行政院國家科學委員會專題研究計畫成果報告

題目：以動力學和基因定點突變研究黃質菌 -葡萄糖甘酵素之催化機構

(Kinetic and site-directed mutagenesis study on the catalytic mechanism of Flavobacterium -glucosidase)

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一、中文摘要

本計畫針對自黃質菌中純化得之 -葡萄糖甘酵素進行分子層級之反應機構研究。研究顯示該酵素對反應基質之醣基部份有極佳之特異性，但卻可催化多種葡萄糖甘化合物。由氫核磁共振光譜觀測酵素分解 PNPG **反應可知其產物為 -形葡萄糖，可見酵素催化屬保留形之雙取代反應機構。另將酵素對不同基質所得之** k_{cat} **以** Bronsted equation **可得一向下彎曲之雙相圖** (biphasic plot)**，顯示其為兩步驟之催化反應。另** 136E/Q、137D/N **和** 169H/A **等突變酵素亦經定點突變法完成，其中** 169H/A **與原型酵素比較其** K_m **值相當，但反應性至少下降** 100 **倍以上，足見** H-169 **對此酵素催化之重要性。**

關鍵詞：黃質菌、 -葡萄糖甘酵素、反應機構、定點突變

Abstract

A *J*glucosidas from *Flavobacterium meningosepticum* has been purified and characterized. The enzyme exhibited a high specificity on the glycon portion of aryl-= -glycosides. NMR spectroscopy revealed the enzyme catalyzes hydrolysis of *p*-nitrophenyl - -glucoside with the retention of anomeric configuration, indicating that a double displacement mechanism was involved. The Bronsted relationship showed a concave-downward plot, which is consistent with the two-step mechanism. A series of site-directed mutagenesis experiments, which include 136E/Q, 137D/N, and 169H/A, have been performed to approach the essential groups of this enzyme. Kinetic studies showed the catalytic activity of 169H/A was at least 100-fold weaker than those of wild type enzyme. However, the Michealis constant for the enzyme toward *p*-nitrophenyl- -glucoside were quite similar. The preliminary results revealed that H169 of the enzyme deeply involve in the catalytic activity.

Keywords: *F. meningosepticum*; -glucosidase; mechanism; Bronsted relationship; site-directed mutagenesis

二、緣由與目的

-Glucosidase is a member of cellulases. The main function of this enzyme is to hydrolyze the product, cellobios, of other glycohydrolases in cellulose degradation system. The synergistic actions of these cellulolytic enzymes ensure the hydrolysis of cellulose effectively. In our previous studies, we have purified a -glucosidase from *Flavobacterium meningosepticum* to homogeneity. The gene encoding for this enzyme have also been cloned and sequenced. The comparison of amino acids sequence revealed that this -glucosidase is a new member of Family b3 enzyme. Since the catalytic mechanisms of the enzymes in this group have not yet been studied, we plan to elucidate the mechanistic action of this cloned enzyme in molecular level. In order to eliminate the laborious situation, the overexpression of the enzyme and the optimized purification process are the first issue in our project. Substrate syntheses are the key step in this research. More than 10 aryl- -D-glucosides will be prepared for investigating the mechanism of this enzyme.

三、結果與討論

In order to study the catalytic mechanism of the cloned −glucosidase at least 10 substrates with various leaving phenols (pK_a 4 ~ 10) were synthesized according to literatures (1~3).

Substrate specificity

A variety of glycosides, including ~-PNPG, -PNPG, -ONPG, -PCPG, -PG, -CNPG, -DNPG, -PNPM, -PNPGal, -PNPAG, -PNPX, and -PNPA, were tested as substrates. Of all the substrates investigated only the aryl -D-glucopyranosides were hydrolyzed effectively. The -*D*-pyranoside configuration is essential for effective catalysis. PNPM and PNPAG are virtually inactive. Clearly, the C-2 hydroxy group on glucopyranoside is also important. Effect of hydroxyl group inversion at C-4 (being galactosides) weakens the catalytic efficiency by a factor of 160-fold (based on relative V_{max}/K_m value). Though it is highly specific with regard to the glycone moiety, the purified enzyme shows a broad tolerance for the aglycone portion. It can accommodate various substrates, such as 2,4-dinitrophenyl, 2,5-dinitrophenyl, 3,4-dinitrophenyl, 4-chloro-2-nitrophenyl, *p*-nitrophenyl, *o*-nitrophenyl, m-nitrophenyl, 4-cyanophenyl and phenyl- -D-glucosides, with similar Michaelis constant (K_m). However, the k_{cat} values can be different up to 2.6-order of magnitude.

Extented Bronsted Plot

The extended Bronsted plot has been shown to be a valuable tool to rationalize mechanistic actions of enzymes (4~6). Based on the k_{cat} values of arylglucosides (in Table 1), an extended Bronsted plot can be constructed by plotting the logarithmic form of the relative k_{cat} values ($\log^r k_{cat}$, see Table 2) of aryl- -*D*-glucosides against the pK_as of leaving phenols. A biphasic characteristic with a concave-downward trend was clearly seen (Fig. 1). This provides an indication for a two-step mechanism: formation and breakdown of glucosy-enzyme intermediate or alternative carbonium-enzyme intermediate.

^{1}H Nuclear magnetic resonance (^{1}H-NMR) analysis of the stereoselectivity of the hydrolysis

^{1}H-NMR spectroscopy has been used to investigate the stereoselectivity of various glycohydrolases such as cellulases and xylanase (7,8). Studies revealed that glycohydrolases often present a double-displacement mechanism, which involves the retention of the anomeric configuration. However, interestingly, a -xylosidase purified from *Clostridium cellulolyticum* was shown to act by inverting the - anomeric configuration (9). To better understand the catalytic function of this purified −glucosidase, a NMR study of stereoselectivity of the enzyme was carried out. Results were shown in Figure 2. In a glucose ^{1}H-NMR study two doublets were found to be centered at ¢= 4.48 ppm (*J*=7.8 Hz) and ¢= 5.07 ppm (*J*=3.3 Hz), corresponding to the =and the ~ anomeric protons, respectively. The ratio of /~=is 64/36 when they are equilibrated. The C1 proton of PNPG was shown to be a doublet centered at 5.12 ppm (*J*=7.8 Hz). When *F. meningosepticum* -glucosidase was added to PNPG, the anomeric signal increased. At 18 minutes, a new doublet generated by mutarotation process and centered at ¢= 5.07 ppm (*J*=3.3 Hz) emerged. The ratio of -glucose to ~-glucose was 90:10. At 35 minutes, the =and ~ anomeric signals constantly increased. The ratio of to ~ was 81:19. The anomeric proton of PNPG was extinguished completely in 70 min (data not shown). This NMR study

clearly showed that *F. meningosepticum* -glucosidase catalyzes the hydrolysis of PNPG with the retention of anomeric configuration.

Possible reaction mechanism

NMR study suggested that the catalytic action of this -glucosidase involve a double displacement mechanism. Bronsted plot also supported a two-step mechanism involved. A tentative mechanism (shown in Fig. 3) was proposed for the hydrolysis catalyzed by the purified -glucosidase. The observed retention of the configuration at C-1 can be explained either by a stereospecific hydration of the carbonium-enzyme intermediate (step 2a) or by a hydrolysis of the glucosyl-enzyme intermediate in an inversed displacement manner (step 2b). In spite of the uncertainty of the transition state structure of the reaction, the bottleneck step can be unequivocally predicted from the extended Bronsted relationship. Since substrates with good leaving phenols (such as *2',4'*-dinitrophenol, *p*-nitrophenol) show no significant dependence of their reactivity on the leaving phenols' pK_as, the reaction rate-limiting step (*r.l.s.*) of the good substrates is therefore the breakdown of the intermediate whereas the *r.l.s.* of the poor substrates such as PG is the formation of the intermediate. Similar result can be seen in -glucosidase from sweet almond (10) and *A. faecalis* (11).

Site-directed mutagenesis

A series of site-directed mutagenesis experiments, which include 136E/Q, 137D/N, and 169H/A, have been performed to approach the essential groups of this enzyme. All mutants were sequenced to confirm the correct colony selection. Kinetics studies showed the catalytic activity of 169H/A was at least 100-fold weaker than those of wild type enzyme. However, the Michealis constant for the enzyme toward *p*-nitrophenyl- -glucoside were quite similar. The preliminary results revealed that H169 of the enzyme deeply involve in the catalytic activity.

四、計畫成果自評

The mechanistic study of -glucosidase from *F. meningosepticum* has been performed successfully. This enzyme is a new member of Family B glucosidase and the catalytic action in molecular level has not yet been studied. We have investigated the catalytic reaction by structure co-relation, NMR and site-directed mutagenesis. The results unequivocally demonstrated the catalytic action of the enzyme involving a two-step mechanism via most likely the formation of a glucosyl-enzyme intermediate. The amino acids mediated the enzyme function are very likely H-169 and Asp-137. The more sophisticated study is still going.

Table 1. Km and relative k_{cat} values

substrate phenol substituent	pK_a	K_m (mM)	$^{r}k_{cat}$ (M)	$\log {}^{r}k_{cat}$	$\log {}^{r}k_{cat} / K_m$
2,4-dinitro	3.96	0.372	1.57E-6	-5.80	-2.38
2,5-dinitro	5.15	0.325	1.10E-6	-5.99	-2.51
3,4-dinitro	5.36	0.150	7.00E-7	-6.15	-2.33
4-chloro-2-nitro	6.45	0.788	1.42E-6	-5.85	-2.74
4-nitro	7.18	0.815	5.48E-7	-6.26	-3.17
2-nitro	7.22	1.470	5.62E-7	-6.25	-3.42
4-cyano	8.49	0.801	3.22E-8	-7.49	-4.39
H	9.99	1.100	4.19E-9	-8.38	-5.42

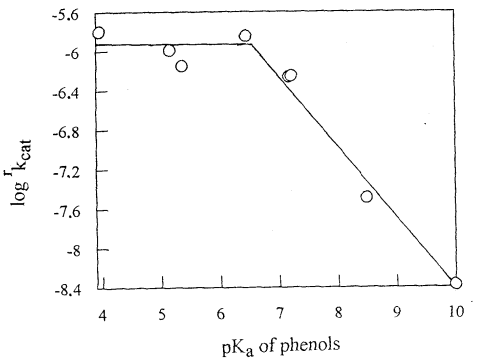

Fig. 1. Bronsted plot displays a concave-downward trend.

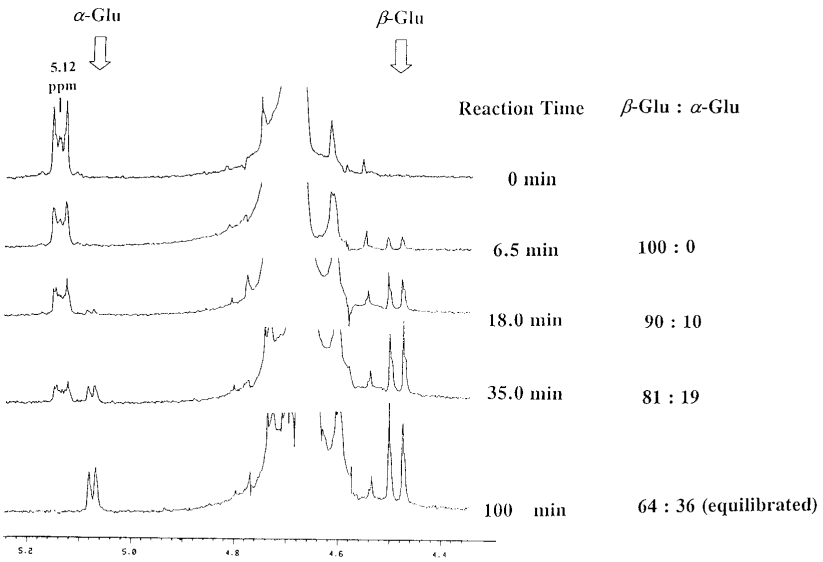

Fig.2 ^{1}H NMR analysis of the stereoselectivity of the reaction catalyzed by β-glucosidase from *F. meningosepticum*. A suitable amount of enzyme was applied on 2.5 mM PNPG at pD = 7.1. The reaction was monitored about every 10 minutes after addition of the enzyme.

1.

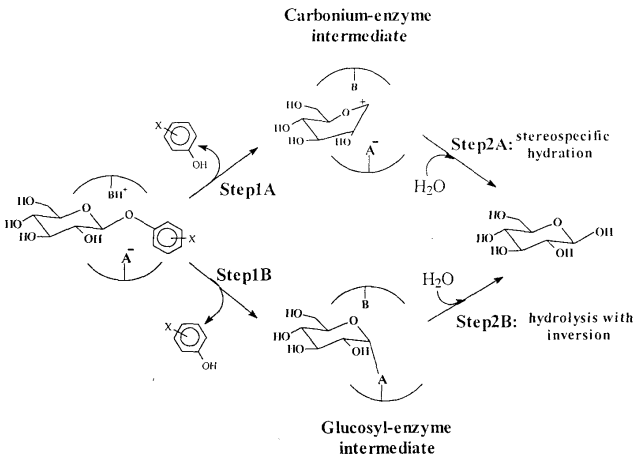

Fig. 3. Proposed two-step reaction mechanism of β-glucosidase hydrolysis. The pathways A and B lead to the formation of carbonium-enzyme and glucosyl-enzyme intermediate, respectively. Step 1 is the formation of the intermediate while step 2 is the breakdown of the intermediate.

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