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Enzymatic deglycosylation of enfumafungin, a triterpene glycoside natural product, and its chemically synthesized analogues

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Abstract

A panel of 27 commercial enzymes was screened for the deglycosylation of a triterpene glycoside (enfumafungin), an antifungal natural product with a novel mechanism of action that bears an ester, a hemi-acetal and a carboxylic acid functionalities in its structures. Only one enzyme, a recombinant β-D-glucosidase, was identified which catalyzed the desired deglycosylation with the formation of the aglycone moiety that also bears an ester, a hemi-acetal and a carboxylic acid functionalities. Several chemical analogues of this natural product, whose ester moieties had been removed by chemical hydrolysis, were also screened and shown to be susceptible to deglycosylation by eight of the enzymes in the panel. These enzymes included one β-glucuronidase and six thermophilic glycosidases in addition to the recombinant β-D-glucosidase. Scaled-up synthesis of the intact aglycones, using recombinant β-D-glucosidase and β-glucuronidase, reaffirmed the catalytic utility of selected enzymes in large-scale organic synthetic reactions.

Keywords: Triterpeneglycosides; Antifungal natural products; Enzymatic screening; Deglycosylation; Glycosidases

1. Introduction

Merck scientists have recently identified an antifungal natural product, enfumafungin [1], with a novel mechanism of action [2]. This compound, a triterpene glycoside (glucoside) with glucose at C-3 position, is acetylated at C-2 and bears a cyclic hemi-acetal and carboxylic acid functionality at the C-10 and C-14 positions, respectively. After preparation and antifungal activity evaluation of numerous analogues, including those shown in Scheme 1, of the parent natural product, it became apparent that the deglycosylated form of the glycoside, where the acetate, cyclic hemi-acetal and carboxylic acid functionalities remained intact, would be a desired starting material for the synthesis of more derivatives [3]. To this end, a panel of commercial enzymes including various hydrolases and meso- and thermophilic glycosidases were selected and screened for the direct deglycosylation activity. Here we report the identification of one enzyme, a recombinant β-D-glucosidase, which catalyzed the hydrolysis of the glycosidic linkage of the parent glycoside with no loss of the labile acetate. We also report the identification of six thermostable enzymes and one β-glucuronidase from a mollusk (*Helix pomatia*) which could only deglycosylate the deacetylated forms of the parent glycoside.

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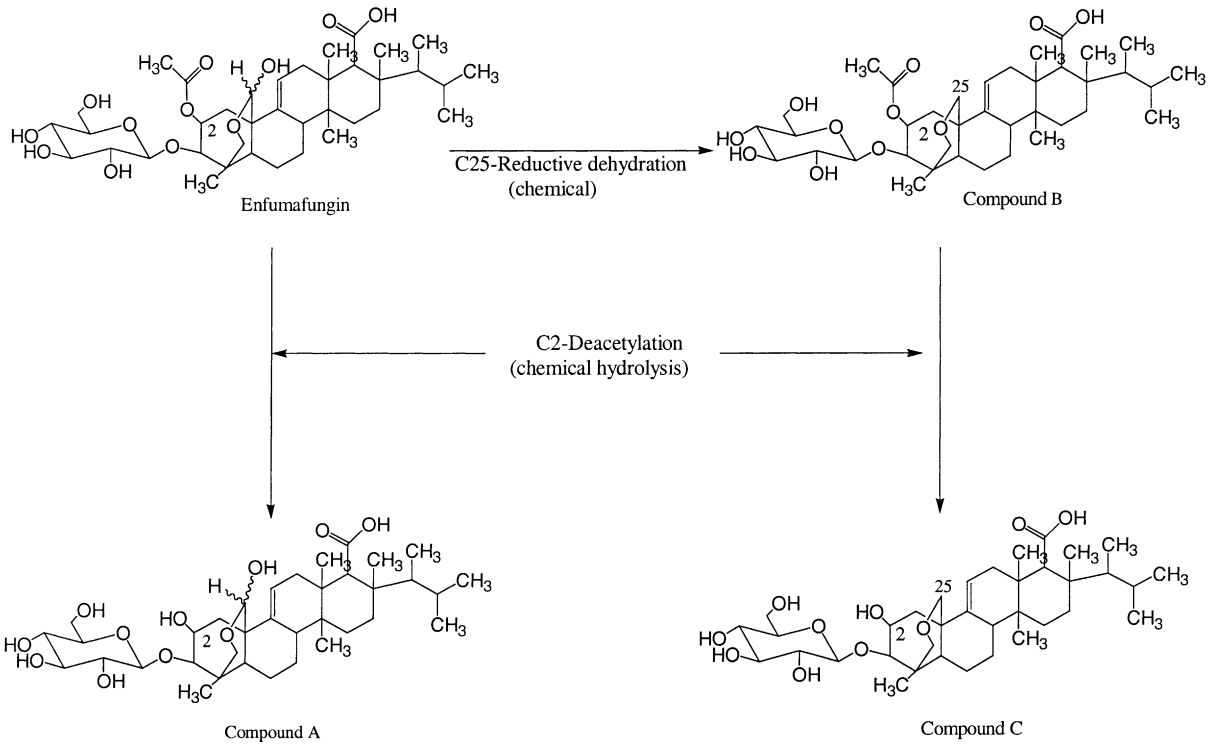


Scheme 1. Structures of enfumafungin and its chemically synthesized analogues.

2. Experimental

2.1. Enzyme sources

Seventeen commercial enzymes consisting of α- and β-glucosidases, β-glucuronidase, α-amylase and others were purchased from various enzyme suppliers. A recombinant β-glucosidase, expressed in *Escherichia coli*, was obtained from Calbiochem (LaJolla, CA). A CloneZyme library (Diversa Inc., San Diego, CA) consisting of 10 thermostable glycosidases (thermopile), identified as Gly001-01 to Gly001-10, were purchased from Sigma.

2.2. Assay development

2.2.1. Commercial hydrolases

An aliquot of each enzyme was dissolved in 0.99 ml of 0.1 M Na acetate buffer, pH = 6. An amount of 10 μl of DMSO solution (50 mg/ml) of the desired substrate was then added to the solutions of various enzymes and the resulting mixtures were incubated in a waterbath at 35°C. At various time intervals, an aliquot from each of the reaction mixtures was withdrawn and used to evaluate the formation of the desired product by HPLC system.

2.2.2. Thermostable glycosidases

In this case, the enzymatic assays were carried out according to the manufacturers' suggested procedures with appropriate modifications. Briefly, each enzyme was dissolved in 0.99 ml of MES buffer (2-(*N*-morpholine)ethanesulfonic acid), pH = 6. An amount of 10 μl of DMSO solution (10 mg/ml) of the desired substrate was then added to each vial containing enzyme solution. The mixtures were incubated and evaluated for product formation as described above.

2.3. HPLC monitoring of the enzymatic catalysis

For enzymatic screening, an aliquot of each enzymatic reaction mixtures was analyzed on an analytical

C-8 reverse-phase HPLC column (Inertsil, Analytical, Mahawa, NJ). The column was developed with either isocratic (65% aqueous acetonitrile plus 0.1% TFA) or a linear gradient solvent systems. In the gradient solvent system, the concentration of acetonitrile (0.1% TFA) in water (0.1% TFA) was raised from 50 to 95% in 20 min. In both isocratic and gradient a flow rate of 1 ml/min at the column temperature of 55°C was used. The elution from the column was monitored by a Diode Array equipped detector at 210 nm.

2.4. Large-scale enzymatic synthesis of deglycosylated enfumafungin and its chemically synthesized analogues

2.4.1. Deglycosylation of enfumafungin

An amount of 0.1 ml DMSO solution of compound enfumafungin (200 mg/ml) was suspended in 8.9 ml of 0.1 M Na acetate buffer, pH = 6, in a 50 ml conical flask and sonified. To the resulting suspension was then added 1 ml of 0.1 M Na acetate buffer solution of a recombinant β-glucosidase (10 mg, 14 U/mg, Cat. no. 347350, CalBiochem, San Diego, CA). The reaction mixture was incubated at 37°C on a shaker with 220 rpm gyratory motion. After 54 h of incubation, the content of the reaction flask was brought to 50% in acetonitrile, and after pH adjustment to 5, applied on an activated C-18 reverse-phase semi-preparative column (Applied Separation, Allentown, PA). The desired deglycosylated enfumafungin was eluted with 75% aqueous acetonitrile with 45% recovery. The product was characterized by proton and carbon NMR, and mass spectrometry.

2.4.2. Deglycosylation of compound A

Two enzymes, namely, the recombinant β-glucosidase from Calbiochem and the β-glucuronidase from *H. pomatia* (Sigma, St. Louis, MO) were found to be suitable for the scale-up of the deglycosylation of compound A. Reaction with recombinant β-glucosidase (10 mg) was carried out in 0.1 M Na acetate, pH = 6 (19.75 ml) containing 50 mg of compound A which was added as DMSO solution (0.25 ml). The final reaction mixture was incubated, worked-up and its extract was purified by similar procedures as described for enfumafungin. The purified deglycosylated form of compound A was recovered from 75 to 100% acetonitrile cut with 36% recovery.

Reaction with the β-glucuronidase from *H. pomatia* was run under condition similar to the reaction of recombinant β-glucosidase except in this case 7 ml of the enzyme solution was used. The purified deglycosylated product was recovered in the 75–100% acetonitrile cut with 48% recovery.

2.4.3. Deglycosylation of compounds B and C

Similar to the previous reaction, both the recombinant β-glucosidase (5 mg) and β-glucuronidase (2 ml) catalyzed the deglycosylation of compounds B and C. For scale up in this case, 5 mg of compound B and 8 mg of compound C, each dissolved in 0.1 ml DMSO, was added to 4.9 ml of 0.1 M Na acetate buffer, pH = 6, and the reaction was carried out and worked-up as before. The deglycosylated products were recovered from 100% methanol wash of the C-18 reverse-phase column after washing the column with 100% acetonitrile, with 54 and 42% recovery, respectively.

2.4.4. Mass spectrometry and NMR analysis

LC–MS analysis was performed on an LCQ instrument (Finnigan Co., San Jose, CA) using electrospray ionization (ESI). NMR spectra were recorded on a Varian XL 300 or Unity 500 NMR spectrometers. The chemical structures of all the enzymatically synthesized products that are reported in this paper were characterized by proton NMR and mass spectrometry.

3. Results and discussion

Enzymes show a wide variety of catalytic selectivities under various reaction conditions including aqueous and organic solvents. To exploit these properties for the specific deglycosylation of enfumafungin, a panel of 17 enzymes, consisting of glycosidases, glucuronidases and other hydrolases, were obtained from various enzyme vendors. Ten thermostable glycosidases that are reported [4] to function at high temperatures with promising and sometimes unexpected catalytic potentials were also included in the panel (Table 1).

3.1. Enzyme screening

Our panel of enzymes was screened for their deglycosylation potential of enfumafungin, utilizing

Table 1
List of the hydrolytic enzymes that were used to screen for the specific deglycosylation of enfumafungin and its chemical derivatives

Enzymes	Source/specificity	Supplier
Gly001-01	β-D-Glucoside	Diversa (Sigma)
Gly001-02	β-D-Glucoside	Diversa (Sigma)
Gly001-03	β-D-Glucoside	Diversa (Sigma)
Gly001-04	β-D-Glucoside	Diversa (Sigma)
Gly001-05	β-D-Glucoside	Diversa (Sigma)
Gly001-06	β-D-Glucoside	Diversa (Sigma)
Gly001-07	β-D-Glucoside	Diversa (Sigma)
Gly001-08	β-D-Glucoside	Diversa (Sigma)
Gly001-09	β-D-Glucoside	Diversa (Sigma)
Gly001-10	β-D-Glucoside	Diversa (Sigma)
β-Galactosidase	Bovine testes	Boehringer Mannheim
α-Glucosidase	Recombinant *S. cervisiae*	Boehringer Mannheim
Amyloglucosidase	α-Linked polysaccharide/*Aspergillus niger*	Boehringer Mannheim
Lysozyme	α-Linkage/hen egg white	Boehringer Mannheim
β-Glucosidase	Recombinant, *E. coli*	Calbiochem
α-Amylase	Porcine pancreas	Calbiochem
β-Glucuronidase	*H. pomatia*	Calbiochem
α-Glucosidase	Baker's yeast	Sigma
β-Glucosidase	Almonds	Sigma
β-Glucosidase	Recombinant	Sigma
β-Glucuronidase	Bacterial	Sigma
β-Glucuronidase	Recombinant	Sigma
Lyticase	Poly(β-1,3-glucose)	Sigma
Lyticase	Recombinant	Sigma
Naringinase	*Penicillium decumbens*	Sigma
Laminarinase	*Penicillium* species	Sigma
β-Glucuronidase (glucurase)	Bovine liver	Sigma

the standard assay conditions for their natural substrates. From the 27 enzymes that were screened, only one, a recombinant β-glucosidase mediated the deglycosylation of enfumafungin. This enzyme, which is produced by recombinant technology in *E. coli*, is reported [5] to have a molecular weight of 210 kDa and catalyses the hydrolysis of β-D-glucoside to D-glucose and alcohol. This enzyme was therefore selected and the constituents and conditions of the assay, namely, buffer, protein concentration, pH, temperature and time of incubation, were optimized for large-scale synthesis of the intact aglycone of enfumafungin with HPLC as monitoring system. As shown in Figs. 1 and 2, this enzyme has a pH optimum at 6–7 and shows its highest activity at temperatures from 37 to 47°C in 0.1 M acetate buffer. Using the assay condition thus established, the protein concentration and time of incubation were also optimized for maximum product formation. Fig. 3 illustrates the time course of the aglycone formation when 2 mg of β-D-glucosidase

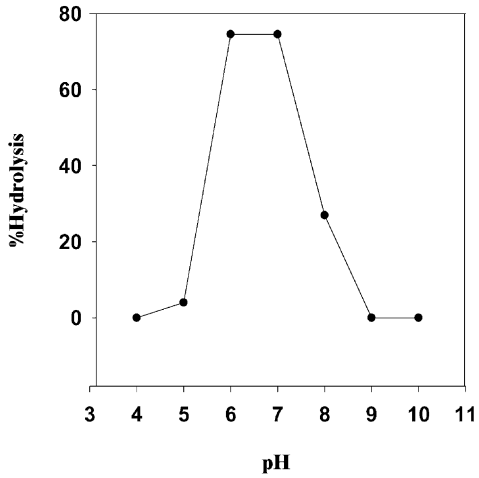


Fig. 1. Optimization of pH for the enzymatic deglycosylation of enfumafungin. Product of the enzymatic reaction mixtures were evaluated by HPLC after incubation for 46 h at 37°C. Each assay mixture contained 2.5 mg enzyme, 1 mg substrate in 1 ml of 0.1 M acetate buffer.

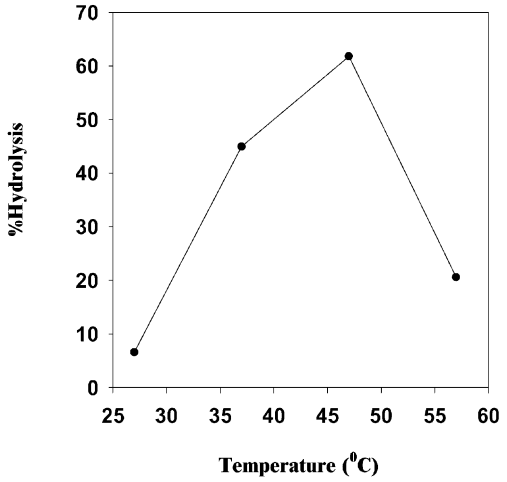


Fig. 2. Temperature optimization for the deglycosylation of enfumafungin. Product of the enzymatic reactions were evaluated after 46 h of incubation at pH = 6. Each assay mixture contained 2.5 mg enzyme and 1 mg substrate in 1 ml of 0.1 M acetate buffer.

was used in the assay system. As shown, at about 60 h into incubation, the product concentration reached its maximum and thereupon stayed constant for the remaining of the incubation time. The reason for this catalytic behavior cannot be explained at this time as the stability and substrate/product inhibition of the recombinant β-D-glucosidase have not been studied in this work. We, however, speculate that the enzyme is probably losing its catalytic potential after about 60 h under described assay condition, which means that catalytic rate and therefore rate of product formation can be enhanced by simple addition of more enzyme.

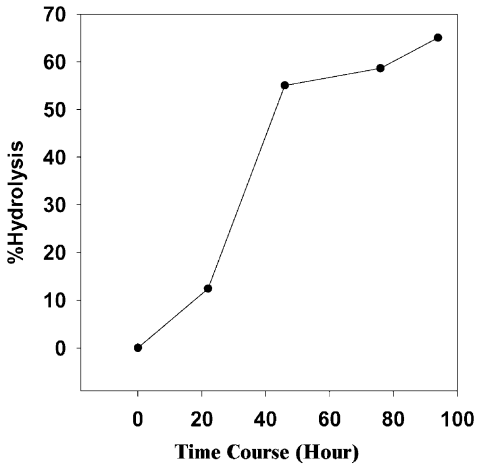


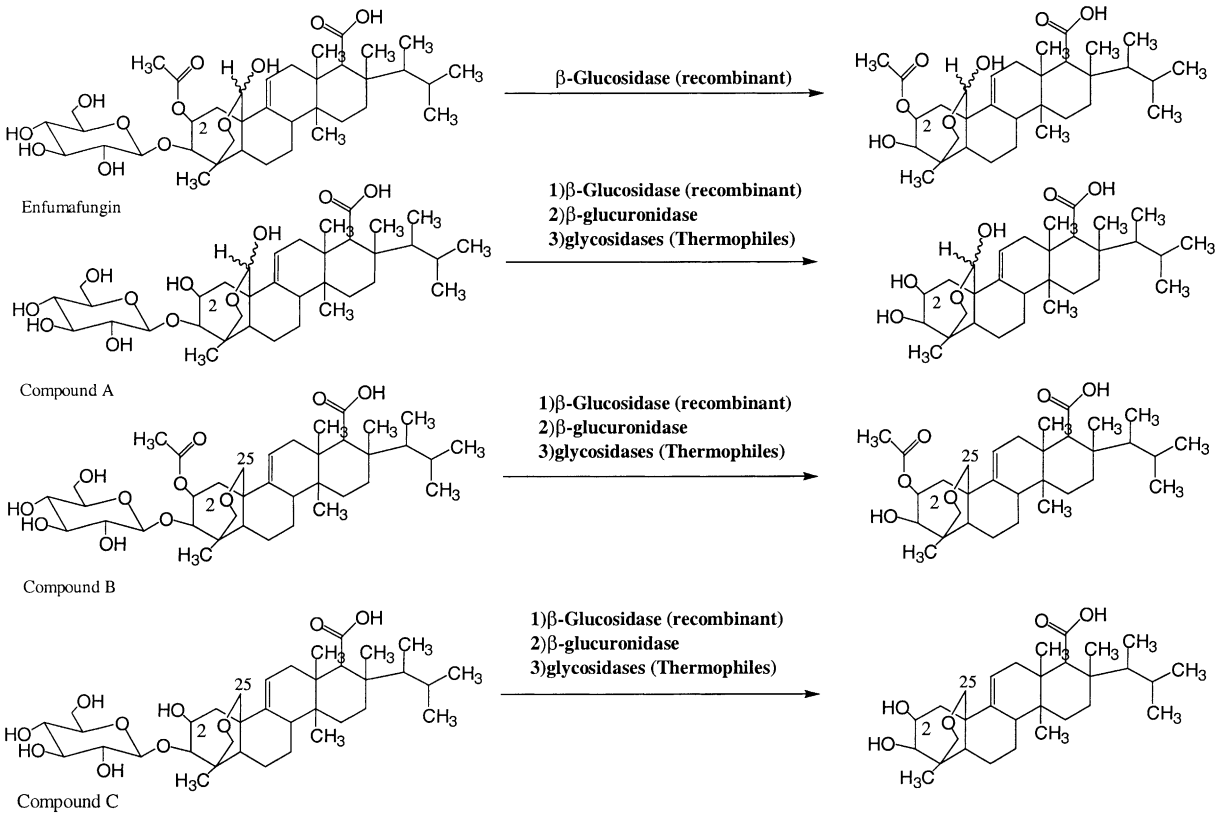
Fig. 3. Time course of the deglycosylation of enfumafungin. The product of the enzymatic reactions were evaluated by HPLC after incubation of the reaction mixtures at 37°C. Assay mixture contained 2.5 mg enzyme and 1 mg substrate in 1 ml of 0.1 M acetate buffer, pH = 6.

3.2. Substrate specificity of the recombinant β-D-glucosidase

After selection of the recombinant β-D-glucosidase and establishment of the optimal assay condition for enfumafungin as the substrate, the catalytic potential of this enzyme in the deglycosylation of compounds A–C was then examined. As shown in Scheme 2, all three compounds were deglycosylated upon catalysis by recombinant β-D-glucosidase under conditions similar to those used in the deglycosylation of enfumafungin. No other enzyme of our enzyme panel was found to show such catalytic potential where aglycone moiety was acetylated. On the contrary, six of the thermostable enzymes, namely, Gly001-01, Gly001-02, Gly001-03, Gly001-04, Gly001-08 and Gly001-09, catalyzed the deglycosylation of those analogues in which C-2 acetyl group had been removed. Similarly, β-glucuronidase from mollusk was found which could only catalyze the deglycosylation of deacetylated analogues similar to the six thermostable enzymes.

3.3. Scale-up synthesis of aglycones

The potential of enzymes in catalyzing a wide variety of fascinating reactions have been well established. However, to have practical use as catalysts, especially in an industrial setting, enzyme availability, stability, cost, and condition of the reactions are some of the issues that must initially be taken into consideration. In the present work, after identification of several enzymes with desired catalytic potential, we tried to develop small-scale synthesis in order to prepare enough material to satisfy our need. Toward this goal, two of the enzymes, namely, β-D-glucosidase and β-glucuronidase were used and, as reported in this paper, milligrams quantities of various deglycosylated triterpenes were prepared. Based on what is reported



Scheme 2. Enzymatic deglycosylation of the antifungal natural product, enfumafungin, and its chemical analogues.

here, the scale of the reaction can be increased by simply using more enzyme in a larger reaction volume. Using this strategy, a larger scale enzymatic reaction, in which 150 mg substrate had been utilized, has successfully been carried out. In this case, in order to keep the reaction volume to a minimum, the substrate had to be extensively sonicated, however.

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