from *Aspergillus niger* and its expression in *Aspergillus kawachi*. *J. Ferment. Bioeng.* 79, 422–428.
- Knapfel, D., Vats-Mehta, S., Aumont, F., Sharck, F., Morosoli, R., 1990. Purification and characterization of a new xylanase (xylanase B) produced by *Streptomyces halicus* 66. *Biochem. J.* 267, 45–50.
- Knapfel, D., Daignault, N., Morosoli, R., Sharck, F., 1992. Purification and characterization of a new xylanase (xylanase C) produced by *Streptomyces halicus*. *Appl. Microbiol. Biotechnol.* 36, 626–631.
- Kruegel, U., Dijkstra, B.W., 1996. Three-dimensional structure of endo-1,4- β -xylanase I from *Aspergillus niger*: molecular basis for its low pH optimum. *J. Mol. Biol.* 263, 70–78.
- Krishnamurthy, S., Vithayathil, P.J., 1989. Purification and characterization of endo-1,4- β -xylanase from *Pseudomonas variol* Bailer. *J. Ferment. Bioeng.* 67, 77–82.
- Kubota, B.K., Horita, H., Kawai, K., Takamizawa, K., Suzuki, T., 1992. Xylanase I of *Aeromonas caviae* MB-1 isolated from the intestine of a herbivorous insect (*Semiothisa pyralis*). *Biochem. Biotechnol. Biochem.* 56, 1463–1464.
- Kubota, B.K., Suzuki, T., Ito, Y., Naito, H., Kawai, K., Takamizawa, K., Horita, H., 1997. Cloning and expression of xylanase I gene (*xynA*) of *Aeromonas caviae* MB-1 in *Escherichia coli*. *J. Ferment. Bioeng.* 85, 292–295.
- Kumar, P.R., Sivarajamurthy, S., Vithayathil, P.J., Viswanatha, M.A., 2000. The tertiary structure at 1.99 Å resolution and the proposed amino acid sequence of a family-11 xylanase from the thermophilic fungus *Pseudomonas variol* Bailer. *J. Mol. Biol.* 295, 581–593.
- La Grange, D.C., Pretorius, L.S., van Zyl, W.H., 1996. Expression of a *Trichoderma reesei* β -xylanase gene (XYN2) in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 62, 1036–1044.
- Lee, J., 1997. Biological conversion of lignocellulosic biomass to ethanol. *J. Biotechnol.* 56, 1–24.
- Lee, S.F., Forsberg, C.W., Ratnay, J.B., 1987. Purification and characterization of two endoxylanases from *Chaetomium acetobutylicum* ATCC 824. *Appl. Environ. Microbiol.* 53, 644–650.
- Li, X.L., Zhang, Z.Q., Dean, J.F., Erickson, K.E., Ljungdahl, L.G., 1993. Purification and characterization of a new xylanase (AFX-II) from the fungus *Aureobasidium pullulans* Y-2311-1. *Appl. Environ. Microbiol.* 59, 3212–3218.
- Li, X.L., Ljungdahl, L.G., 1994. Cloning, sequencing, and regulation of a xylanase gene from the fungus *Aureobasidium pullulans* Y-2311-1. *Appl. Environ. Microbiol.* 60, 5160–5166 published online in: *Appl. Environ. Microbiol.* 60 (1994) 4647.
- Li, X.L., Chen, H.Z., Ljungdahl, L.G., 1997. Mesocentric and polycentric anaerobic fungi produce structurally related cellulases and xylanases. *Appl. Environ. Microbiol.* 63, 628–635.
- Löbbeck, P.D., Panfil, L., Degraf, Y., Löbbeck, M., Alekhina, I., Bulat, S.A., Collinge, D.B., 1997. PCR cloning, DNA sequencing and phylogenetic analysis of a xylanase gene from the phytopathogenic fungus *Acremonium pin* Lb. *Physiol. Mol. Plant Pathol.* 51, 377–389.
- Mast, J., Rom, M., Verbeke, J., Stam, H., Santos da Silva, M.J., Bono, M., Egmond, M.R., Hagenaars, M.L.D., van Gerven, R.F.M., Henning, J.G.M., van den Hondel, C.A.M.J.J., van Rotterdam, C.O., 1992. Xylanases and their application in bakery. In: Visser, J., Beldman, G., Kusters-Van Someren, M.A., Voragen, A.G.J. (Eds.), *Xylans and Xylanases*. Elsevier, Amsterdam, pp. 349–360.
- Mary-Servais, C., Maron, A., Gerard, C., Dourst, J., 1994. Cloning and nucleotide sequence of a xylanase-encoding gene from *Streptomyces* sp. strain EC3. *DNA Sequence* 6, 147–158.
- McCarthy, A.A., Morris, D.D., Bergquist, P.L., Baker, E.N., 2000. Structure of XynB, a highly thermostable β -1,4-xylanase from *Dictyoglomer thermophilum* RM46B.1, at 1.8 Å resolution. *Acta Cryst. D* 56, 1366–1375.
- Millward-Sadler, S.J., Poole, D.M., Howland, R., Haskewood, G.P., Clarke, J.H., Gilbert, H.J., 1994. Evidence for a general role for high-affinity non-catalytic cellulose binding domains in microbial plant cell wall hydrolases. *Mol. Microbiol.* 11, 375–382.
- Millward-Sadler, S.J., Davidson, K., Haskewood, G.P., Black, G.W., Gilbert, H.J., Clarke, J.H., 1995. Novel cellulose-binding domains, NodB homologues and conserved modular architecture in xylanases from the aerobic soil bacteria *Pseudomonas fluorescens* subsp. *cellulosa* and *Cellulibacter* *subsp.* *Biochem. J.* 312, 39–48.
- Morris, D.O., Gibbs, M.D., Chiu, C.W.J., Koh, M.H., Wong, K.K.Y., Allison, R.W., Nelson, P.J., Bergquist, P.L., 1998. Cloning of the *xynB* gene from *Dictyoglomer thermophilum* Rt 46 B.1 and action of the gene product on kraft pulp. *Appl. Environ. Microbiol.* 64, 1759–1765.
- Morris, D.O., Gibbs, M.D., Ford, M., Thomas, J., Bergquist, P.L., 1999. Family 10 and 11 xylanase genes from *Caldwellia* *sp.* strain Rt46B.1. *Extremophiles* 3, 103–111.

cat
190

- Nagashima, M., Okumoto, Y., Okazaki, M., 1989. Nucleotide sequence of the gene of extracellular xylanase in *Streptomyces* sp. No.36 a and construction of secretion vectors using xylanase gene. *Trends Biotechnol.* 9, 91–94.
- Nakai, R., Wakabayashi, K., Asano, T., Aono, R., Horikoshi, K., Nakamura, S., 1994. Nucleotide sequence and mutational analysis of the gene encoding the novel alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M-1. *Nucleic Acids Symp. Ser.* 31, 235–236.
- Nakamura, S., Aono, R., Wakabayashi, K., Horikoshi, K., 1992. Alkaline xylanase produced by newly isolated alkaliphilic *Bacillus* sp. In: Visser, J., Heldman, G., Kusters-Van Sommeren, M.A., Voragen, A.G.J. (Eds.), *Xylans and Xylanases*. Elsevier, Amsterdam, pp. 443–446.
- Nieland, H., Engelbrecht, J., Brunk, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Prot. Eng.* 10, 1–6.
- Oka, T., Roy, C., Watson, D.C., Wakarchuk, W., Campbell, R., Yaguchi, M., Jurasek, L., Pake, M.G., 1993. Amino acid sequence and thermostability of xylanase A from *Schizophyllum commune*. *FEBS Lett.* 334, 296–300.
- Pake, M.G., Bombacinski, R., Desrochers, M., Jurasek, L., Yaguchi, M., 1986. A xylanase gene from *Bacillus subtilis*: nucleotide sequence and comparison with *Bacillus pumilus* gene. *Arch. Microbiol.* 144, 201–206.
- Prestegard, W., Shimmyo, A., Kinnabla, S., Okada, H., 1983. Purification and properties of endoxylanase produced by *Bacillus pumilus*. *Agric. Biol. Chem.* 47, 957–963.
- Paradis, F.W., Zhu, H., Krell, P.J., Phillips, J.P., Forsberg, C.W., 1993. The xynC gene from *Fibrobacter succinogenes* S85 codes for a xylanase with two similar catalytic domains. *J. Bacteriol.* 175, 7666–7672.
- Perez-Gonzalez, J.A., de Graaf, L.H., Visser, J., Ramon, D., 1994. Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes. *Appl. Environ. Microbiol.* 61, 2179–2182.
- Sabini, R., Schenck, G., Dauter, M., Dauter, Z., Jorgensen, P.L., Schalko, M., Dupont, C., Davies, G.J., Wilson, K.S., 1999. Catalysis and specificity in enzymatic glycoside hydrolysis: a ^3D conformation for the glycosyl-enzyme intermediate revealed by the structure of the *Bacillus agaradhaerens* Shafly 11 xylanase. *Chem. Biol.* 6, 483–492.
- Sakita, K., Kojima, Y., Kondo, T., Karita, S.-I., Ohtsuka, K., Shimada, K., 1993. Nucleotide sequence of the *Clavibacter thermophilus* xynA gene encoding xylanase A: identification of catalytic and cellulose binding domains. *Biochem. Biotechnol.* 57, 273–277.
- Sakita, K., Kojima, Y., Kondo, T., Karita, S.-I., Shimada, K., Ohtsuka, K., 1994. Purification and characterization of xylanase A from *Clavibacter thermophilus* F-9 and a recombinant *E. coli*. *Biochim. Biotechnol. Biochem.* 58, 1496–1505.
- Schleicher, A., Holmann, K., Hayn, M., Sauer, W., Schwab, H., 1994. Cloning and characterization of the gene from the thermostable xylanase XynA from *Thermomyces lanuginosus*. *J. Biotechnol.* 40, 211–218.
- Shareck, F., Roy, C., Yaguchi, M., Morosoli, R., Knapp, D., 1991. Sequences of three genes specifying xylanases in *Streptomyces* *sp.* Genes 107, 75–82.
- Shibuya, H., Kaneko, S., Hayashi, K., 2000. Enhancement of the thermostability and hydrolytic activity of xylanase by random gene shuffling. *Biochem. J.* 349, 651–656.
- Suma, A., Antranikian, G., 1997. Xylanolytic enzymes from fungi and bacteria. *Crit. Rev. Biotechnol.* 17, 39–67.
- Törönen, A., Mach, R.L., Messner, R., Gonsky, R., Kallén, N., Harkid, A., Kuback, C.P., 1992. The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. *Bio/Technol.* 10, 1461–1465.
- Törönen, A., Harkid, A., Rouvinen, J., 1994. Three-dimensional structure of endo-1,4- β -xylanase II from *Trichoderma reesei*: two conformational states in the active site. *EMBO J.* 13, 2493–2501.
- Törönen, A., Rouvinen, J., 1995. Structural comparison of two major endo-1,4-xylanases from *Trichoderma reesei*. *Biochemistry* 34, 847–854.
- Törönen, A., Rouvinen, J., 1997. Structural and functional properties of low molecular weight endo-1,4- β -xylanase J. *Biotechnol.* 57, 137–149.
- Tsujibo, H., Miyamoto, K., Kudo, T., Minami, K., Sakamoto, T., Hasegawa, T., Isumori, Y., 1992. Purification, properties and partial amino acid sequences of thermostable xylanase from *Streptomyces thermotolerans* OPC-520. *Appl. Environ. Microbiol.* 58, 371–375.
- Tsujibo, H., Ohtsuka, T., Ho, T., Yamanaka, I., Miyamoto, K., Sugiyama, M., Isumori, Y., 1997. Cloning and sequence analysis of genes encoding xylanases and xylanase xylanase from *Streptomyces thermotolerans* OPC-520. *Appl. Environ. Microbiol.* 63, 661–664.
- Turunen, O., Brunk, K., Fenei, P., Vekmannper, J., Wu, X., Rouvinen, J., Leisola, M., 2001. A combination of weakly stabilizing mutations with a disulfide bridge in the α -helix region of *Trichoderma reesei* endo-1,4- β -xylanase II increases the thermal stability through synergism. *J. Biotechnol.* 88, 37–46.
- Vinik, C., De Bolla, X., Deplacit, E., Feytmans, E., 1995. Knowledge-based modelling of the D-lactate dehydrogenase three-dimensional structure. *Prot. Struct. Funct. Genet.* 21, 307–318.
- Wakarchuk, W.W., Campbell, R.L., Sung, W.L., Davoodi, J., Yaguchi, M., 1994a. Mutational and crystallographic analysis of the active site residues of the *Bacillus circulans* xylanase. *Protein Sci.* 3, 467–475.
- Wakarchuk, W.W., Sung, W.L., Campbell, R.L., Cummings, A., Watson, D.C., Yaguchi, M., 1994b. Thermostabilization of the *Bacillus circulans* xylanase by the introduction of disulfide bonds. *Prot. Eng.* 7, 1379–1386.
- Wong, K.K.Y., Saddler, J.N., 1992. *Trichoderma* xylanases, their properties and application. *Crit. Rev. Biotechnol.* 12, 413–435.
- Wu, S.-C., Kaufman, S., Durvill, A.G., Albersheim, P., 1995. Purification, cloning and characterization of two xylanases from *Magnaporthe grisea*, the rice blast fungus. *Mol. Plant-Microbe Interact.* 8, 506–514.

191

- Yaguchi, M., Roy, C., Ujda, M., Watson, D.C., Wakarchuk, W., 1992a. Amino acid sequence of the low-molecular-weight xylanase from *Trichoderma viride*. In: Visser, J., Beidman, G., Kusters-Van Sommeren, M.A., Voragen, A.G.J. (Eds.), *Xylans and Xylanases*. Elsevier, Amsterdam, pp. 149–154.
- Yaguchi, M., Roy, C., Watson, D.C., Rollin, F., Tan, L.U.L., Senior, D.J., Saddler, N., 1992b. The amino acid sequence of the 20KD xylanase from *Trichoderma harzianum* B58. In: Visser, J., Beidman, G., Kusters-Van Sommeren, M.A., Voragen, A.G.J. (Eds.), *Xylans and Xylanases*. Elsevier, Amsterdam, pp. 435–438.
- Yang, R.C.A., Mackenzie, C.R., Narang, S.A., 1988. Nucleotide sequence of a *Bacillus circulans* xylanase gene. *Nucleic Acids Res.* 16, 7187.
- Yoshino, S., Oishi, M., Moriyama, R., Kato, M., Taniyoshi, N., 1994. Two family G xylanase genes from *Chaetomium gracile* and their expression in *Aspergillus nidulans*. *Curr. Genet.* 29, 73–80.
- Yu, J.H., Park, Y.S., Yum, D.Y., Kim, J.M., Kong, I.S., Bai, D.H., 1993. Nucleotide sequence and analysis of a xylanase gene (*xynS*) from alkali-tolerant *Bacillus* sp. YA-14 and comparison with other xylanases. *J. Microbiol. Biotechnol.* 3, 139–145.
- Zappa, H., Jones, W.A., Woods, D.R., 1990. Nucleotide sequence of a *Clavibacter acetabulariae* P262 xylanase gene (*xynS*). *Nucleic Acids Res.* 18, 2179.
- Zhang, J.-X., Flint, H.J., 1992. A bifunctional xylanase encoded by the *xynA* gene of the rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 comprises two dissimilar domains linked by an asparagine/glutamine-rich sequence. *Mol. Microbiol.* 6, 1013–1023.
- Zhang, J.-X., Martin, J., Flint, H.J., 1994. Identification of non-catalytic conserved regions in xylanases encoded by the *xynB* and *xynD* genes of the cellulolytic rumen anaerobe *Ruminococcus flavefaciens*. *Mol. Gen. Genet.* 249, 260–264.
- Zhu, H., Paradise, F.W., Krill, P.J., Phillips, J.P., Forsberg, C.W., 1994. Enzymatic specificities and modes of action of the two catalytic domains of the XynC xylanase from *Fibrobacter succinogenes* S83. *J. Bacteriol.* 176, 3885–3894.

Acidophilic adaptation of family 11 endo- β -1,4-xylanases: Modeling and mutational analysis

FRÉDÉRIC DE LEMOS ESTEVES,¹ VIRGINIE RUELLE,² JOSETTE LAMOTTE-BRASSEUR,¹ BIRGIT QUINTING,³ AND JEAN-MARIE FRÈRE¹

¹Centre d'Ingénierie des Protéines, Institut de Chimie, B6a, and ²Laboratoire de Spectrométrie de Masse, Université de Liège, Sart Tilman, B-4000 Liège, Belgium

³Centre d'Ecologie Evolutive et Fonctionnelle, Division Immunologie Animale, Maricla, B-6900, Belgium

(Received December 10, 2003; Final Revision January 22, 2004; Accepted February 1, 2004)

Abstract

Xyl1 from *Streptomyces* sp. S38 belongs to the low molecular mass family 11 of endo- β -1,4-xylanases. Its three-dimensional structure has been solved at 2.0 Å and its optimum temperature and pH for enzymatic activity are 60°C and 6.0, respectively. *Aspergillus kawachi* xylanase XynC belongs to the same family but is an acidophilic enzyme with an optimum pH of 2.0. Structural comparison of Xyl1 and XynC showed differences in residues surrounding the two glutamic acid side chains involved in the catalysis that could be responsible for the acidophilic adaptation of XynC. Mutations W20Y, N48D, A134E, and Y193W were introduced by site-directed mutagenesis and combined in multiple mutants. Trp 20 and Tyr 193 are involved in substrate binding. The Y193W mutation inactivated Xyl1 whereas W20Y decreased the optimum pH of Xyl1 to 5.0 and slightly increased its specific activity. The N48D mutation also decreased the optimum pH of Xyl1 by one unit. The A134E substitution did not induce any change, but when combined with N48D, a synergistic effect was observed with a 1.4 unit decrease in the optimum pH. Modeling showed that the orientations of residue 193 and of the fully conserved Arg 131 are different in acidophilic and "alkaline" xylanases whereas the introduced Tyr 20 probably modifies the pK_a of the acid-base catalyst via residue Asn 48. Docking of a substrate analog in the catalytic site highlighted striking differences between Xyl1 and XynC in substrate binding. Hydrophobicity calculations showed a correlation between acidophilic adaptation and a decreased hydrophobicity around the two glutamic acid side chains involved in catalysis.

Keywords: endo- β -1,4-xylanase; acidophilicity; site-directed mutagenesis; structural analysis; hydrophobicity; docking

Xylan, the major constituent of hemicellulose, is a heterogeneous polysaccharide consisting of a β -1,4-linked D-xylane backbone substituted to varying degrees with *O*-acetyl, α -L-arabinofuranosyl, 4-*O*-methylglucuronic acid groups or α -1,2-linked glucuronic acids (Singh et al. 2003). Its complete breakdown requires a set of enzymes among which

endo- β -1,4-xylanases (EC 3.2.1.8) are crucial for depolymerization into xyloligomers of various lengths. Xylanases are widely used in many industrial applications such as pulp biobleaching in the paper industry, baking, saccharification of lignocellulosic biomass, wine making, and fruit juice clarification (Sunna and Antranikian 1997; Wouters et al. 2001). On the basis of amino acid sequence similarities and hydrophobic cluster analysis, xylanases have been classified into families 10 and 11 of glycoside hydrolases although psychrophilic xylanases have been recently discovered and classified into family 8 of glycoside hydrolases (Wong et al. 1988; Gilkes et al. 1991; Hemsley

Reprint requests to Jean-Marie Frère, Centre d'Ingénierie des Protéines, Institut de Chimie, B6a, Université de Liège, Sart Tilman, B-4000 Liège, Belgium; e-mail: jmf@ulb.ac.be; fax 00-32-4-346-33-64.

Article and publication are at <http://www.protein-science.org/cgi/full/10.1110/j.1093-4548.2004.0036104>.

234
193

and Davies 1997; Collins et al. 2002). Family 10 xylanases and some cellulases exhibit molecular masses higher than 30 kD and (α/β)₂ barrel folds whereas the M_r values of family 11 are generally lower and their structures consist of a single catalytic domain with a sandwichlike fold. This fold resembles a partly closed right hand composed of two β -sheets and one α helix, which form a large cleft where 3 to 7 xylane subunits can be accommodated (Torronen and Rouvinen 1995; Gruber et al. 1998). Family 11 members are highly similar; they show closely related three-dimensional structures and catalytic site geometries (Torronen et al. 1993; Torronen and Rouvinen 1997). Catalysis occurs by a double displacement mechanism whereby the anomeric configuration of the glycosidic oxygen is retained. It is thought to involve two conserved glutamic acids, functioning, respectively, as nucleophile and as general acid-base catalyst. However, the optimum pH is highly variable within this family and xylanase activity can be observed at pH values ranging from 2.0 to 11.0. Acidophilic and "alkaline" xylanases are active in pH ranges 2.0–6.0 and 4.0–11.0, respectively (Torronen and Rouvinen 1997; Joshi et al. 2000). It is of interest to understand these features in relation to industrial applications where endo- β -1,4-xylanases are widely used under extreme pH and temperature conditions. Recent studies have partly clarified the basis of acidophilicity, and it appears that the pH profile is influenced by the nature of residues surrounding the catalytic glutamic acids whose pK_a s are finely tuned by a complex hydrogen bond network (Torronen and Rouvinen 1997). Indeed, comparison of primary and tertiary structures have shown that, in the catalytic site of acidophilic endo- β -(1,4)-xylanases, an aspartic acid is hydrogen bonded to the acid/base catalyst whereas it is replaced by an asparagine in the alkaline xylanases (Torronen and Rouvinen 1995). Moreover, site-directed mutagenesis studies have shown the importance of neighboring residues in the fine-tuning of the pK_a s of these glutamates, in particular, that of the acid-base catalyst (Krugel and Dijkstra 1996; Fushinobu et al. 1998; Joshi et al. 2001). Interestingly, conformational changes induced by either substrate binding or pH modification have been observed in the catalytic site (Torronen and Rouvinen 1995). Indeed, the acid-base catalyst can adopt two positions that might correspond to different pK_a values so that it can accept or donate a proton (Torronen et al. 1994). Moreover, crystal structures and molecular dynamic simulations of the catalytic pathway have suggested an opening-closing movement of the active site proceeding by way of three different conformations (Mullin et al. 1998).

Xyl1 from *Streptomyces* sp. S38 is a family 11 endo- β -1,4-xylanase. It is one of the most active xylanases presently described (Gendis et al. 2000). Its optimum temperature is 60°C and its optimum pH about 6.0. It exhibits interesting properties for bleaching and delignification and its structure

has been solved at 2.0 Å (Wouters et al. 2001). In an attempt to better understand the adaptation of endo- β -1,4-xylanases to acidic environments, the Xyl1 structure was compared to those of two acidophilic xylanases, and features potentially responsible for acidophilicity were identified and introduced in Xyl1 by site-directed mutagenesis.

Results

Comparison of Xyl1 with acidophilic endo- β -1,4-xylanases

Alignment of the amino acid sequence of Xyl1 with those of other family 11 endo- β -1,4-xylanases highlighted two groups, one containing so-called alkaline xylanases possessing basic pIs and the second acidophilic xylanases with acidic pIs and lower optimum pH values (Fig. 1). Xyl1 shares 51.5% and 45.9% identity, respectively, with the acidophilic *Aspergillus kawachi* XynC and *Trichoderma reesei* Xyn1 enzymes. Comparison of the three-dimensional structures of the enzymes and sequence alignment highlighted the following interesting differences:

1. At the edge of the cleft of Xyl1, residues 48 and 134 are Asn and Ala, respectively (Fig. 2). Asn48 is conserved in the alkaline xylanase group and is assumed to be, in part, responsible for the pH adaptation (Fig. 1; Torronen and Rouvinen 1995; Fushinobu et al. 1998). As seen in Table 1, Asn 48 is located at a bonding distance of 3.6 Å from the acid/base catalyst Gln 191 (distance between heavy atoms; Table 1; Wouters et al. 2001). The corresponding residues in acidophilic xylanases is an Asp making a shorter hydrogen bond with the acid-base catalyst; the distances between AspOD1 and the acid/base catalyst Gln(A/B)OE1 are 2.8 Å and 2.9 Å in XynC and Xyn1, respectively (Fig. 1; Table 1). In the acidophilic xylanases, the residue corresponding to Ala 134 is a Gln located in the thumb at the bottom and pointing into the active site. Together with the Asp 48 residues at the lower boundary of the cleft, Gln 134 confers a significantly more negative environment along the edge of the acidophilic enzyme cleft (Fushinobu et al. 1998).
2. Arg 131, fully conserved among family 11 xylanases, points towards the thumb in Xyl1, whereas it protrudes toward the cord in acidophilic enzymes (Fig. 2).
3. The compared structures present aromatic residues nicely lined up inside the active site and at the edge of the cleft. In particular, Tyr 16, Tyr 83, Tyr 95, and Tyr 185 are conserved among all family 11 xylanases, whereas Trp 148 is only conserved in alkaline xylanases and is usually replaced by Phe or Ile in the acidophilic group (Fig. 1). Tyr 127 is substituted by Trp in Xyn1 and by a Cys forming a disulfide bridge with Cys 106 in

Acidophilic adaptation of xylanases

[illegible]

Figure 1. Sequence alignment of family 11 xylanases. The three acidophilic enzymes are from *Aspergillus fumigatus* (XynC; Ito et al. 1992), *Aspergillus niger* (Xyl1; Krings et al. 1996), and *Trichoderma reesei* (XYN1; Teemoran et al. 1992). The so-called "alkaline" enzymes are from *Trichoderma reesei* (XYN2; Teemoran et al. 1992), *Trichoderma harzianum* (Wang and Saddler 1992), *Aspergillus niger* (XynB; Krings and Dijkstra 1996), *Streptomyces* sp. 538 (Xyl1; Goeds et al. 2000), *Streptomyces fidus* (XynB; Chappell et al. 1990), *Streptomyces* H33 (xylanase C; Masy-Servais et al. 1996), *Bacillus* D3 (Harris et al. 1997), and *Bacillus cereus* (XynA; Yang et al. 1999). The residues referred to in the text are in bold, the catalytic Glu residues underlined, and the mutated residues in bold and underlined. The individual numbering of each enzyme is shown. In the text, the standard numbering resulting from the alignment is used consistently. n.d.: Not determined.

XynC. Interestingly, in acidophilic xylanases, Trp 20 and Tyr 193 are replaced by Tyr and Trp, respectively. Tyr 20 in Xyn1 is involved in a stacking interaction with the xylose ring at subsite -2 (Torronen and Rouvinen 1997). In the same way, Trp 193 in Xyn1 is assumed to determine subsite +2 (Torronen and Rouvinen 1997). This difference between acidophilic and alkaline xylanases is conserved among all members of the two groups (Fig. 1).

4. Two salt bridges are found in XynI and three in XylI and XynC (Table 2). One (A) is common to the three enzymes and is the only one shared by XylI and the acidophilic enzymes. Salt bridge B, located in the thumb is only found in this latter group. It cannot be formed in XylI because of the E134A substitution. Salt bridge C, only found in XynC, is replaced by a hydrogen bond in XylI. Salt bridges D and E are specific to XylI.

36
195

de Lencastre et al.

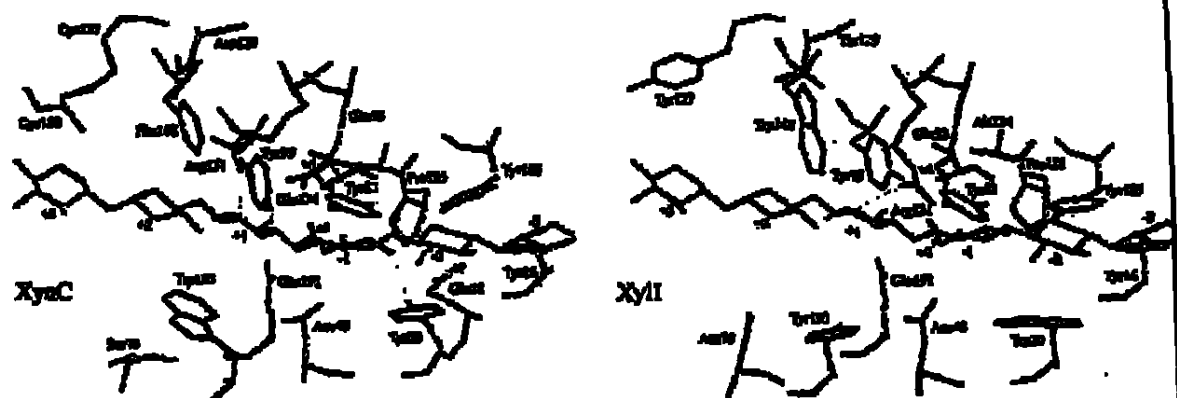


Figure 2. Docking of a chain of eight xylose residues in the active site of XynC and XylI. Six of the eight xylose residues were accommodated in the catalytic site of XylI and XynC. The geometry of the complex was optimized by energy minimization.

Site-directed mutagenesis

On the basis of these observations, four residues that could be responsible for the acidophilic adaptation were chosen and mutated in the wild-type XylI enzyme. Four variants were constructed with mutations W20Y, N48D, A134E, and Y193W and three combinations W20Y/Y193W, N48D/A134E, and W20Y/N48D/A134E, made in order to study the possible interdependence of these mutations and partly reproduce the catalytic site of an acidophilic xylanase. All substitutions were located in the catalytic site, in the neighborhood of the two Glu residues involved in the catalysis.

Preliminary characterization of the recombinant proteins

The modified proteins were expressed in *Streptomyces parvulus* as previously described (Georis et al. 2000). All recombinant strains exhibited xylanolytic activity when plated on 1% onto spelt plates with the exceptions of those pro-

ducing the Y193W and W20Y/Y193W mutants. Secretion of the two proteins was verified by Western blot, but no activity was detected in the culture supernatants.

Thermophilicity and thermostability

Thermophilicity of the active recombinant proteins N48D, A134E, W20Y, N48D/A134E, and W20Y/N48D/A134E was evaluated and the temperature dependence of their activities established. All the purified proteins exhibited maximal activity at 60°C, similar to the wild-type enzyme (not shown).

Thermal unfolding curves were established at pH 6.0 between 35°C and 85°C. The curves were obtained by following the decrease of fluorescence at 323 nm induced by a temperature gradient of 1 °C/min. Thermal denaturation of the wild-type enzyme XylI is irreversible and its apparent T_m was previously evaluated at 61.1 ± 0.1 °C under the same conditions (Georis et al. 2000). The apparent T_m values

Table 1. Some important interatomic distances in the crystallographic (XynA, XylI-WT, XYN1, and XynC) and modeled (XylI mutants) structures of the proteins

	XynA	XylI	A134E	W20Y/N48D/A134E	N48D	W20Y	N48D/A134E	XYN1	XynC
Optimum pH	6	6	6	5.3	5	5	4.7	3.5	2
N48D/A134E-Glu191OH1	3.59	3.4	3.53	3.45	3.09	2.95	3.47	2.9	2.8
Glu191OH2-(Wad1)O	2.97	2.99	2.99	2.85	2.71	2.7	2.88	2.65	2.59
(Wad1)O-Tyr95OH	2.94	2.99	2.95	2.7	2.85	2.76	2.71	2.66	2.67
Tyr95OH-Glu93OH1	2.77	2.75	2.76	2.76	2.82	2.82	2.77	2.75	2.71
Glu93OH2-(Wad2)O	2.84	2.82	3.1	3.38	3.41	2.82	3.39	3.33	3.37
Glu93OH2-Tyr95OH	2.76	2.75	2.76	2.74	2.77	2.75	2.75	2.74	2.76
Glu134OH1-(Wad2)O	—	—	2.6	2.74	—	—	2.75	2.75	2.77
Glu191OH2-(Wad1)O	8.7	8.69	8.72	8.31	8.33	8.28	8.15	8.08	7.97
Arg131NH1-Glu191OH1	7.16	8.99	8.20	8.27	8.85	8.69	8.26	8.08	10.12
Arg131NH1-N48D/A134E	4.51	6.98	6.28	6.25	6.96	6.50	6.14	6.08	10.48

XynA: *Bacillus subtilis*; XYN1: *Trichoderma reesei*; XynC: *Aspergillus fumigatus*; XylI: *Streptomyces* sp. D36; Wad1 and Wad2: water molecules.

Acidophilic adaptation of xylose

Table 2. Side-chain interactions in the wild-type enzymes

Salt bridge	XYNI	XynC	XylI	Std. numbering
A	R20-R153	R24-R138	V92-W144	90-186
B	R109-R112	R115-R118	R121-L124	151-184
C	N707/R109	R113-R115	T719/F121	128-131
D	Q116A	T716A	K128-K131	138-141
E	-T7103	N902/Q109	R30-D115	104-125

Salt bridges in the wild-type enzymes are in bold. The corresponding residues that cannot form salt bridges are shown in italic. The individual numbering of each enzyme is used, but the last column shows the standard numbering of Figure 1.

of W20Y was higher whereas those of N48D, A124E, N48D/A134E and W20Y/N48D/A134E were slightly lower (Table 3).

Acid stability

The wild-type enzyme and the mutants were tested for their stability by measuring their residual activities at optimum pH after incubation at pH 1.0, 3.0, and 6.0 at 20°C. Whatever the pH, 100% activity was retained after a 24-h incubation. Moreover, far- and near-UV CD spectra were recorded between 195 and 260 nm from pH 1.0 to 6.0 for the wild-type enzyme and the mutants after 10 min and 24 h of incubation at 20°C. No change in the spectra was observed, suggesting that all the proteins were quite stable and that the low pH did not induce major changes in the native structure (not shown).

pH profiles of the purified proteins

The activity of the five purified proteins, W20Y, N48D, A134E, N48D/A134E, and W20Y/N48D/A134E, was assayed at pH values between 2.0 and 9.0. The wild-type enzyme optimum pH was close to 6.0 (Fig. 3). Its activity was reduced by 95% at pH 3.0 and was negligible at pH 2.0. N48D and W20Y were optimally active at pH 5.0. Moreover, they showed, respectively, 33% and 30% of their

maximum respective activities at pH 3.0 (Fig. 3). The A134E mutant showed an activity profile similar to that of the wild-type enzyme although slightly narrower. Interestingly, the double mutant N48D/A134E was optimally active at pH 4.7 and retained 37% of the maximum activity at pH 3.0, suggesting that the A134E mutation lowers the optimum pH of this mutant but not that of the wild-type enzyme. However, the triple mutant showed an optimum pH of about 5.0 like N48D and W20Y, and thus it seems that the effects of some of the mutations are not additive.

The specific activity of the wild-type enzyme was evaluated at 2100 ± 200 IU/mg at 60°C (Georis et al. 1999). With the exception of the W20Y protein, which presented a slightly higher specific activity, all mutants showed decreased specific activities at their respective optimum pH values (Table 3).

Modeling of the mutants

All distances referred to in the text are summarized in Table 1.

In the wild-type enzyme, the Trp 20 side chain does not interact with other residues. Modeling of the W20Y mutant is relatively straightforward, leading to the formation of a Tyr20OH-Asn48OD1 hydrogen bond, which itself induces a shortening of the Asn48ND2-Gln191OE1 distance from 3.6 Å to 2.95 Å.

The replacement of Ala 134 by Glu can give rise to several conformations of the latter side chain. All starting conformations lead to models in which Arg 131 is strongly hydrogen bonded to Glu 134 and Wat2 is also hydrogen bonded to the same residues. However, the formation of the salt bridge only slightly reorients the Arg side chain with respect to its position in the wild-type XylI. The Arg131NH1-Asp48OD1 and Arg131NH-Gln191OE1 distances decrease from 6.98 Å to 6.28 Å and from 8.99 Å to 8.20 Å, respectively.

The distances between Wat1 and Tyr 95 on the one hand and Glu 191 on the other are similar to those found in the wild-type enzyme. By contrast, a shortening of the Tyr95OH-(Wat1)O-Glu191OE2 distances is also observed (2.95 Å/2.85 Å and 2.99 Å/2.71 Å) in the N48D model, and this is even more obvious in the cases of the N48D/A134E and W20Y/N48D/A134E mutants.

The side chain of Tyr 193 is not involved in hydrogen bonding interactions with other residues of the wild-type enzyme, and is located in a position that does not impair binding of the substrate. The Y193W mutation yields two possible stable conformations of the residue. In the first one, a hydrogen bond is formed between Trp193NE1 and Glu191OE2 (3.27 Å) whereas in another one, 10 kcal/mole less stable, a Trp193NE1-Gln194OE2 hydrogen bond (3.25 Å) is observed. In both cases, the Trp side chain protrudes

Table 3. Properties of the purified wild-type and mutant proteins

Mutant(s)	Optimum pH	Specific activity (IU/mg)	T_m (°C)
Wild type	6	2100 ± 200	61.1 ± 0.1
N48D	5	1180 ± 90	60.9 ± 0.1
W20Y	5	2340 ± 200	63.7 ± 0.2
A134E	6	530 ± 60	61 ± 0.1
N48D/A134E	4.7	850 ± 70	59.1 ± 0.3
W20Y/N48D/A134E	5.3	580 ± 40	59.2 ± 0.2

Specific activities (IU/mg) of the purified recombinant and wild-type proteins and T_m values obtained by fluorescence (°C).

In both structures, the xylose ring in subsite +1 interacts with Arg 131 and Tyr 95. In turn, the xylose in subsite -1 interacts with the carbonyl group of the conserved Pro 135, whereas the OH group of Tyr 183 is hydrogen bonded to xylose at subsite -2.

However, some interactions are different. At subsite -1 the xylose residue is hydrogen bonded to the guanidinium group of Arg 131 in Xyl1, an interaction that is not found in the case of XynC. In addition, some hydrogen bond interactions between xylose at -1 (Tyr 20) and -2 (Gln 18) are not observed with Xyl1, due to the following differences: Gln 18 in XynC is replaced by Ser in Xyl1 and XynC Tyr 20 is a Trp in Xyl1. In this latter case, the stacking interaction between the ring of residue 20 and the xylose moiety at subsite -1 is, however, conserved.

Discussion

Xyl1 from *Streptomyces* sp. 538 belongs to family 11 of endo- β -1,4-xylanases. Comparison of its structure with those of acidophilic xylanases of the same family revealed that acidophilic adaptation could be partly explained by replacements of residues surrounding the two glutamic acids involved in the catalysis.

The environment of the nucleophile Glu 93 is more conserved than that of the acid-base catalyst Glu 191. As previously described for *Bacillus circulans* XynA, the N48D mutation improved the acidophilic performance of Xyl1 by lowering its optimum pH (6.0 to 5.0) but at the cost of a decrease of nearly 50% in specific activity, whereas in the case of *B. circulans* enzyme, this mutation yielded an enzyme slightly more active than the wild type (Joshi et al. 2000). However, the decreased specific activity observed here might be due to the utilization of a different substrate. This result agrees with observations made by Fushinobu et al. (1998) on the mutational analysis of the *Aspergillus kawachi* XynC in which the inverse (D $\rightarrow$ N) mutation drastically increased the optimum activity pH from 2.0 to 5.0.

In the wild-type Xyl1 enzyme, residue Asn 48 is connected to the acid-base catalyst Glu 191 and makes a 0.6 Å longer bond than the corresponding hydrogen bond between Asp 48 and Glu 191 in the acidophilic XynC (3.6 Å versus 3 Å). Indeed, as previously suggested, Asn 48 and Glu 191 may function together as the general acid-base catalyst using a "reverse protonation" mechanism (Joshi et al. 2000). In this context, the Q146E mutation performed in the *B. circulans* XynA lowered its optimum pH by 0.6 units. Residue 146 is situated near the nucleophile Glu 93 and the introduction of a carboxylic group in position 146 lowers the pK_a of Glu 93. These mutations highlight the importance of the close neighboring residues in determining the pH activity range.

Xylanases of the acidophilic group present a glutamic acid in the thumb (Glu 134). In XynC, this residue is assumed to contribute to the negative charge at the edge of the cleft and to partly determine its low optimum pH (Fushinobu et al. 1998). The neighboring Arg 131 is highly conserved in family 11 xylanases, underlining its importance (Sapag et al. 2002). In Xyl1, this residue is assumed to be close enough to maintain the low pK_a of the nucleophile Glu 93 (Wouters et al. 2001). Interestingly, Joshi et al. showed that the R131N mutation in *B. circulans* XynA resulted in a dramatic activity loss accompanied by an increase of the pK_a of the two glutamates but especially of the acid-base catalyst Glu 191, whereas mutation R131A in *Thermomyces lanuginosus* xylanase YNA was predicted to increase the pK_a of the nucleophile without affecting the acid-base catalyst (Gruber et al. 1998; Joshi et al. 2001). The environment of residue Arg 131 is different in Xyl1 and in XynC and leads to a different orientation of its lateral chain. In Xyl1, this residue points toward the thumb, whereas, in the acidophilic xylanase, it protrudes towards the cord. In XynC, this residue is connected to Asp 129 and Glu 134 by two salt bridges. In Xyl1, none of these salt bridges can be formed because residue 129 and 134 are Thr and Ala, respectively. Moreover, Arg 131 fills the void space available due to the low steric hindrance of the shorter lateral chain of Ala 134. Docking experiments showed that, in Xyl1, this residue binds the substrate at subsites -1 and +1, whereas in XynC it only binds the xylose ring at subsite +1. The A134E mutation in the catalytic site of Xyl1 had no effect on the optimum pH of Xyl1 but decreased its specific activity by 75%. This substitution allows the putative formation of the salt bridge Arg131ND1-Glu134OE1 as found in XynC but not the formation of the second one. Thus, because of the lack of the second salt bridge, complete reorientation of Arg 131 in the mutant A134E probably could not occur as in XynC and the binding to the xylose ring in subsite -1 remains the same as in the wild-type Xyl1. In fact, this residue makes a bond network with neighboring residues and the substrate that is much of interest and probably is an important key in the acidophilic adaptation of xylanases. However, combination of mutations A134E and N48D led to a larger decrease of the optimum pH (1.4 pH unit) than when these mutations were separately introduced or with the additional W20Y substitution. This synergistic effect still remains obscure and it is not understood why the triple mutant does not present the largest decrease in optimum pH. Solving the three-dimensional structures of mutants will be necessary to understand and explain the exact effects of these mutations.

In the active site cleft of XynC, five subsites (+3, +2, +1, -1, and -2) have been identified where Tyr 20 and Trp 193 are located at subsites -2 and +1, respectively (Fushinobu et al. 1998). In this way, the structure of the inactive mutant E191C of *B. circulans* XynA complexed with xylobiose

10
179

revealed a stacking interaction of Trp 20 with the xylose ring at subsite -2 (Wakarchuk et al. 1994). Moreover, in *Trichoderma reesei* XYN1 and XYN2 xylanases, residue 20 is assumed to be part of subsites -2 and residue 193 of subsites +1 and +2, respectively (Tien and Rouvinen 1993, 1997). The W20Y mutation lowered the optimum pH of Xyl1 by one unit pH and slightly increased its specific activity, whereas Y193W inactivated the enzyme. We assume that the introduced Tyr 20 acts in the same way as Asp 48 to decrease the optimum pH of Xyl1. Actually, modeling showed that Tyr 20 attracts residue Asn 48 in a rocking motion by formation of a hydrogen bond between Tyr20OH and Asn48OD1 and induces a shortening of the distance between Glu191OB1 and Asn48ND2 from 3.6 Å to 2.95 Å. Interestingly, the orientation of residue 193 is different in acidophilic and in alkaline xylanases. In Xyl1, Tyr 193 protrudes into the solvent, whereas, in XynC, Trp 193 bends into the cleft. The Y193W substitution in Xyl1 may sterically affect neighboring residues, in particular residue Asn 48, which is very close, but also Asn 76, which may hinder the correct positioning of the Trp ring and thus probably trigger a reorganization of the hydrogen bond network. It remains noteworthy that, in XynC, Trp 193 influences the hydrophobicity around the Glu residues involved in catalysis and may probably influence the pK_a of the acid/base catalyst. With the exception of the triple mutant W20Y/N48D/A134E, calculations have shown that hydrophobicity around the nucleophile and the acid-base catalyst decreased with increasing acidophilic activity. This observation was also made in the case of the alkalophilic variants of the *Neocallimastix patriciarum* xylanase (Chen et al. 2001), where mutations increased hydrophobicity and alkalophilicity of the enzyme.

Materials and methods

Bacterial hosts strains and plasmids

Streptomyces parvulus DMET41380 and the *Streptomyces* plasmid vector pJ486 were gifts from Drs. D.A. Hopwood and T. Kloser (John Innes Institute, Norwich, UK). pDML11 and pDML1012 were used as previously described (Goris et al. 1999). *Escherichia coli* DH5 α was used as host strain. The *E. coli*-*Streptomyces* shuttle plasmid resulted from the subcloning of pDML1011 derivatives (obtained by site-directed mutagenesis) in the unique HindIII site of the pJ486.

Media, antibiotics, and culture conditions

Streptomyces liquid cultures were grown in YEME (Hopwood et al. 1985) and M13 media (Morosoli et al. 1986) on rotary shaker at 220 rpm and 28°C. R2YE or minimal agar medium (Hopwood et al. 1985) were used for plating. The *E. coli* strain was grown at 37°C in Luria-Bertani broth. Tetracycline (Squibb) was used at final concentrations of 25 μ g/mL in liquid and solid media,

and 50 μ g/mL in overlays. Ampicillin (Sigma Chemical) was used at 100 μ g/mL.

After transformation by shuttle plasmids carrying different mutations, Xyl1 recombinant proteins were produced as follows: 25 mL of 48-h cultures of *S. parvulus* in Tryptic Soy Broth (TSB) were used to inoculate 1 L of M13 medium with 1% xylose as carbon source and 25 μ g/mL of tetracycline (Squibb). Cultures were grown in 1-L Erlenmeyer flasks containing a stainless steel spring to improve oxygen transfer and 250 mL of medium. They were incubated for 72 h at 28°C and 220 rpm on a rotary shaker. The production of xylanase was monitored at 8-h intervals.

Cloning, mutagenesis, and DNA sequencing

Site-directed mutagenesis was performed on pDML1012 (Goris et al. 2000) by using the Quick-Change Site Directed Mutagenesis Kit from Stratagene according to the protocol recommended by the manufacturer and the following primers (Pharmacia LKB): W20Y (up): GGCCTACTACTCTGTTCTACACCGACGGGG GCG; W20Y (down): CCCCOCCTGCTGTTTATAGAACGAGTA GTAGTAGCC; N48D (up): GCGGCGACTCTGTCGACGCCA GGG; N48D (down): CCTTGCCTGCGACGAAGTCCGCC GC; A134E (up): ACCCGGGTCAACGACCGCTCCCTTCGA AG; A134E (down): CTTCGACCGACGGCTCTTGAACCCGC GT; Y193W (up): GGCGACGAGCGCTGCGACGACGACGT GGGAGC; Y193W (down): GCTCCCACTGCTCTGCCAGGCC TCCCTCGCC. The polymerase chain reaction (PCR) conditions were 5 min at 95°C, 15 times (30 s at 95°C; 1 min at 55°C; 8 min at 72°C) and 3 min at 72°C. pDML1012 derivatives were isolated using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech Inc.) and nucleotide sequences were determined by double-strand sequencing using the dideoxy-nucleotide method and the Thermosequenase fluorescent DNA sequencing kit (Amersham) with universal and reverse primers. Experiments were performed on an ABI automated fluorescent DNA sequencer (Pharmacia).

pH-dependence of xylanase activity

Endo- β -1,4-xylanase (EC 3.2.1.8) activity was measured by the dimethylbenzylidene acid method (DNS; Miller 1959). Aliquots (40 μ L) of diluted enzyme were mixed with 360 μ L of a 1% suspension of o-nitrophenol xylan (Sigma Chemical Co.) at different pH and incubated for 10 min at 50°C. Buffers were 0.05 M HCl/KCl (pH 1.0 to 2.0), 0.05 M sodium citrate (pH 2.5 to 6.0), 0.05 M sodium phosphate (pH 6.0 to 7.0) and 0.05 M Tris/HCl (pH 7.5 to 9.0). One international unit (IU) is defined as the amount of enzyme that releases 1 μ mol of reducing sugar per minute.

Proteins purification and analysis

Culture supernatants (1 L) were harvested by continuous centrifugation at 10,000g for 20 min. They were microfiltered on 0.4 μ m Millipore filters (Millipore) and diluted 10-fold with water. After adjustment to pH 5.0, 300 g of moist carboxymethylcellulose (CMC) equilibrated in 10 mM sodium citrate buffer (pH 5.0) was added. The exchanger was washed with 3 L of the same buffer and harvested by deswelling. Elution of recombinant enzymes was performed batchwise with 1 L of 10 mM citrate buffer (pH 5.0) containing 1.5 M NaCl. Solutions were concentrated to 35 mL, dialyzed against 10 mM citrate buffer (pH 5.0) and loaded onto a Sulfolipoyl Sepharose Fast Flow column (CK 26/40, 150 mL;

Amerham Pharmacia Biotech). The enzyme was eluted with a linear NaCl gradient (450 mL) from 0 to 0.2 M. Fractions exhibiting xylanase activity were pooled, concentrated to 5 mL, and applied onto a molecular sieve column of Sephacryl S100 HR (2.6 × 95 cm, 500 mL; Amerham Pharmacia Biotech) equilibrated in 20 mM phosphate buffer (pH 7.0). The fractions containing xylanolytic activity were collected and concentrated to 0.5 mg/mL. M_r values were estimated by SDS-polyacrylamide gel electrophoresis (Laemmli 1970).

Fluorescence study and circular dichroism spectroscopy

Tryptophan fluorescence was monitored at a protein concentration of 25 µg/mL using a SLM-AMINCO model 8100/2 spectrofluorimeter (Spectronic Instruments) with excitation and emission wavelengths of 284 and 333 nm, respectively. Bandwidths were 1 nm for excitation and 4 nm for emission. Thermal denaturation of the wild-type enzyme and the mutants was recorded by following the fluorescence at 323 nm at a constant heating rate of 1°C/min from 35 to 85°C and pH 6.0. The temperature in the thermostated cell was monitored with a differential thermometer and denaturation curves were established as described by Pace (1986).

Far- and near-UV CD spectra were obtained using a JASCO model J-810 spectropolarimeter by scanning from 195 to 260 nm and 250 to 330 nm, respectively, with 0.2 nm steps and an averaging time of 6 s/run, at 25°C. All values were corrected for the contribution of the solvent, and quartz cells of 0.1 and 1 cm were used for far- and near-UV, respectively, at a protein concentration of 0.2 mg/mL.

Amino acid numbering

The standard numbering used in the text results from the alignment numbering in Figure 1.

Modeling of protein structures

The crystallographic structures of the *Streptomyces* sp. S38 xylanase XylI (Wooten et al. 2001) was used. Models of the mutant proteins were obtained from the X-ray structure of the wild-type protein, and the local conformational space of the mutated amino acids was searched by a minimum perturbation approach (Shih et al. 1985). The protein structures were manipulated with InsightII (Accelrys Inc.). The geometries of the *B. circulans* XynA (PDB code 1BVV, from which the inhibitor molecule was removed), *T. reesei* XYN1 (PDB code 1XYN) and *A. kawachii* XynC (PDB code 1BK1) xylanases were optimized by energy minimization. As the PDB structure file of XynC did not contain the coordinates of water molecules, a Monte Carlo water bath was generated around the molecule, and their atomic positions were refined together with the enzyme coordinates. The minimization scheme was the same as in the case of the XylI models.

Hydrophobicity

According to Mehler and Garnier (1999), a quantitative measure of the hydrophobicity of the microenvironment is needed because the response of the residue to changes in the local environment is the quantity of interest. On the basis of these considerations, the Reichert Fragmental Hydrophobic Constants were selected to evaluate the hydrophobicities of the microenvironments in the present

calculations (Reichert 1977, 1979). The microenvironment of any group is defined as the region that lies within a sphere of radius 4.25 Å from any (nonhydrogen) atom belonging to the group. This radius was chosen because it essentially includes the first shell around each atom, but does not extend to atoms beyond this. Atoms satisfying this distance criterion are included in the calculation of the total hydrophobicity around the given glutamic acid group, according to the equation 1 (Mehler and Garnier 1999):

$$H_{\text{PTA}} = \sum_b \sum_A \frac{N_A}{N_b} R_{\text{FHC}_b} (r_{ab} \leq 4.25 \text{ Å}) (B + A) \quad (1)$$

where R_{FHC_b} is the contribution of atom b to the fragmental hydrophobic constant, N_A is the number of atoms in group A , and $A_b = 0$ or 1 if atom b has been counted or not, respectively.

Modeling of the substrate

The structure of a xylane backbone fragment containing eight sugars was built using the biopolymer module of the INSIGHTII (MSI). The crystal structure of the *B. circulans* xylanase complexed with xylanase (PDB code 1BCK; Wakarchuk et al. 1994) and 2-fluoro-xylane (PDB code 1BVV; Shih et al. 1999), as well as that of *B. agaradhaerans* xylanase complexed with xylanopyranoside (PDB code 1QHT; Sahini et al. 1999) were used as references for positioning the substrate in the active cleft of the *Streptomyces* sp. S38 XylI enzyme. The structures of the four xylanases were superimposed by minimizing the root mean square deviation between the carbons of 12 residues bordering the active site. The two central units of the substrate were superimposed on the mean position of the two xylane molecules in the complexes. The six other xyloses (three in the direction of the nonreducing end and three in the direction of the reducing end) were manually oriented to cover hydrophobic areas in the binding cleft and to allow possible hydrogen-bonding interactions. The geometries of modeled enzyme-substrate complexes were optimized using the AMBER all atom force field (Pearlman et al. 1995) first by steepest descent energy minimization of atoms with forces above 500 kcal mol⁻¹ Å⁻¹ and then by conjugate gradient energy minimization until the root mean square gradient was less than 0.1 kcal mol⁻¹ Å⁻¹.

Acknowledgments

This work was supported in part by Ministry of Walloon Region, Department of Technology Research and Energy (conventions Bioval I no. 981/3860 and no. 981/3709) and by the Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture (FRIA, Brussels, Belgium). F.d.L.E. is a fellow of FRIA. The purchase of the Jasco 810 equipment was supported in part by a grant from the Fonds de la Recherche Fondamentale et Collective (F.N.R.S., contract no. 24545.01). We thank Drs. J. Geric, M. Gallani, A. Matagne, and F. Bouillonne for their helpful advice.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Chen, Y.L., Tang, T.Y., and Chang, K.J. 2001. Directed evolution to produce an alkaliphilic variant from a *Neocallimastix pasteuriana* xylanase. *Can. J. Microbiol.* 47: 1083-1094.

de Lencastre et al.

- Collins, T., Morris, M.A., Sola, I., Chagoyan, M., Feller, G., and Garbay, C. 2002. A novel family 8 xylose, functional and physicochemical characterization. *J. Mol. Chem.* 277: 8513-8519.
- Fukushima, S., In, K., Kozono, M., Wakagi, T., and Muramatsu, H. 1998. Crystallographic and mutational analysis of an extremely acidophilic and acid-stable xylose: Bimodal distribution of acidic residues and importance of Asp27 for catalysis at low pH. *Protein Eng.* 11: 1121-1128.
- Garcia, J., Glanville, F., Lencastre-Bauer, J., Doreau, B., Van Soest, J., Garcia, B., and From, J.M. 1999. Sequence, overexpression and purification of the family II endo- β -1,4-xylosidase encoded by the xylII gene of *Saccharomyces sp. 533*. *Chem. Eng.* 127: 123-133.
- Garcia, J., de Lencastre, B., Lencastre-Bauer, J., Benguer, V., Doreau, B., Glanville, F., Garcia, B., and From, J.M. 2000. An additional conserved interaction improves the thermostability and acidophilicity of a mesophilic family II xylose: Structural basis and molecular study. *Protein Sci.* 9: 466-473.
- Gilman, N.R., Norrish, B., Kilian, D.G., Miller, L., R.C., and Warren, R.A. 1991. Dimeric structure of the xylose: Sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55: 305-315.
- Gutierrez, X., Kirschner, G., Hays, M., Schindler, A., Scher, W., and Kistly, C. 1998. Thermophilic xylose from *Thermotoga lanthanum*: High-resolution X-ray structure and modeling studies. *Biochemistry* 37: 13475-13485.
- Harris, G.W., Fichengill, R.W., Cameron, I., Dobson, P., Town, J.P., Brown, C., and Punt, S. 1997. Structural basis of the properties of an industrially relevant thermophilic xylose. *Protein* 29: 71-84.
- Henderson, J., and Davies, G. 1997. Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.* 7: 637-644.
- Hopwood, D.A., Bida, M.J., Chant, K.P., Kwan, T., Brown, C.J., Kilian, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M., and Shrago, E. 1993. Genetic manipulation of *Saccharomyces*. A laboratory manual. The John Innes Foundation, Norwich, UK.
- Ho, K., Swadlow, K., and Jerns, K. 1992. Cloning and sequencing of the xylC gene encoding acid xylose of *Aspergillus niger*. *Biotechnol. Biochem.* 133: 1338-1340.
- Jacob, M.D., Sella, G., Fox, L., Boyer, G.D., Wilson, S.G., and McIntosh, L.P. 2000. Hydrogen bonding and catalysis: A novel explanation for how a single amino acid substitution can change the pH optimum of a glycosidase. *J. Mol. Biol.* 299: 255-279.
- Jacob, M.D., Sella, G., Wilson, S.G., Boyer, G.D., Wilson, S.G., and McIntosh, L.P. 2001. Disrupting the electrostatic interactions and pH-dependent activity of a family II glycosidase. *Biochemistry* 40: 10115-10129.
- Khopshi, D., Vito-Molina, S., August, F., Shew, F., and Marston, R. 1990. Purification and characterization of a new xylose (xylose B) produced by *Saccharomyces* *sp.* *Microb. J.* 267: 45-50.
- Krupel, U., and Dijkster, H.W. 1994. Three-dimensional structure of endo-1,4- β -xylosidase I from *Aspergillus niger*: Molecular basis for its low pH optimum. *J. Mol. Biol.* 233: 70-78.
- Lancaster, U.K. 1970. Cleavage of structured proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- Mary-Servais, C., Morvan, A., Gourd, C., and Doreau, J. 1996. Cloning and nucleotide sequence of a xylose-encoding gene from *Saccharomyces sp.* *Environ. Microbiol.* 38: 147-151.
- Milne, E.L., and Gussard, F. 1999. A self-consistent, microenvironment-mediated potential approach to calculate pH-dependent electrostatic effects in proteins. *Biochem. J.* 347: 3-12.
- Muller, G.J. 1999. Use of differential scanning calorimetry for determination of melting points. *Anal. Chem.* 71: 426-431.
- Murphy, R., Bernard, J.L., Morvan, F., Shew, F., and Kloppe, D. 1994. Purification and properties of a xylose from *Saccharomyces* *sp.* *Biochem. J.* 299: 587-591.
- Mullis, J., Tournier, A., Pankaj, M., and Rothermel, J. 1998. Functional conformational changes of endo-1,4-xylosidase II from *Trichoderma reesei*: A molecular dynamics study. *Protein Sci.* 434-444.
- Pace, C.N. 1986. Determination and analysis of rate and equilibrium hydrolytic dissociation curves. *Methods Enzymol.* 131: 265-290.
- Pandey, D., Cui, D.A., Caldwell, J.A., Ross, W.S., Choudhary, T.E., Pandey, D.M., Sella, G.I., Singh, U.C., Weber, P.K., and Kishore, P.A. 1995. AMBER 4.1. University of California, San Francisco, CA.
- Richter, R.P. 1977. The hydrophobic fragment of a protein. *Environ. Microbiol.* 1977. The hydrophobic fragment of a protein: An extension to a 1000 dalton polypeptide. *Rev. J. Mol. Chem.* 14: 479-483.
- Schell, M., Schellhammer, G., Dörner, M., Dörner, Z., Jorgensen, P.L., Schell, M., Dörner, C., Dörner, G.I., and Wilson, K.S. 1999. Catalysis and specificity in enzymatic glycoside hydrolysis: A 2.5 Å resolution structure of the glycosyl-enzyme intermediate revealed by the structure of the *Bacillus pumilus* family II xylose. *Chem. Biol.* 6: 483-493.
- Schell, M., Wilson, K.S., Dörner, Z., Schell, M., and Dörner, G.I. 2001. Glycosyl-enzyme binding to family II xylose: Both covalent interaction and water product complex display (2.5 Å) conformation at the active site. *Acta Crystallogr. D Biol. Crystallogr.* 57: 1344-1347.
- Singh, A., Warren, J., Lambert, C., de Lencastre, B., Sengupta, J., and Doreau, B. 2002. The endoxylanase from family II: Computer analysis of protein sequences reveals important structural and phylogenetic relationships. *J. Biochem. Microbiol.* 109-131.
- Slon, H.H., Sella, J., and Karpis, M. 1981. Structure of proteins with single-site mutations: A minimum perturbation approach. *Protein Anal. Acad. Sci.* 68: 1697-1700.
- Sella, H.H., Sella, J.G., Nguyen, N.T., Mahesh, L.P., Zhou, L., and Boyer, G.D. 1999. Sugar ring distortion in the glycosyl-enzyme intermediate of a family II xylose. *Biochemistry* 38: 5344-5354.
- Singh, A., Mathias, A.M., and Prid, R.A. 2001. *Thermotoga lanthanum*: Properties of xylose and their kinetic studies. *FEBS Microbiol. Rev.* 27: 3-16.
- Soma, A., and Asanuma, G. 1997. Xylanase enzymes from fungi and bacteria. *Crit. Rev. Microbiol.* 17: 39-87.
- Tournier, A., and Rothermel, J. 1995. Structural comparison of two major endo-1,4-xylosidases from *Trichoderma reesei*. *Biochemistry* 34: 847-856.
- , 1997. Structural and functional properties of low molecular weight endo-1,4- β -xylosidase. *J. Biochem.* 57: 137-149.
- Tournier, A., Math, R.L., Metzger, R., Kishore, N., Haddad, A., and Kishore, C.P. 1992. The two major xylosidases from *Trichoderma reesei*: Characterization of both enzymes and genes. *Biochemistry* 31: 1461-1465.
- Tournier, A., Kishore, C.P., and Haddad, R. 1991. Active site residues of endo-1,4- β -xylosidase and family II cellulase revealed by cloning analysis. *FEBS Lett.* 281: 329-339.
- Tournier, A., Haddad, A., and Rothermel, J. 1994. Three-dimensional structure of endo-1,4- β -xylosidase II from *Trichoderma reesei*: Two conformational states in the active site. *EMBO J.* 13: 2499-2504.
- Wakabayashi, W.W., Campbell, R.L., Song, W.L., Doreau, J., and Yaguchi, M. 1994. Molecular and crystallographic analysis of the active site residues of the *Bacillus circulans* xylose. *Protein Sci.* 3: 467-473.
- Wang, K.K.Y., and Sella, J.N. 1992. *Trichoderma reesei* xylose, their properties and application. *Crit. Rev. Microbiol.* 12: 413-433.
- Wang, K.K., Tan, L.J., and Sella, J.N. 1992. Molecular biology of β -1,4-xylosidase in microorganisms: Functions and applications. *Microbiol. Rev.* 56: 303-317.
- Worrest, J., Garcia, J., Hughes, D., Vandenberg, L., Doreau, J., From, J.M., Doreau, B., and Chant, P. 2001. Crystallographic analysis of family II endo- β -1,4-xylosidase XylII from *Saccharomyces sp. 533*. *Acta Crystallogr. D Biol. Crystallogr.* 57: 1813-1819.
- Yang, R.C., MacKinnon, C.R., Brown, D., and Narsing, S.A. 1999. Hyperexpression of a *Bacillus cereus* xylose gene in *Escherichia coli* and characterization of the gene product. *Appl. Environ. Microbiol.* 65: 1192-1195.