Biotechnology Reports 17 (2018) 55–62

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

# Improvement of *Aspergillus flavus* saponin hydrolase thermal stability and productivity *via* immobilization on a novel carrier based on sugarcane bagasse

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ARTICLE INFO

*Keywords:*
Saponin hydrolase
Immobilization
Sugarcane bagasse
*Aspergillus flavus*

ABSTRACT

Soyasapogenol B (SB) is known to have many biological activities such as hepatoprotective, anti-inflammatory, anti-mutagenic, antiviral and anticancer activities. Enzymatic conversion of soyasaponins to SB was carried out using saponin hydrolase (SH) extracted from *Aspergillus flavus*. The partially purified enzyme was immobilized on different carriers by physical adsorption, covalent binding or entrapment. Among the investigated carriers, Eupergit C and sugarcane bagasse (SCB) activated by DIC and NHS were the most suitable two carriers for immobilization (the immobilized forms recovered 46.5 and 37.1% of the loaded enzyme activity, respectively). Under optimized immobilization conditions, immobilized SH on Eupergit C and on activated SBC recovered 87.7 and 83.3% of its original activity, respectively. Compared to free SH, immobilized SH on Eupergit C and on activated SCB showed higher optimum pH, activation energy, half-lives and lower deactivation constant rate. Also, their SB productivities were improved by 2.3- and 2.2-folds compared to free SH (87.7 and 83.3 vs. 37.5%, respectively). Hence, being SCB more sustainable and an inexpensive material, it can be considered a good alternative to Eupergit C as a support for SH immobilization. SH immobilization on industrially applicable and inexpensive carrier is necessary to improve SB yield and reduce its production cost. The chemical structure of SCB and the resulting cellulose derivatives were studied by ATR-IR spectroscopy. The thermal analysis technique was used to study the chemical treatment of SCB and coupling with the enzyme. This technique confirmed the removal of lignin and hemicellulose by chemical treatment of SCB.

## 1. Introduction

Saponin hydrolase (SH) is an enzyme that catalyzes the hydrolysis of natural saponins to more biologically active compounds; mainly their aglycones. For example, conversion of glycyrrhizin, Licorice saponin, by *Aspergillus niger* hydrolase into glycyrrhetinic acid, which is widely used as antiulcer, antiallergic, antiviral, antibacterial and hepatoprotective agent [1]; conversion of major ginsenosides, ginseng saponins, to more active minor ginsenosides by intestinal bacteria [2]; and conversion of yellow ginger saponins by *Trichoderma harzianum* CGMCC 2979 to diosgenin, which acts as precursor for human steroidal drugs [3]. Also, hydrolysis of soyasaponins by *Aspergillus flavus* SH resulted in a production of soyasapogenol B (SB) [4], which is a candidate therapeutic agent for chronic hepatitis [5]. SB (3 β, 22 β, 24-trihydroxyolean-12(13) ene) has been reported to have other biological activities, such as platelet aggregation suppressing effect, therapeutic activities for immune diseases, anti-inflammatory activity, and growth suppression effect on human breast cancer, colon cancer, ovarian cancer and hep G2 cells [6–8].

Enzymatic production of SB from soybean saponin is often advantageous as compared to acid hydrolysis due to its high selectivity, specificity, efficiency, eco-friendly status and minimum production of byproducts. Consequently, microbial enzymes are considered as a competitive tool for clean manufacturing of valuable pharmaceutical compounds on an industrial scale. However, industrial applications required a selection of an efficient technique that permits to improve the enzyme features to provide extended active lifetime [9]. Enzyme immobilization has been reported to improve many enzyme properties

*Abbreviations:* SH, saponin hydrolase; SB, soyasapogenol B; SCB, sugarcane bagasse

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https://doi.org/10.1016/j.btre.2017.12.007
Received 14 July 2017; Received in revised form 14 December 2017; Accepted 27 December 2017
Available online 28 December 2017

including; enzyme stability, specificity, selectivity, activity, and reduction of inhibition by products or medium [10]. Moreover, immobilization permits easy biocatalyst–product separation and facilitates recovery and reusability of enzymes, hence, providing cost-effectiveness use of enzymes in continuous biocatalytic processes [11]. Methods of immobilization could be classified into four main methods; namely: physical adsorption, ionic bonding, covalent bonding and entrapment [12]. Only one research on SH immobilization has been reported [13], but there are no reports on SB production from soybean saponin by immobilized SH.

In the context of our research on the microbial production of SB from soyasaponins by SH enzyme as a biocatalyst, the present work investigates *Aspergillus flavus* SH immobilization on different carriers using different methods (physical adsorption, covalent binding, and entrapment). In addition, optimization of the immobilization conditions was carried out targeting to develop an improved biocatalyst for SB production. Comparison between the immobilized and free SH regarding their catalytic properties and their thermal stabilities has been also studied.

## 2. Materials and methods

### 2.1. Materials and carriers for enzyme immobilization

Rice straw and corn cob were supplied by local farmers. SCB was obtained from Egyptian Sugar and Integrated Industries Company (ESII), El-Hawamdia, Giza, Egypt. Sawdust was supplied by local wood processing factories. Cotton and loafa were obtained from local markets. Eupergit C was supplied by Röhm Pharma Polymers (Darmstadt, Germany). Sodium alginate was supplied by BDH chemicals Ltd., Poole, England. Polyethyleneimine (branched, 10000 MW) (PEI), 50% Glutaraldehyde (GA), *N-N*'-diisopropylcarbodiimide (DIC) and N-hydroxysuccinimide (NHS) were bought from Alfa-Aesar (Karlsruhe, Germany). Chitosan was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

### 2.2. Preparation of SH enzyme

SH preparation was obtained from *A. flavus* by a method described in a previous paper [14]. The concentrated culture filtrate was fractionated by 75% acetone. This partially purified enzyme (specific activity 12.7 mU/mg protein) was lyophilized and used for the preparation of the immobilized enzyme.

### 2.3. Immobilization methods

Cellulosic agricultural leftovers (SCB, saw dust, rice straw, corn cob and loafa) were treated with 1.25% (w/v) sulphuric acid and 1.25% (w/v) sodium hydroxide at 100 °C for 1 h each time, followed by washing with distilled water. Then it was dried in hot air oven at 100 °C for 2 h.

#### 2.3.1. Physical adsorption

*A. flavus* SH immobilization by physical adsorption was carried out by loading 1 ml of enzyme solution (20 mg of lyophilized partially purified enzyme was dissolved in 1 ml of 20 mM phosphate buffer (pH 7), which equivalent to 2 mg protein/ml) on 0.5 g of non-activated cellulosic carriers.

#### 2.3.2. Entrapment in calcium alginate

1 ml of 4% sodium alginate solution was mixed with 1 ml of an enzyme solution (20 mg/ml). In another set of experiments, 4% gel solution was treated with GA (0.2%, w/v) before the addition of the enzyme sample. The whole mixture was made into beads by dropping the alginate solution in 0.1 M $CaCl_2$ solution. The resulting beads (1.0–1.5 mm in diameter) were collected and washed with phosphate buffer (20 mM, pH 7) to remove the unbound enzymes [15].

#### 2.3.3. Covalent binding

SH immobilization on Eupergit C (acrylic epoxy-activated resins). Unless stated otherwise, 0.5 g of Eupergit C was covered with 1 ml of an enzyme solution (20 mg/ml) in 20 mM phosphate buffer (pH 7) and stirred (150 rpm) for 2 h at room temperature.

SH immobilization on different activated carriers: SH covalent linkage to different cellulosic carriers or chitosan was performed using four different coupling agents' procedures as follows.

a) Activation of the carrier by glutaraldehyde (GA). 0.5 g of each support was suspended in 5 ml of 1% (w/v) GA in 50 mM phosphate buffer pH 8 and gently stirred for 2 h, at room temperature. The activated carriers were then filtered and washed 3 times with distilled water before drying. GA is a bi-functional reagent that can react with different enzyme moieties, mainly involving primary amino groups of proteins. Nevertheless, it is known that it can also react with other groups (thiols, phenols, and imidazole) [16].
b) Activation of the carrier by sodium periodate (PI). 0.5 g of different carriers was soaked in 20 ml of PI solution (30 mg/ml) in 0.1 M acetate buffer (pH 4.0). The mixture was placed in the dark and stirred at ambient temperature for 96 h. The oxidized carriers were then washed with distilled water several times to remove the oxidant and used for the immobilization of SH without drying.
c) Activation of the carrier by DIC and NHS. This was carried out using two different procedures [17]. In the first case, carriers (0.5 g each) were immersed in 5 ml of 0.1 M, pH 3.5 MES buffer and dried at 40 °C under vacuum. Afterwards, carriers were immersed in dimethylformamide (DMF) containing 2 (or 4) mM DIC and 5 (or 10) mM NHS and kept shaken for 2 h at 150 rpm. Then, the activated carriers were centrifuged to remove excess solvent (DMF) and washed with buffer 20 mM phosphate buffer, pH 7.0 to ensure that there no remaining DMF. In another procedure carriers were dipped directly in 5 ml of 0.1 M, pH 3.5 MES buffer containing 2 (or 4) mM DIC and 5 (or 10) mM NHS and kept shaken for 2 h at 150 rpm. Then washed with 20 mM phosphate buffer, pH 7.0 and dried overnight under vacuum.

A specified amount of activated carriers (0.5 g) was stirred separately with 1 ml of an enzyme solution (20 mg/ml) in 20 mM phosphate buffer (pH 7) for 2 h at 150 rpm and 4 °C. The carriers with the immobilized enzyme were separated by filtration and washed thoroughly with bi-deionized water. The carriers with immobilized enzyme designated as immobilized SH were dried in a desiccator at room temperature and finally, activities were assayed from hydrolysis of soyasaponins. Supernatants and washings were assayed for SH activity and protein.

### 2.4. Enzyme activity and protein determination

1% soyasaponins suspended in 0.2 M acetate (pH 5) was added to the carriers with the immobilized enzyme or the control supports or the free lyophilized enzyme, and the mixtures were allowed to react at 40 °C for 1 h. The reaction product (SB) was found to increase linearly up to 4 h. Reaction products were extracted with double its volume ethyl acetate. The quantity of SB in the sample was analyzed by high-pressure liquid chromatography (HPLC). One unit of enzyme activity is defined as the amount of free or immobilized enzyme that produces one µmole of aglycone (SB) *per* min from the substrate. Recovered activity was used for evaluation of immobilization efficiency and it was calculated as follows:

$$RA\ (\%) = A_I/A_F*100 \quad (1)$$

Where $A_F$ and $A_I$ refer to the activities of free and immobilized SH,