Bacillus subtilis 的木聚素水解酵素的基因選殖及 DNA 混合突變

Cloning and DNA shuffling of xylanase genes from Bacillus subtilis

中文摘要

木聚素是半纖維素的主要組成成分，並且是自然界中含量僅次於纖維素的多醣。所有植物的木聚素是由 D-xylopyranosyl 組成主幹而以 b-1,4 鍵結，並帶有許多像是醣醛酸此種的單糖側鏈。木聚素水解酵素能夠水解木聚素的主鏈。木聚素水解酵素是作用在木聚素主幹的內部鍵結而產生木寡糖。許多微生物包括真菌和細菌都能產生超過一種的木聚素水解酵素。

木聚素水解酵素在造紙工業上被當成漂白劑來使用。在造紙過程中，紙漿中所含的木質素會使紙漿帶有顏色因此必須被除去。現今用來漂白的物質爲對環境有害的氯化合物。若紙漿先經由木聚素水解酵素的處理則可減少氯化合物的使用。由於在造紙場的作業環境爲高溫且具有高鹼性，因此所使用的木聚素水解酵素也要具有在此環境下能作用的能力。本篇論文的目地即是以 DNA 混合突變來針對木聚素水解酵素作改變，希望得到具有熱穩定的酵素。

從基因序列，知道 Bacillus subtilis 會產生五種木聚素水解酵素。首先以聚合酵素鏈鎖反應 (PCR) 來對 Bacillus subtilis 產生的木聚素水解酵素作基因選殖，得到長度分別爲 0.68 kb 和 1.28 kb 的 xynA 和 ynfF 基因片段。將此兩段基因利用 pKK-223-3 質體來表現，於 SDS-PAGE 上顯示其產生的蛋白質分別爲 22 和 42 kDa 且從 zymogram gel 上可看出此二個蛋白質皆具有木聚素水解酵素的活性。從 zymo-
gram gel 上顯示 ynfF 蛋白質有被蛋白酵素分解的現象。把 ynfF 基因片段從 C 端 Hind III 限制酵素認得的序列切除 127 個胺基酸後，在 SDS-PAGE 上並無發現到蛋白質的表現。推測可能切除後造成其蛋白質的折疊錯誤而很快地就被蛋白酵素水解掉。

xynA 基因片段以 DNase I 將其剪切成小於 300 bp 的片段，再以 PCR 將 DNA 重組。在二種含木聚素的培養皿上顯示這些帶有突變基因的細菌只有 30% 還具有木聚素水解酵素的活性。經由測量木聚素培養皿上產生的透明區域的大小，發現一株菌產生的透明區域特別大，將此株菌命名爲 xynA-1。比較 xynA 與 xynA-1 的核酸序列發現 xynA-1 有三個地方的核苷酸與 xynA 不同，而所轉譯的胺基酸只有一個與 xynA 蛋白質不同，就是在第 41 個胺基酸由 glycine 變成 serine。比較 xynA 及 xynA-1 蛋白質不同溫度的反應能力，顯示 xynA-1 蛋白質有比 xynA 蛋白質更寬廣的溫度作用範圍。在熱穩定的實驗中，xynA-1 蛋白質的解鏈溫度 (Tm) 從 xynA 蛋白質的 45oC 提高到 xynA-1 蛋白質的 49oC。而 xynA 與 xynA-1 蛋白質的最佳反應 pH 值均爲 7。

Bacillus circulans xylanase (BCX) 的結構被用來對 xynA-1 蛋白質作 3-D modeling，因爲 BCX 的胺基酸序列與 xynA 蛋白質只有一個地方不同。BCX 的

結構有如半開的手掌。由電腦模擬所得的結構推測 xynA-1 蛋白質的 Tm 值會較高是因爲在拇指處靠近 N 端的第一個 b-turn 上由突變所產生的第 41 個胺基酸 serine 和第 39 個胺基酸 aspartic acid 之間的側鏈可能形成的氫鍵所造成。此氫鍵會固定這個 b-turn 進而加強形成拇指狀的第一個 b-sheet 和第二個 b-sheet 之間的作用力。比較 xynA 與 xynA-1 蛋白質的催化能力，從 initial rate，Vmax，和 Km 値的相似性得知此二個蛋白質的催化能力並無不同，這是可預測的，因爲突變所改變的胺基酸並不是靠近酵素的活化區，因此沒有改變活化區的結構。

英文摘要

Xylan is a major component of hemi-cellulose and is characterized by a ß-1,4-linked D-xylopyranosyl main chain that carries a variable number of neutral or uronic monosaccharide substituents. The hydrolysis of its characteristic backbone involves ß-1,4-xylanases and ß-xylosidases. Xylanases attack internal xylosidic linkages on the backbone to produce several xylooligomers. Microorganisms including fungi and bacteria often produce more than one type of xylanase.

Xylanases are used as bleach boosters in the pulp industry. In paper production, the pulp must be treated to remove the lignin component which gives the unwanted colour. The standard method of paper whitening uses environmentally unfriendly chlorine bleaches. Xylanases pretreatment reduces the quantity of chloride needed. As industrial pulping is conducted under conditions of high temperatures and high pH, the ideal xylanase for pulp bleaching would be thermostable and active at alkaline pH. So in this experiment, the specific aim is to obtain a thermostable xylanase by manipulating its gene with DNA shuffling.

From the genome sequence, a Bacillus subtilis might encode 5 different xylanases. Firstly, we used PCR to clone two xylanase genes, xynA and ynfF; which are 0.68 kb and 1.28 kb, respectively. Over-express these two genes in pKK223-3 vector and showed the molecular weight of xynA protein is 22 kDa and that of ynfF protein is 42 kDa in SDS-PAGE. Also these two proteins showed xylanase activity in zymogram gel. On the zymogram gel the ynfF protein showed discrete protease processed bands. Truncated ynfF protein with deletion of 127 amino acid at C- terminus from the HindIII recognition site did not showed protein band in SDS-PAGE. The deletion might cause the truncated protein folded wrongly and was degraded by protease immediately.

The xynA DNA was fragmented by DNaseI to produce DNA pieceses smaller than 300 bp and reassembled by PCR. In xylan-plate and RBB-xylan plate showed only 30 % of these mutated clones had xylanase activity. By measuring the size of clear zones in xylan or RBB-xylan plate, one clone, named xynA-1, had the largest clear zone. Comparing the DNA and amino acid sequence between xynA and xynA-1 showed

there are three nucleotides changes in xynA-1 DNA sequence and only one amino acid change in xynA-1 protein. The changed amino acid is the 41th amino acid from glycine to serine. In temperature effect experiment xynA-1 protein exhibited broadened optimal temperature ranges than xynA protein. Then in the thermostability experiment, the Tm value shifted from 45oC of xynA protein to 49oC of xynA-1 protein (Tm is the temperature at which 50 % inactivation occurs after heat treatment for 30 min). These two proteins have the same pH optimum at 7.

Bacillus circulans xylanase (BCX) was used as the structure model for xynA-1 protein because it has only one amino acid difference from xynA protein. The BCX structure resembles a partially closed right-hand. The increased thermostability of xynA-1 protein might be due to the newly formed hydrogen bond between the side chains of the 41th Ser and the 39th Asp residues in the first b-turn. This bonding might stabilize this b-turn which in turn can strengthen the structure of b-sheets 1 and 2 that formed the thumb. The Km, initial rate and Vmax of xynA-1 protein are similar with the wild-type protein so it shows this mutant did not affect the catalytic site. This could be predicted because the changed amino acid in xynA-1 protein is not close to the active site.