UNIVERSITI PUTRA MALAYSIA

PURIFICATION AND CHARACTERIZATION OF ENDOXYLANASES CLONED FROM *Fibrobacter succinogenes* S85 AND EXPRESSED IN *Escherichia coli* HB 101

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Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Science and Environmental Studies, Universiti Pertanian Malaysia.

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Dedicated To :

My Parents, brother and sisters who have been understanding and supportive throughout the duration of this project.

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LIST OF ABBREVIATIONS

The following abbreviations were used in the text:

CM	carboxymethyl
CMC	carboxymethylcellulose
%C	percentage of bis-crosslinkaged to acrylamide
°C	degrees centrigrade
Da	dalton
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
EDTA	ethylenediamine tetra acetic acid
EGTA	ethyleneglycol bis-β-aminoethylether tetra acetic acid
h	hour
kbp	kilo basepair
kDa	kilodalton
K_m	Michaelis constant
L	litre
mA	milliampere
M	molar
mg	milligram
min	minute

ml	millilitre
mM	millimolar
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
RBB	remazol brilliant blue
R_f	relative mobility
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TCA	trichloro acetic acid
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TLC	thin layer chromatography
%T	percentage of acrylamide
µg	microgram
µl	microlitre
µmol	micromole
V	volt
V_{max}	maximal velocity of a enzyme reaction
wt/vol	weight per volume
YT	yeast trypton

Abstract of the thesis submitted to the Senate of Universiti Pertanian Malaysia in fulfilment of the requirements for the degree of Master of Science.

PURIFICATION AND CHARACTERIZATION OF ENDOXYLANASES CLONED FROM *Fibrobacter succinogenes* S85 AND EXPRESSED IN *Escherichia coli* HB101

By

SOONG CHEE LEONG

April 1996

Chairman : Assoc. Prof. Abdullah Sipat, Ph.D.

Faculty : Science and Environmental Studies

The xylanase enzyme from *Escherichia coli* HB101 containing the xylanolytic recombinant plasmid pBX6 was purified to homogeneity using ultrafiltration, DEAE-Sepharose, CM-Sepharose and Sephadex G-200 chromatography. Three xylanases, namely, Xyn A, Xyn BI and Xyn BII were obtained and were found to have the same molecular weight and optimum pH which were estimated to be 60.3 kDa and pH 7.0 respectively. The optimum assay temperature for both Xyn A and Xyn BI was 50°C, while for Xyn BII, it was 40°C. The xylanases were stable up to 45°C at pH 7.2 for 30 min. Approximately 80% of the enzyme activity was retained at the pH range of 5.0 to 8.0.

The isoelectric point for Fraction A, Fraction BI and Fraction BII was 8.2, 8.5 and 5.5, respectively. The respective apparent K_m and V_{max} value on oat-spelt xylan was 12.2 mg/ml and 47.9 µmol xylose/min/mg protein for Xyn A; 10.8 mg/ml and 52.1 µmol xylose/min/mg protein for Xyn BI; 8.7 mg/ml and 54.2 µmol xylose/min/mg protein for Xyn BII. From the hydrolysis products of oat-spelt xylan analysed on thin-layer chromatography, the xylanases hydrolysed xylan through an endo-acting mechanism as no xylose, xylobiose or arabinose was detected. Thus, the xylanases

were classified as an endoxylanase. The xylanases showed no activity toward carboxymethylcellulose (CMC), crystalline cellulose (Avicel) and cellulose filter paper. The xylanases were not affected by potassium chloride, EDTA and EGTA at concentrations of 10 mM. Calcium chloride and magnesium chloride at the same concentrations enhanced the xylanase activities by 50%. Mercury chloride at 1.0 mM concentration completely inhibited the activities of all the purified xylanases.

From zymogram analysis and characteristics of the xylanases investigated, multiplicity of xylanases in *E. coli* HB101 (pBX6) was probably due to post-translational modification of a single gene product.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai memenuhi syarat untuk mendapatkan Ijazah Master Sains.

PENULENAN DAN PENCIRIAN ENZIM ENDOXILANASE YANG DIKLON DARIPADA *Fibrobacter succinogenes* S85 DAN DIEKSPRES OLEH *Escherichia coli* HB101

OLEH

SOONG CHEE LEONG

April 1996

Pengerusi : Prof. Madya Abdullah Sipat, Ph.D.

Fakulti : Sains dan Pengajian Alam Sekitar

Enzim xilanase dari bakteria *Escherichia coli* HB101 yang mengandungi plasmid rekombinan xilanolitik pBX6 telah ditulenkan melalui penurasan ultra, DEAE-Sepharose, CM-Sepharose dan kromatografi Sephadex G-200. Tiga jenis xilanase telah diperolehi iaitu Xyn A, Xyn BI dan Xyn BII dan didapati mempunyai berat molekul dan pH optima yang sama yang dianggarkan lebih kurang 60.3 kDa dan pH 7.0. Suhu optima untuk Xyn A dan Xyn BI adalah 50°C, manakala untuk Xyn BII adalah 40°C. Enzim-enzim xilanase stabil sehingga 45°C pada pH 7.2 selama 30 min. Pada julat pH 5.0-8.0, enzim-enzim ini masih mengekalkan lebih kurang 80% aktiviti enzim.

Titik isoelektrik untuk Fraksi A, Fraksi BI dan Fraksi BII adalah 8.2, 8.5 dan 5.5, masing-masing. Nilai-nilai K_m dan V_{mak} untuk substrat 'oat-spelt' xilan adalah 12.2 mg/ml dan 47.9 µmol xilosa/min/mg protein bagi Xyn A; 10.8 mg/ml dan 52.1 µmol xilosa/min/mg protein bagi Xyn BI; 8.7 mg/ml dan 54.2 µmol xilosa/min/mg protein bagi Xyn BII. Hasil hidrolisis oleh enzim-enzim xilanase ke atas 'oat- spelt' xilan yang dianalisis melalui kromatografi lapisan nipis (TLC) menunjukkan bahawa substrat xilan dihidrolisiskan melalui mekanisma endo, di mana xilosa, xilobiosa dan arabinosa tidak

dihasilkan Maka enzim-enzim xilanase ini dikelaskan sebagai jenis endoxilanase Enzim-enzim xilanase ini tidak bertindak ke atas substrat karboksimetilselulosa (CMC), selulosa kristal (Avicel) atau kertas turas selulosa Kalium klorida, EDTA dan EGTA pada kepekatan 10 mM tidak mempengaruhi aktiviti enzim-enzim xilanase. Kalsium klorida dan magnesium klorida pada kepekatan yang sama merangsangkan aktiviti enzim sebanyak 50%. Merkurik klorida pada kepekatan 1.0 mM pula merencatkan kesemua aktiviti enzim-enzim xilanase

Penganalisaan zimogram dan ciri-ciri enzim menunjukkan kewujudan kepelbagaian enzim xilanase dalam bakteria *E. coli* (pBX6), berkemungkinan besar disebabkan oleh modifikasi selepas proses translasi ke atas hasilan gen tunggal.

CHAPTER I

INTRODUCTION

Hemicellulose is the second most abundant polysaccharide in nature after cellulose. Xylan, the major polymeric component of hemicelluloses, consists of a β-1,4-linked D-xylosyl backbone, with branches containing xylose and other pentoses, hexoses, and uronic acids (Timell, 1967). It is found in the cell walls of terrestrial plants and constitutes more than 30% of the dry weight (Joseleau et al., 1992).

The utilization of xylan polymer necessitates its breakdown into monomers. There are two ways to achieve this, namely using acid and enzymes (Panbangred et al., 1983a). Acid hydrolysis is rapid and simple, but is not specific and produces many unwanted byproducts which are difficult to separate. Enzymatic hydrolysis of xylan on the other hand is a more specific process, resulting in the production of xylose or other xylooligosaccharides.

There are at least two types of xylanase enzymes; β-1,4-D-endoxylanase and β-xylosidase (Dekker and Richards, 1976). There is a commercial interest in the application of xylanases for the production of xylose which can be then bioconverted to many useful substances (Ohsugi et al., 1970 ; Detroy et al., 1982; Saddler et al., 1982; Weimer, 1985). Xylanases can also be used as a biobleaching agent in the pulp and paper industry to help reduce the use of hazardous chemical bleaching agents (Nissen et al., 1992).

Xylanases are mainly produced by fungi and bacteria. Some microorganisms produce multiple forms of xylanase (Berenger et al., 1985; Mitsuishi et al., 1987; Tsujibo et al., 1990; Matte and Forsberg, 1992).

The degradation of cellulose and hemicellulose plays a significant role in the feed digestion in ruminant animals. Their breakdown provides the carbon and energy requirements of the animal (Forsberg et al., 1981). The major cellulolytic bacteria in bovine rumen is *Fibrobacter succinogenes*. This bacterium is also highly xylanolytic (Forsberg et al., 1981). A gene coding for xylanase activity has been previously cloned from *F. succinogenes* S85 and expressed in *Escherichia coli* HB 101 (Sipat et al., 1987). The primary xylanolytic recombinant plasmid, named pBX1, was further subcloned into pUC19 and after several deletions, the resulting derivative named pBX6 was obtained (Sipat et al., 1987). *E. coli* containing this recombinant plasmid (pBX6) produces active xylanases. This thesis is a report on the purification and characterization of the endoxylanases produced by the recombinant *E. coli* (pBX6).

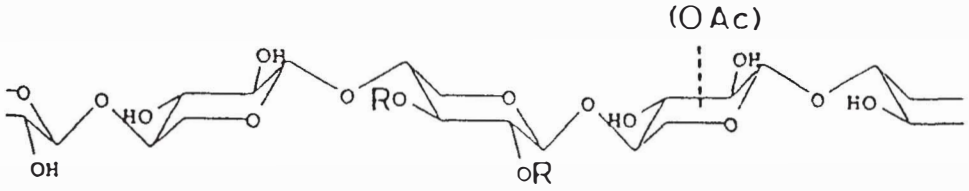
CHAPTER II

LITERATURE REVIEW

Structure and Physicochemical Properties of Xylan

Xylan is a major component of the hemicellulose complex found in plants and it accounts for approximately 15 to 30% of the total dry weight (Whistler and Richards, 1970). Xylan can be divided into two classes (Whistler and Richards, 1970; Bastawde, 1992). Homoxylan is normally found in esparto grass and consists of β-(1→4) linked D-xylopyranose units. Heteroxylan is a polymer of β-1,4-linked D-xylopyranose units and is highly substituted by mono- or oligosaccharides such as α-1,2-linked 4-O-methyl-D-glucoronic acid or 4-O-methylester and α-1,3-linked L-arabinofuranose (Figure 1).

Most of the D-xylan polysaccharides in nature are heteroxylan and are acetylated. The acetylated xylan of hardwoods have single 4-O-methyl-α-D-glucuronic acid residues attached to the C-2 of O-acetyl-4-O-methylglucuro-xylan. The acetyl substituents occur in about 70% of the xylosyl residues in hardwood xylans (Timell, 1967).



R : α-D-GlcpA(1→2)Xyl...
4-OMe-α-D-GlcpA(1→2)Xyl...
α-L-Araf(1→3)Xyl...
α-L-Araf(1→2)Xyl...
β-D-Galp(1→5)α-L-Araf(1→3)Xyl...
β-D-Xylp(1→2)α-L-Araf(1→3)Xyl...
α-L-Araf(1→2, 1→3 and 1→2,3 Araf)$_n$(1→3)Xyl...
Feruloyl
p.coumaroyl
Lignin

Figure 1 : The Primary Structure of Xylan with Side Chains Attached (after Joseleau, 1992)

There are also other substituents which include arabinosyl, O-acetyl and uronyl groups (Biely and Schneider, 1985). The arabinosyl substituents in arabinoxylan of softwoods occur in about 12% of the xylosyl residues in softwood xylans (Timell, 1967). Some of the arabinosyl substituents are esterified with ferulic and coumaric acids (Smith and Hartley, 1983).

Deacetylated xylan is insoluble in water, but soluble in alkaline solutions and can be easily hydrolysed by acids. Acetylated xylan is soluble in water and can be extracted by hot water. It is easily degraded by microbial enzymes. Xylan in solution does not reduce Fehling's solution (Bastawde, 1992).

There are other xylose-containing polysaccharides found in the primary cell wall of dicotyledonous plants. These polysaccharides have β-1,4-glucan backbones substituted with α-1,6-xylosyl residues, some of which are further linked to arabinosyl, galactosyl, or fucosyl residues (Goodwin, 1985). In algae, β-1,3- linked xylans and β-1,3; β-1,4-linked xylans have been found (Chen et al., 1986). The β-1,3-xylan has been purified from the green seaweed, *Caulerpa racemosa* (Yamaura et al., 1990).

Xylans are therefore a complex polysaccharide containing a linear xylosyl backbone to which are attached other sugars and acid residues. Its complete degradation also requires the action of several enzymes.

Degradation of Xylan by Xylanase

Xylan is degraded naturally by xylanases which hydrolyse the β-(1→4)-D-xylopyranosyl linkages of the β-(1→4)-D-xylans and the side branches containing arabinoxylan, arabinoglucuronoxylan, arabino-4-O-methyl-D-glucuronoxylan and glucuronoxylan (Dekker and Richards, 1976). Xylanases are classified into three categories (Reilly, 1980; and Bastawde, 1992), namely,

(1) Endo-β-(1→4)-D-Xylanase [β-(1→4)-D-xylan xylano hydrolase, EC 3.2.1.8]. This enzyme acts randomly on xylan to produce large amounts of xylooligosaccharide of various chain lengths. This category is further divided into four types:

(a) Non-arabinose Liberating Endoxylanases (producing simple sugars).

These cannot cleave L-arabinosyl initiated branch points, and produce mainly xylobiose and xylose as the final products. These enzymes can breakdown xylooligosaccharides into small units such as xylobiose.

(b) Non-arabinose Liberating Endoxylanases (producing short xylooligosaccharides).

These cannot cleave branch points and produce mainly xylooligosaccharides larger than xylobiose. They are generally inactive on xylotetrose and smaller substrates.

(c) Arabinose Liberating Endoxylanases (producing simple sugars).

These cleave the xylan chain at the branch points and produce mainly xylobiose, xylose and arabinose.

(d) Arabinose Liberating Endoxylanases (producing short xylooligosaccharides).

These cleave branch points and produce mainly xylooligosaccharides of intermediate size and arabinose.