行政院國家科學委員會補助專題研究計畫成果報告

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※　以定向演化研究 -葡萄糖甘酵素之活性區及其非　※
※　　　　　自然演化之糖甘類酵素　　　　　　　　※
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執行單位：交通大學應用化學系

中　華　民　國　89　**年**　10　**月**　28　**日**

摘要

利用定向演化 (directed evolution) 的技術我們已將β-葡萄糖甘酵素改變成β-半乳糖甘酵素，並經多支層析管柱分離一分子量約為 80,000 的蛋白質，此新酵素已完全喪失原先之葡萄糖甘酵素活性。在β-葡萄糖甘酵素之反應機構研究方面，我們利用 NMR、基質反應性 (substrate reactivity)、二級同位素效應 (secondary kinetic isotope effect) 並結合定點突變 (site-directed mutagenesis) 之蛋白質基因工程技術，已成功的解析此酵素之催化機構且確認天冬門酸-247 (Asp-247) 為主要胺基酸之一，於催化中扮演親核性攻擊(nucleophilic attack) 的角色。由研究結果顯示，因產物為保留型式，且 Bronsted plot 呈向下之雙相圖 (biphasic concave-downward) 此酵素之機構為兩步驟反應:第一步為酵素共價糖基化，第二步為糖基化酵素之去糖基反應，此亦為核磁共振研究得到証實。第一步反應為 S_N2-like，反應達過渡狀態時離去基與葡萄糖之鍵應已幾乎斷裂，且天冬門酸應幾乎已與糖基形成共價鍵，亦即其為 late transition state; 第二步反應為 S_N1-like，亦即達過渡狀態時糖基之碳一 (C1) 應呈正電狀態，即反應過渡態為 carbocation (或其共振態 oxocarbonium ion)。

Abstract

Directed evolution is a new technique, which is developed to enhance the enzyme activity or convert the cloned enzyme to another. We now apply this new technique on the study of enzyme active site topology. Plasmid DNA (designated as pCR/bgl) was constructed with a insertion of 2.7 kb DNA fragment containing 2.2 kb -glucosidase gene. This plasmid was transformed in *XL1-red E. coli* strain and cultured for several generations, which was then subjected to screening the mutant strain with -galactosidase activity by X-gal LB-plate. 8 colonies were found to possess strong activity. One of the mutant enzymes with high activity were expressed, purified, and characterized. Also, The mechanistic study of the wild-type -glucosidase was performed by means of NMR spectroscopy, substrate reactivity, secondary kinetic isotope effect. The stereochemistry of enzyme catalyzing the hydrolysis of *p*-nitrophenyl- *JD*-glucopyranoside was unequivocally identified as occurring with retention of anomeric configuration. Based on the k_{cat} values of a series of arylglucosides, a Bronsted plot was constructed with the =-0.85 (Bronsted constant) for poor substrates (pK_a of leaving phenols > 7) and ~0 for good substrates. Indicate that a two-step mechanism is involved in catalytic rection. The secondary deuterium kinetic isotope effects with 2,4-dinitrophenyl- -D-glucopyanoside, *o*-nitrophenyl- -D-glucopyanoside, and *p*-cyanophenyl- -D-glucopyanoside as substrates are 1.17 0.02,

1.19 ±0.02, and 1.06 ±0.02, respectively. An S_N1-like mechanism for deglucosylation step and an S_N2-like mechanism for glucosylation step are proposed. The site-directed mutagensis study for investigation of the essential amino acid residue was also performed. The reactivity (k_{cat}/K_m) of D247N mutant is 200,000-fold weaker than that of wild type, whereas, the D247E mutant remains 20% of the wild type activity indicating that Asp-247 is likely to be one of the essential amino acids which function as a nucleophile in the catalysis. The irreversible inactivation of the enzyme by condurito B-epoxide also supports this conclusion.

Introduction

*J*glucosidase (EC 3.2.1.21) catalyzes the hydrolysis of the *J*glucosidic linkages of glucosides. A large extent of these enzymes from bacteria (1-3), fungi (4-7), and plant (8-10) have been purified and studied. Many of them have been cloned (11-21). By computer analysis of protein sequences, -glucosidase can be classified into two families: 1 and 3 (22,23) [or family A and B correspondently by Roja's classification (24,25)]. In addition to the biotechnological importance of this group of enzymes (26-29), study of their catalytic mechanisms provides a way to understand the catalytic power of the enzyme designed by nature.

Many mechanistic studies on -glucosidase have been performed (30-34). However, the studies were largely focused on the family 1 enzyme. Perhaps the most extensive study on family 1 -glucosidase (from *Agrobacterium faecalis*) was carried out by Withers' group. The techniques employed for elucidating the mechanism of the enzyme and the topology of the active site include pH dependences, inhibitions, secondary deuterium isotope effect, and structure-reactivity (33), essential amino acid labeling with fluorosugar (35), reaction with deoxy substrate analogues (36), and site-directed mutagenesis (37,38). On the basis of their studies and many of others, the general conclusions of the family 1 enzymes were re-strengthened. In summary, this group of enzymes is retaining enzymes, which catalyze the hydrolysis of their substrates with retention of the anomeric configuration. The catalytic reaction is identified as a two-step, double displacement mechanism involving two essential carboxylic acid residues, functioning as general acid/base and nucleophile. Though the

action of family 3 enzymes is thought to be similar to that of family 1, a detail kinetic investigation combining with the inspection of essential residue by site-directed mutagenesis was much less reported, presumably, at least partially, due to the unavailability of a suitable, cloned bacterial enzyme and the x-ray structure.

Recently, the family 3 hydrolases gained a great potential for making progress on the mechanistic study owing to the complete resolution of a three-dimension structure of a barely -glucosidase (39). On the basis of the structure, Asp-285 and Glu-491 were suggested to be the candidates for the nucleophile and the general acid/base catalyst, respectively. Thought the function as nucleophile of Asp-285, which is conserved in the SDW sequences of family 3 enzymes, was supported by the active site affinity labeling (20, 30), it has not yet been confirmed by means of site-directed mutagenesis. For better understnading the detail mechanism and the active topology of the Family 3 hydrolases, a cloned and expressed enzyme is essential. The *J*glucosidase from *Flavobacterium meningosepticum* is one of the suitable candidates. The complete purification of the *F. meningosepticum* *J*glucosidase (denote as fbgl)(40) and gene cloning of the enzyme (41) has reported. In this study, we combined the physical and chemical studies to investigate the catalytic mechanism of the enzyme in molecular level and proposed the Asp-247 functioning as the essential nucleophile by site-directed mutagenesis. We also applied DNA shuffling and random mutagenesis techniques to directly approach the active site topology of the enzyme by converting this -glucosidase into -galactosidase. The un-nature -galactosidase was expressed, purified and characterized.

Results and discussions

(A) Directed evolution for trnasforming -glucosidase into -galactosidase The plasmid containing of β-glucosidase gene was transformed in *XL1-red E. coli* strain and cultured for several generations, which was then subjected to screening the mutant strain with -galactosidase activity by X-gal LB-plate. 8 colonies were found to possess strong activity. One of the mutant enzymes with high activity were expressed, purified, and characterized. The un-nature -galactosidase was purified by several chromatographic steps including Q, SP, HIC, and G200.

Interestingly, the mutated enzyme barely binds to Q or SP columns but tightly to HIC. Clearly, its protein surface property is totally different from its original -glucosidase. The molecular mass is comparable to the original -glucosidase (80 kDa). Its catalytic activity is totally converted into -galactosidase without any detectable -glucosidase activity. It also possesses significant ~-*L*-arabinosidase activity (10% that of -galactosidase). Unfortunately, the plasmid DNA is unstable. The identification of the gene sequence was delayed. Figure 1 showed the methodology of screening this new -galactosidase from the mutated DNA library and the result after transformation. Blue colonies were thought to bare the mutated NDA with -galactosidase coding gene.

(B) Mechanistic study of -glucosidase

Substrate specificity and reactivity

A variety of glycosides, including PNP-Man, PNP-Gal, PNP-Ac-Gln, PNP-Xyl, PNP-Ara, ~-PNP-Glu, and a series of aryl -glucosides were employed for investigating the substrate specificity and the reactivity of the expressed wild-type enzyme. Of all the substrates investigated only the aryl -D-glucopyranosides were hydrolyzed effectively. The activities for non-glucoside substrates were less 0.05% of that of PNP-Glu. This is distinguished from that of *Agrobacterium* -glucosidase, a family 1 enzyme, which possesses noticeable activities towards PNP-Gal (2.5% of that of PNP-Glu) and PNP-Ara (4.6%) (33). This broad glycosides specificity was also found in another family 1 -glucosidase from sweet almond. It was considered as a bi-functional enzyme with both -glucosidase and -galactosidase activities (46-48). Though fbgl is highly specific on the glycon moiety of substrate, the enzyme shows a broad specificity for the aglycon portion. It accommodates -*D*-glucosides with various aryl groups for the aglycon moiety. Kinetic parameters for those of aryl -D-glucopyranosides are summarized in table 1. T hough the K_m values for all substrates are comparable, the k_{cat} values are different up to 2.6-order of magnitude. Analyzing the data allow us to investigate the catalytic mechanism of the enzyme by means of linear-free energy relationship, namely, Bronsted relationship. The extended Bronsted plot has been shown to be a valuable tool to rationalize mechanistic actions of enzymes (49-51). Based on the k_{cat} values,

an extended Bronsted plot can be constructed by plotting the logarithmic form of the k_{cat} against the pK_as of the leaving phenols (Figure 2). A biphasic plot with a concave-downward trend was obtained with the slopes ($_{lg}$ value) near zero in the lower pK_a (<7.0) and –0.89 in the higher pK_a (>7.0). Clearly, the enzymatic reaction involves a two-step mechanism with the formation of an intermediate, most likely the glucosy-enzyme intermediate. Since substrates with good leaving phenols (such as DNP-Glu, CNP-Glu) show no significant dependence of their reactivity on the leaving phenols' pK_as, the rate-limiting step (r.l.s.) of the reaction for good substrates is therefore the breakdown of the intermediate whereas the r.l.s. of the poor substrates is the formation of the intermediate. The slope of the leaving-dependent portion of the Bronsted plot ($_{lg}$ = -0.89) indicates a large degree of glycosidic bond cleavage at the transition state.

^{1}H-NMR analysis of the stereoselectivity

1H-NMR spectroscopy has been successfully used for investigation the stereoselectivity of various glycohydrolases. The studies reveal the diversity in mechanism of glycohydrolases(25-27). To better understand the catalytic action of fbgl, the NMR study of stereoselectivity of the enzyme was carried out. Results were shown in figure 3. In a glucose 1H-NMR study two doublets were found to be centered at ¢ = 4.38 ppm (J=7.8 Hz) and ¢ = 4.98 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 2,4-DNPG was shown to be a doublet centered at 5.20 ppm (J=7.8 Hz). When fbgl was added to 2,4-DNPG, the anomeric signal appeared. At 20 min, a new doublet generated by mutarotation process and centered at = 4.98 ppm (J=3.3 Hz) emerged. The ratio of -glucose to α-glucose was 90:10. The anomeric proton of DNPG was extinguished completely in 90 min and the ~ ratio of glucose approached to 64/36. This result clearly showed that fbgl catalyzes the hydrolysis of DNPG with the retention of anomeric configuration and also provided a clue for supporting a two-step mechanism of the enzyme. The catalytic mechanism of family 3 enzymes is likely to be quite similar to those of family 1 glycohydrolases.

Secondary kinetic isotope effect

Kinetic ~-deuterium isotope effect has been considered as a useful tool for distinguishing between S_N1 and S_N2 mechanisms. It provides the way to

investigate changes in hybridization at the substituted site in proceeding from the ground state to the transition-state of the reaction. Isotope effects k_H/k_D >1 are expected for S_N1-like mechanism, while effects near unity were thought to be S_N2-like reaction. Secondary deuterium kinetic isotope effects (k_H/k_D) upon the deglycosylation of various glycosidases, such as: -galactosidase and -glucosidase, were shown to range from 1.25 to 1.09 (28-30). For these enzymes, the transition-state of the reactions was believed to possess substantial carbocation (or alternatively oxocarbonium ion) character. In this study, the secondary deuterium kinetic isotope effects of the cloned -glucosideas were carefully measured with DNP-Glu, ONP-Glu, and PCP-Glu to be 1.17 ±0.03, 1.19 ±0.02, and 1.04 ±0.02, respectively. The large isotope effect for good substrates provides strong evidence that the intermediate of the enzymatic reaction is a covalent glucosy-enzyme intermediate instead of an ion-pair complex, for which an inverse isotope effect is expected. In addition, the large isotope effect also indicates that the hydrolysis of glucosyl-enzyme intermediate is S_N1-like mechanism indicating that relatively large amount of carbocation character occurs at the transition state. In contrary to those of good substrates, a smaller isotope effect was observed for PCP-glu (a poor substrate) suggesting that an S_N2-like mechanism, i.e. a relatively small amount of carbocation charater at the transition state, is involved in the glucosylation step. In combining with the observation of Bronsted plot for higher pK_a range, $_{lg}$ = -0.89, the mechanism of glucosylation step is consistent with a relatively late transition state process. The Nucleophilic essential group of the enzyme has largely associated at the C1 position of substrate when the reaction is approaching to transition state, where the glucosidic bond of substrate is cleaved extensively. In summary of the results described above, the catalytic action of fbgl involving two-step, glucosylation and deglucosylation, mechanism was shown in scheme I. The glucosylation step involes more S_N2-like mechanism and possesses a relatively late transition state. Whereas, the deglucosylation step is more S_N1-like with carbocation (or its resonant oxocarbonium ion) as the transition state (TS 2). The mechanism of fbgl was found to be

highly consistent with that of family 1 enzyme.

Kinetics of Asp-247 mutants

The catalytic reaction of fbgl is identified as a two-step, double displacement mechanism. Two amino acid residues, functioning as general acid/base and nucleophile, are involved. Based on the multialignment of amino acid residues of some family 3 enzymes found that a "SDW", sequence is conserved, shown in figure 4. The aspartate residue has considered as the nucleophile for *A. niger* (20) as well as for *A. wentii* -glucosidase (30) by active site affinity labeling. Though the correspondent sequence in fbgl is "TDY", it is highly homogenous to "SDW". A group of mutant enzymes were constructed for investigating the role of at Asp-247 in fbgl by site-directed mutagenesis. The aspartate residue has changed to asparagin, glutamate, and glycine. These mutations have no significant effect on protein's conformation as indicated in CD spectra shown in figure 5. All purified mutants were assayed against 2,4-DNP-Glu. The kinetic parameters were summarized in table 2. As can be seen, values K_m of each mutant are quite similar with only few folds of variations. The catalytic efficiency (k_{cat}/K_m) of D247G and D247N is largely reduced by a factor of $3x10^4$ and $2x10^5$, respectively. However, the D247E mutant keeps at least 20 % activity, as compared with the wild type enzyme. This activity variation provides an evidence for drawing a reasonable conclusion that Asp-247 plays an important role on the enzymatic catalysis.

Inhibition study.

Conduritol-B-epoxide (CBE) was widely used as active site-directed inhibitor for -glucosidase (52-54). In order to elucidate the catalytic essential amino acid residues, the wild type of fbgl was inactivated by CBE. As presented in figure 6a, the inactivation is concentration–dependent.

Based on the time-dependent inactivation of fbgl with 1 to 5 mM of CBE, the kinetic parameters of k_i, K_i, and k_i/K_i were determined to be 0.014 s^{-1}, 8.9 mM, and 1.6 $M^{-1}s^{-1}$, respectively (figure 6b). However, by changing the Asp-247 residue into glutamate, the inactivation rate of the D247E mutant was extremely slower than that of wild type enzyme, even at the condition of 8.5 mM CBE [data shown at the top of figure 6a]. This result suggested that the Asp-247 residue is involved in catalysis and most likely functions as the nucleophile. The replacement of glutamate residue on fbgl

results in a weaker binding toward ¢-gluconolactone with a loss of binding energy of 2.5 Kcal/mole (see table 3). A slightly weaker binding of ¢-gluconolactone also observed in the case of D247G mutant with 8-fold larger than that of wild type enzyme. However, as compared the reversible inhibition constant, K_i, the binding affinity of CBE on the wild type and mutant enzymes were nearly identical (table 3). The observation may consistent with the fact that Asp-247 residue interacts with ¢-gluconolactone as the meaner similar to the enzymatic reaction when approaching the transition state of the deglucosylation step. The longer side chain derives from the mutation of D247E not only reduces the space in active site but also changes the direction of the carboxylic group, which is responsible for the nucleophilic catalysis at glucosylation step.

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Table 1. Michaelis constants and relative k_{cat} values of aryl- -*D*-glycopyransosides[a]

Substrate	pK_a of leaving phenols	K_m, mM	k_{cat}, s^{-1}	Log k_{cat}
2,4-DNP-Glu	3.9	0.37	36.4	1.56
2,5-DNP-Glu	5.15	0.33	25.7	1.41
3,4-DNP-Glu	5.36	0.45	16.2	1.21
CNP-Glu	6.45	0.79	32.4	1.51
PNP-Glu	7.18	0.65	12.4	1.09
ONP-Glu	7.22	0.47	12.7	1.10
MNP-Glu	8.39	0.82	0.8	-0.10
PCP-Glu	8.49	0.75	0.7	-0.15
PKP-Glu	9.38	0.68	0.25	-0.61
P-Glu	9.99	1.10	0.09	-1.03

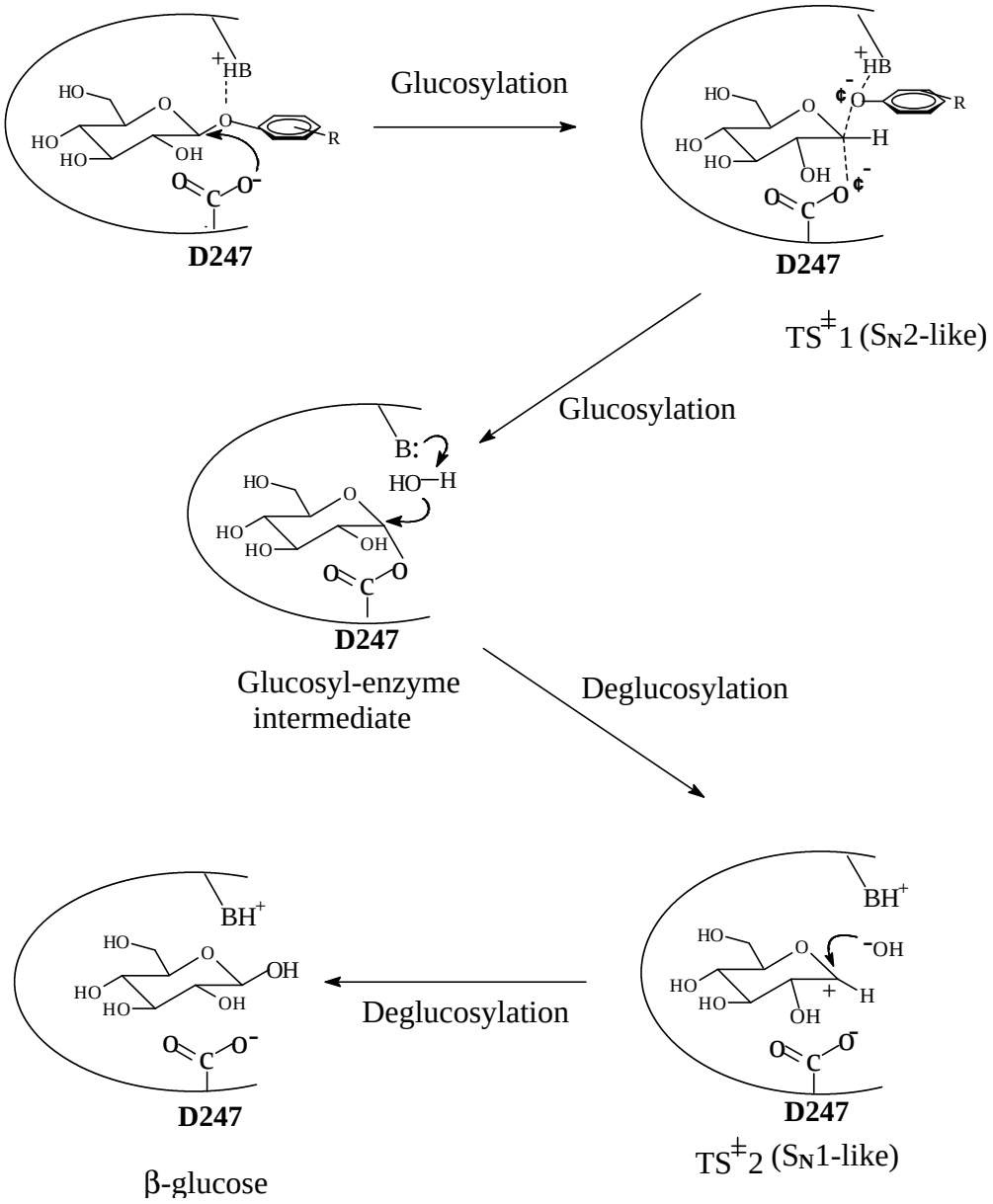
a : K_m values measured at pH 6.8.

Table 2. Apparent activity of wild-type and mutants of -glucosidase towards 2,4-DNP-Glu

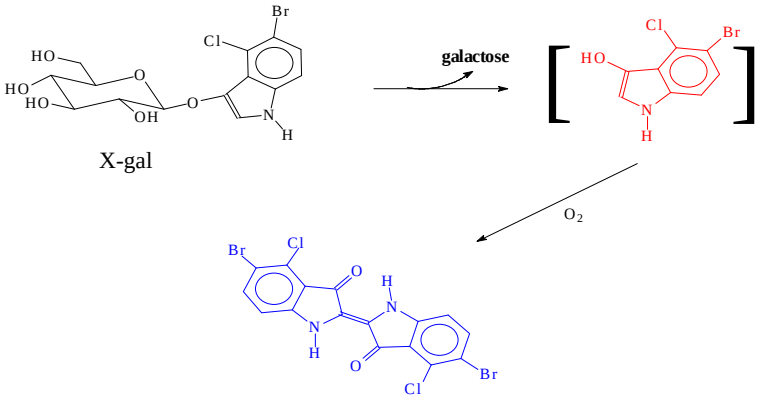
Enzyme	K_m(mM)	k_{cat}(s^{-1})	k_{cat}/K_m($s^{-1}mM^{-1}$)
Wild type	0.37	36.4	98.4
D247G	0.24	4.8	20.0
D247N	1.50	$7.0x10^{-4}$	$4.7x10^{-4}$
D247G	0.23	$7.1x10^{-4}$	$3.1x10^{-3}$

Table 3. Inhibition constants determined for wild-type -glucosidase and D247E mutant

Inhibitor	Wild type	D247E	D247G
δ-gluconolactone	0.0015 mM	0.095 mM	0.012 mM
Conduritol-B-epoxide	8.9 mM (K_i)	9.4 mM	6.2 mM
	$0.014s^{-1}$ (k_i)		

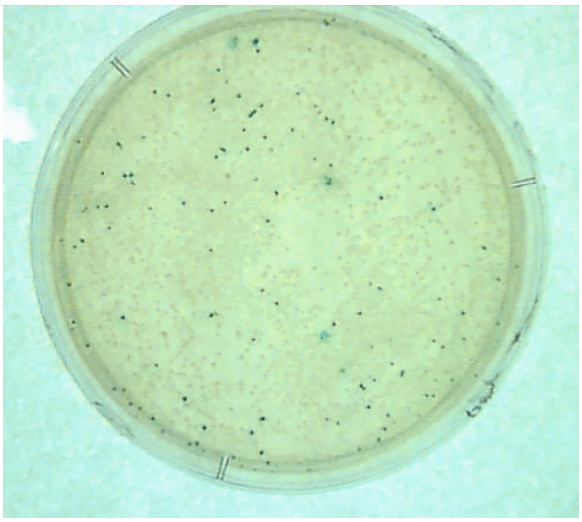

Glucosylation
D247
TS‡1 (SN2-like)
Glucosylation
Glucosyl-enzyme intermediate
Deglucosylation
TS‡2 (SN1-like)
Deglucosylation
β-glucose

Scheme I



X-gal

Indigo Blue



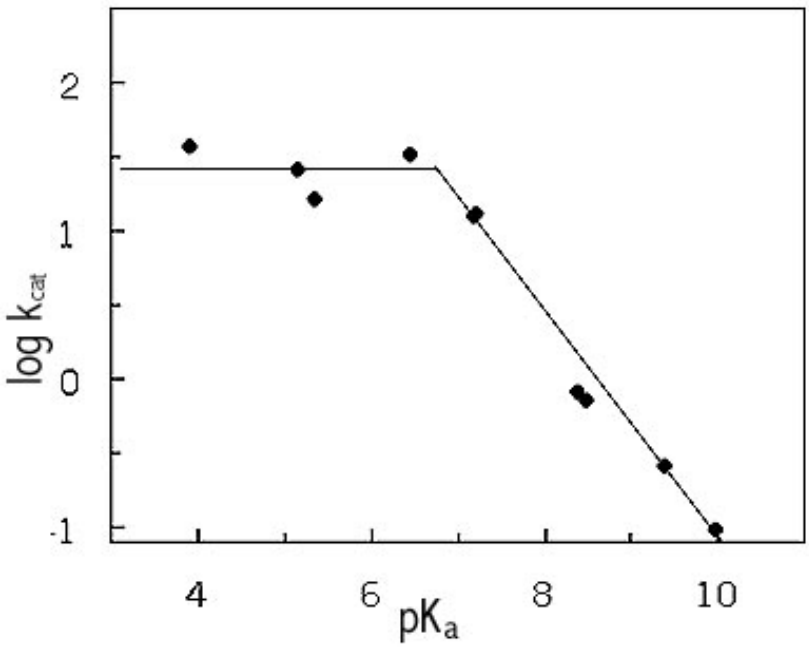


Figure 2.

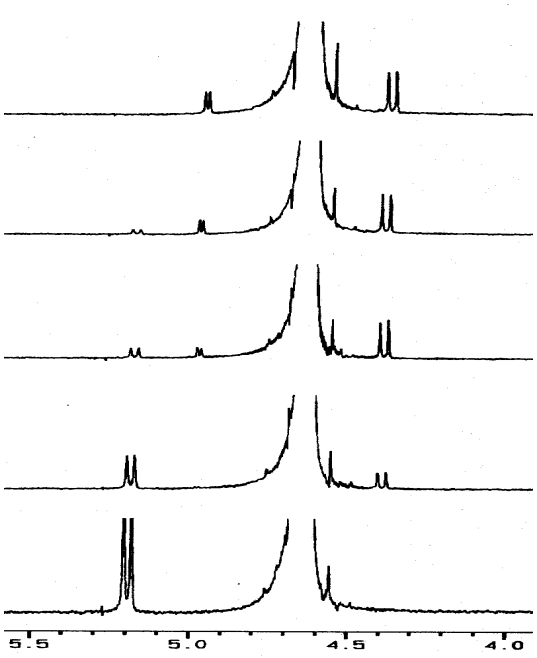


Figure 3.

Kfr	DGMLM**SDW**FGT
Atu	DGVVM**SDW**FGS
Cth	DGFVV**SDW**GAV
Barley	KGFVI**SDW**EGI
Fme	NGFIV**TDY**TGI

Figure 4. Conserved sequence of family3- -glucosidase

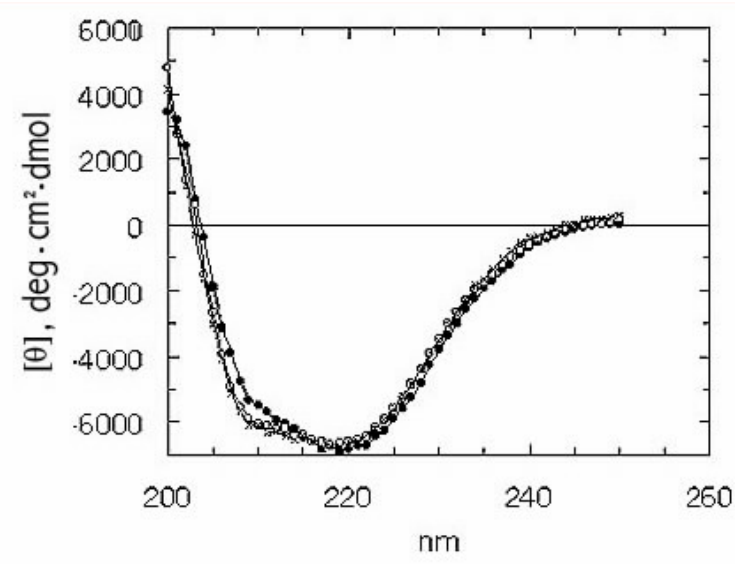


Figure 5. CD spectra of native fbgl and its mutants.

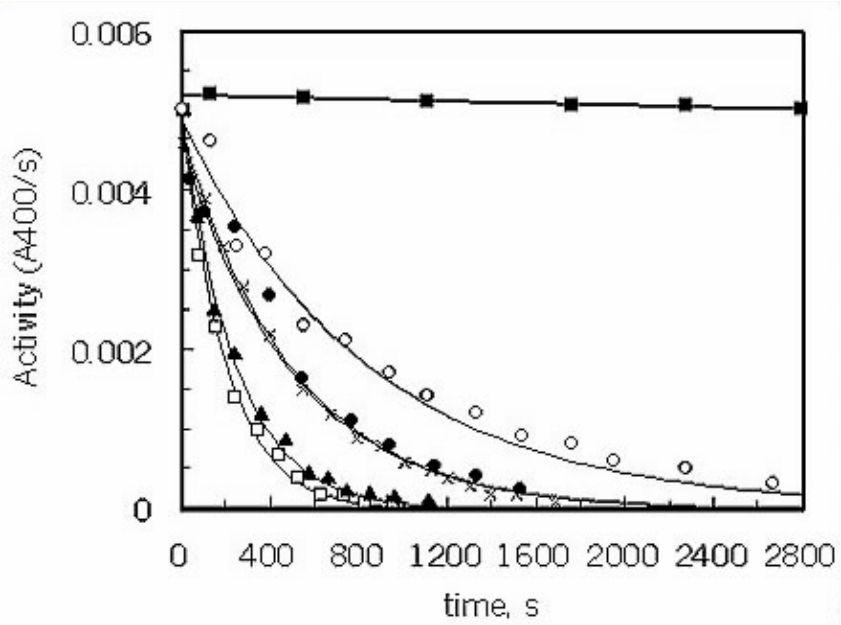


Figure 6a

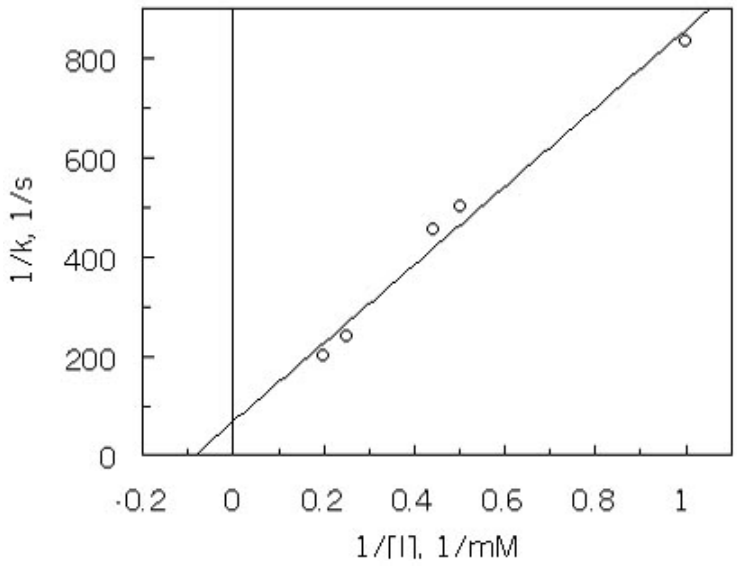


Figure 6b

計畫執行自評：

本計畫中我們以蛋白質基因工程技術結合傳統酵素動力學方法，再輔以化學合成方法，已將 -葡萄糖甘酵素之反應機構解析至分子層級，並已確認催化反應中最重之胺基酸為天冬門酸-247。在新突變酵素方面已得數個半乳糖甘酵素的 clone，並純化得其中一酵素。由於質體 DNA 呈不穩定現象而暫時無法定序以瞭解那些胺基酸的變異可導致酵素持性的變化，此部分仍將繼續努力。