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ARTICLE

Extraction and partial purification of *Aspergillus flavus* cell wall associated saponin hydrolase

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ABSTRACT In spite of the importance of saponin hydrolase (SH) enzyme, in the production of biologically active compounds from natural saponins, it is surprising that many aspects of its nature are unknown. The results of the present work revealed that *Aspergillus flavus* was capable of expressing three SH forms; extracellular, intracellular and cell wall-bound forms. SH cell bound enzyme constituted to more than 75% of the total enzymatic activity in the production medium. The sequential extraction process of SH cell bound enzyme revealed that 47.5% of SH was cytosolic and the rest (52.5%) was associated with the cell wall. The highest SH extraction yield was achieved when 0.25 M Tris-HCl lysis buffer supplemented with 1% Triton X-100 for 24 h at 4-25 °C and pH 8 were applied. Under these optimized conditions, *A. flavus* SH yield increased from 23.6 to 85.83%. The partial purification was achieved by applying successively acetone precipitation, lyophilization, dialysis, and anion exchange chromatography on Fractogel EMD DEAE-650S to the extract. The specific activity of the enzyme extract was 0.27 U/mg after 75% acetone fractionation, while that after anion exchange chromatography was 0.65 U/mg protein. The final enzyme preparation was 7.3-fold purer than the crude extract.
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KEY WORDS

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optimization
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Introduction

Soybean saponins (SS) are oleanane triterpenoid glycosides found in soy and other legumes (Ruiz et al. 1996). These saponins have been divided into group A, B and E saponins according to their aglycone structures; soyasapogenol A, B and E, respectively (Berhow et al. 2002). They have a common structure of glucuronic acid linked to the C-3 site of soyasapogenol A or B as aglycones. Soyasapogenols A and B are generally being more biologically active compared to their glycosides (Gurfinkel and Rao 2003). Soyasapogenol B (SB) is known to have hepatoprotective, antiviral, anti-inflammatory, antimutagenic and growth suppressing effects on cells derived from human ovarian cancer, colon cancer, breast cancer and Hep-G2 cells (Kuzuhara et al. 2006; Watanabe et al. 2006; Zhang and Popovich 2008; Kamo et al. 2014). SB can be obtained from group B SS by hydrolyzing β-D-glucuronic acid residues from SS using the enzyme (β-D-glucuronidase, EC 3.2.1.31) called soybean saponin hydrolase (SH), produced by microorganisms belonging to genera *Neocosmospora, Eupenicillium* and *Aspergillus* (Kudou et al. 1991; Watanabe et al. 2005, 2006). The bioconversion of soyasaponin I (SS I) to SB by SH is shown in Figure 1.

The presence of synthetic as well as hydrolytic enzymes in fungal cell wall was postulated some time ago (Burnett 1979). Extraction procedures were developed including the utilization of diverse salts and hydrophilic and lipophilic solvents to better understand the association of enzymes with the cell wall (Lee and Lin 1995; McDougall and Morrison 1995). Additionally, sequential extraction protocols were elaborated for a more straightforward classification of the enzymes associated with the cell wall. The obtained extracts were sequentially washed with different solutions and/or solvents, resulting in different fractions or degrees of enzymatic association with the cell wall (Sassoon and Mooibroek 2001; Rast et al. 2003; Pérez-de-Mora et al. 2013). Fungal SH is usually known to be present in mycelium as well as in medium (Kudou et al. 1991). Although, many studies were carried out on extracellular SH, little is known about the location and the role of intracellular SH. Nevertheless, the status of SH association with fungal cell wall has remained eventless and not thoroughly characterized.

In a previous research (Amin et al. 2016), it was reported that SH was a cell wall associated glucuronidase, because

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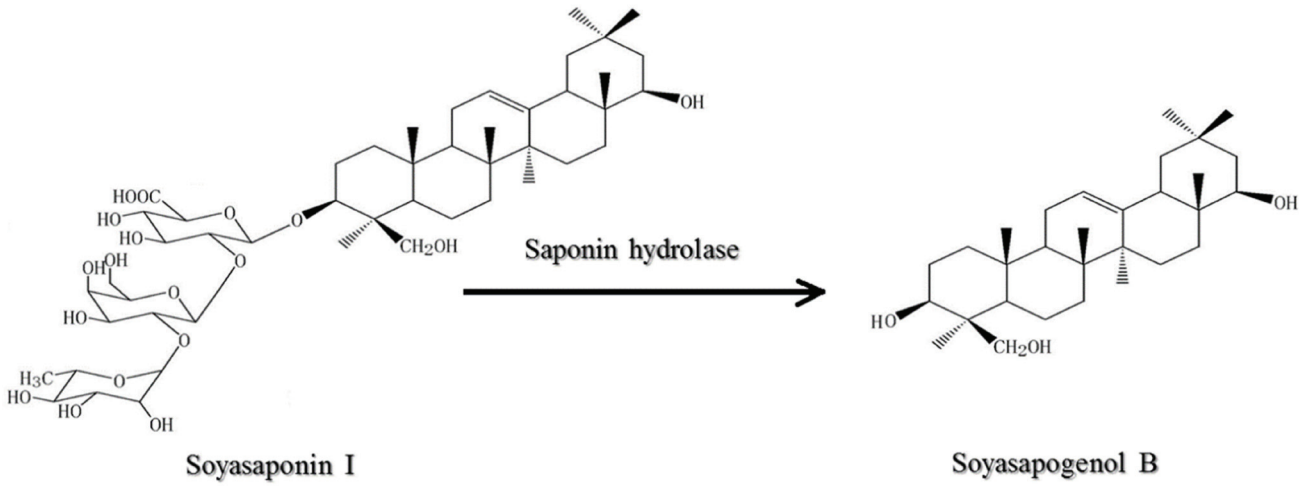

Figure 1. Bioconversion of SS I to SB by SH.

the majority of enzyme activity was detected in the whole cells compared to the extracellular and intracellular enzyme preparations. Here, we studied the activity and association of SH with the cell wall of *Aspergillus flavus* by means of a sequential extraction procedure. Followed by optimization of the extraction procedure in a trial to obtain a complete extraction of cell wall associated SH.

Materials and Methods

Microorganism and cultivation

A. flavus, a fungus isolated from popcorn seeds, was maintained on potato dextrose agar (PDA-Difco). SH production was carried out in production medium, containing (g/l) 20 SS, 40 malt extract, 20 yeast extract, 2 KH_2PO_4, 2 $(NH_4)_2SO_4$, 0.3 $MgSO_4 \cdot 7H_2O$, and 0.3 $CaCl_2 \cdot 2H_2O$ with a pH adjusted to 9 (Amin et al. 2016). Erlenmeyer flasks (250 ml) containing 100 ml of production medium were inoculated separately with 1 ml spore suspension (10^8 spores/flask) of 6 days old *A. flavus* slant culture and incubated for 48 h at 30 °C on a rotary shaker at 150 rpm.

Localization of A. flavus SH enzyme

A. flavus was grown in the previously described production medium for 48 h at 30 °C. The enzyme activity was assayed in cultural filtrate, whole cells and cell extract. For preparation of cell extract, cells were washed twice with saline (0.9%) and grinded with approximately twice its weight of washed cold sand in a cold mortar and extracted by acetate buffer (200 mM acetate, pH 5).

Sequential extraction procedure

The sequential extraction procedure was carried out using a method described previously (Pérez-de-Mora et al. 2013) with the following modifications. Lyophilized fungal cells were grinded with a cooled mortar and pestle under liquid nitrogen and stored at -80 °C until further usages. Three aliquots of cell (50 mg each) were thawed on ice and 2 ml lysis buffer I (250 mM Tris-HCl buffer, pH 8) was added to each tube, then cells were sonicated 5 times at 50% duty cycle for 1 min with Hielscher ultrasonic processor UP200S (Teltow, Germany). Completeness of cell disruption was examined microscopically. The resulting homogenate represents the total cell extract. The sample was clarified by centrifugation at 12000 x g for 10 min at 4 °C. The recovered supernatant was sampled, marked as cytosol (Tris extract). In all subsequent extractions, sonication and centrifugation were performed as per the previously described process. The insoluble pellets were resuspended in 50 mM phosphate buffer, pH 7 (P-buffer). The supernatant was sampled, stored and labeled as P extract. The same procedure was duplicated. The pellet was resuspended using Triton X-100 buffer (P-buffer + 0.5% Triton X-100), the suspension was incubated for 2 h at 4 °C under gentle overhead shaking, after which the suspension was centrifuged and the supernatant sampled (Trit extract). A second extraction using Triton X-100 buffer (P-buffer + 0.5% Triton X-100) was conducted. In a similar fashion, two extractions using 1 M NaCl solution followed (NaCl extract). Enzyme assays were conducted in all extracts in addition to the insoluble pellet after separation of the last extract, which was marked as rest. Enzyme extraction yield (%) was calculated using the following equation (Eq. 1).

Extraction yield (%) = Extraction yield (%) = Cell extract SH activity/Total SH activity x 100 (Eq. 1.)

Optimization of the extraction procedure

The effect of different operational parameters on enzyme extraction was studied including; sonication time and intensity, the use of different detergents (ionic and nonionic ones), varying concentrations of the best detergent, extraction time and extraction temperature. All experiments were conducted using lyophilized grinded *A. flavus* cells suspended in 250 mM Tris-HCl buffer, pH 8 in the presence of a protease inhibitor cocktail (Sigma, St. Louis, Mo), 1 mM EDTA, 2 mM β-mercaptoethanol and 150 mM NaCl and centrifuged at 12000 x g for 10 min at 4 °C.

Partial purification of A. flavus SH enzyme

The crude SH enzyme extracted from *A. flavus* cells under the optimized extraction conditions was centrifuged at 10.000 rpm at 4 °C for 10 min. The supernatant was precipitated using 75% acetone then the precipitate was lyophilized. The lyophilized material was dissolved in minimum volume of 20 mM phosphate buffer, pH 7.5 and dialyzed against the same buffer. Dialyzed enzyme was centrifuged and the supernatant was applied on Fractogel EMD DEAE-650 (1.5 x 20 cm) equilibrated by 10 mM phosphate buffer, pH 7.5. The column was eluted with a 0 to 200 mM NaCl linear gradient in 10 mM phosphate buffer, pH 7.5 (4 h, flow rate 1 ml/min). The active fractions were pooled then dialyzed in cold against water, and after that lyophilized.

Measurement of SH activity

SH activity was measured in cultural filtrate or cell extract as follows: to 1 ml of 2% SS suspended in MUB (modified universal buffer, Skujins 1962), 1 ml of enzyme solution was added, and the mixture was allowed to react at 40 °C for 1 h. The MUB pH was adjusted to achieve a final pH of 6 during the incubations. Reaction products were extracted with 2 ml of ethyl acetate. In case of using whole cells in the reaction mixture, 1 g wet cells was added to 5 ml of 1% SS suspended in 200 mM acetate (pH 5) and the mixture was allowed to react at 40 °C for 1 h. Reaction products were extracted with 5 ml of 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 enzyme that produces 1 µmol of aglycone (SB)/h from the substrate. Cell based specific activity (whole cells or cell extract) can be defined as µmol of aglycone/h/g dry weight cells.

SB analytical methods

Thin layer chromatography (TLC) was carried out on precoated silica gel plates (Merck, silica gel 60F-254). The plates were chromatographed for SB with a solvent system of benzene:ethylacetate:acetic acid (12:4:0.5, v/v/v). SB having an Rf value of 0.35 was detected on TLC plates by acid charring (10% H_2SO_4, 120 °C, 10 min).

HPLC was performed with Waters Alliance E2695 HPLC System (XE Separations Module, Austria) under the following conditions; column: SunFire Prep C18 (5µm, 10 x 150 mm), column temperature: 40 °C; mobile phase: acetonitrile-methanol-water (50:15:35), flow rate: 1 ml/min, and UV detector operating 200 nm. From ethyl acetate 100 µl of containing reaction products was diluted with 900 µl of the mobile phase. From this dilution 10 µl was analyzed by HPLC and the quantity of SB in the sample was determined by comparison with authentic SB (Watanabe et al. 2004). The SB yield (%) in the reaction mixture was calculated using Eq. 2.

$$\text{SB yield (\%)} = \frac{[\text{SB weight} / \text{SBMW}]\times 100}{[\text{SS I weight} / \text{SS I MW}]} \quad \text{(Eq. 2.)}$$

Where, MW is the molecular weight; SS I (soyasaponin I) represents soybean saponins.

Statistical analysis

All the experiments were performed in triplicate, and the values obtained represent the means of duplicate measurements of three independent samples. When ANOVA indicated a significant F-value, least significant difference values at $P<0.05$ were used to separate treatment means.

Results and Discussion

Localization of A. flavus SH enzyme

Hydrolytic enzymes can be classified as (a) intracellular, (b) membrane- or cell wall-associated, and (c) extracellular depending on their location (Alvarez et al. 2004). Data presented in Table 1 revealed that among three different enzyme fraction preparations, the whole cells fraction produced the highest total enzyme activity. Total enzyme activity of this fraction was 3.1 and 4.2 times more active than that of extracellular (cultural filtrate) and intracellular (cell extract) enzyme fractions, respectively (Table 1). In addition, SB yield of the whole cells under mentioned assay conditions was about 6

Table 1. Effect of different SH preparations on SH activity and SB yield.

SH preparations	SH total activity (U/g)	SB yield (%)
Cultural filtrate	22.91 ± 1.14	2.26 ± 0.15
Cell extract	16.73 ± 0.66	4.13 ± 0.23
Whole cells	71.05 ± 3.20	17.55 ± 0.78

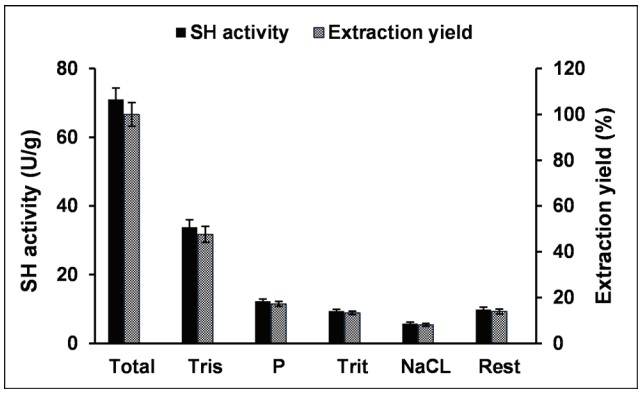

Figure 2. SH activities and percentage of activities in the various fractions of *A. flavus.* Extraction yield (SH %) of each fraction was calculated according to the activity of total cell extract. The experiments were performed in triplicate and the standard deviations were lower than ± 8.1%.

and 4 times that of extracellular and intracellular enzyme preparations, respectively. These results indicated that SH was distributed in three forms, which are extra-, intracellular and cell wall-associated forms. Moreover, cell wall-associated SH contributed to more than 75% of the total enzymatic activity in the production medium.

Sequential extraction procedure

The target enzyme is mainly cell wall-associated one as observed in the results above. The sequential extraction procedure was applied to specify SH associations to the cell wall. Consequently, a suitable protocol could be applied to release it from fungal cells. According to Pérez-de-Mora et al. (2013), this procedure divides enzymes into the following categories: 1) cytosolic (Tris extract), 2) loosely bound (P extract), 3) hydrophobically bound (Trit extract), 4) ionically bound (NaCl) extract, and 5) covalently bound (Rest). Figure 2 shows SH enzymatic compartmentalization among the cytosol (Tris extract) and the cell wall fractions (P extract, Trit extract, NaCl extract, and Rest). The highest proportion of SH activities (47.5% of total activity) was found in Tris fraction (33.77 U/g) and it represented about twice the activity observed in the previous experiment (16.7 U/g) using acetate buffer at pH 5. It was concluded that increasing buffer pH to pH 8 enhanced SH extraction. Schindler et al. (2006) reported that high salt or high pH solutions can separate membrane proteins from membranes. On the other hand, sum of activity in cell wall fractions formed about 52.5% of total activity. This means that 47.5% of SH enzyme is cytosolic and the rest (52.5%) is associated with the cell wall.

Optimization of extraction procedure

According to the above results, more than 50% of SH is associated with the cell wall or membrane protein. Integral membrane proteins need to be solubilized from the lipid bilayer to produce individual proteins before purification. Detergents with amphipathic properties are commonly used to solubilize integral membrane proteins from membranes (Lin and Guidotti 2009). Consequently, the effect of different operational parameters on enzyme extraction was studied including; sonication time and intensity, the use of different detergents (ionic and nonionic ones), application of different concentrations of the best detergent, extraction time and extraction temperature.

Data presented in Table 2 show that not only the extraction yield was affected by the sonication time and intensity, but also the total SH activity. At 25% amplitude, both the total SH activity and the extraction yield increased by time. On the other hand, SH total activity was increased when increasing both the sonication time and intensity up to 3 min at 35% amplitude, whereas the extraction yield was increased up to 5 min at 45% amplitude.

Table 2. Effect of sonication time and intensity on SH total activity and extraction yield.

Sonication condition	Total (U/g)	Cell extract (U/g)	Extraction yield (%)
Amp. 25(%)			
1 min	65.18 ± 4.28	14.81 ± 0.52	22.73 ± 1.37
3 min	77.04 ± 5.23	23.28 ± 1.40	30.21 ± 1.70
5 min	79.16 ± 2.02	29.75 ± 1.88	37.58 ± 2.17
Amp. 35(%)			
1 min	75.72 ± 2.88	20.49 ± 1.31	27.05 ± 1.61
3 min	82.71 ± 3.85	27.93 ± 1.79	33.78 ± 2.04
5 min	71.05 ± 3.17	32.82 ± 2.06	46.20 ± 1.76
Amp. 45(%)			
1 min	82.69 ± 2.22	26.07 ± 0.51	31.53 ± 0.76
3 min	76.41 ± 3.05	31.66 ± 1.35	41.43 ± 2.86
5 min	70.99 ± 4.16	40.05 ± 2.94	56.42 ± 2.36

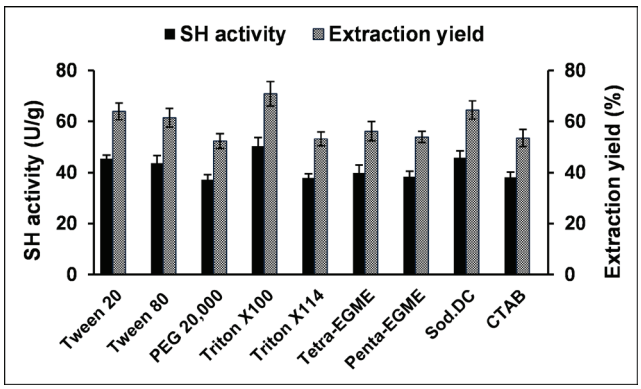

Figure 3. Screening of different detergents for SH activity and extraction from *A. flavus* cells. Extraction yield (SH %) of each fraction was calculated according to the activity of the total cell extract. The experiments were performed in triplicate and the standard deviations were lower than ± 6.8%. PEG: polyethylene glycol 20 000; Tetra-EGME: tetra-ethylene glycol mono *n*-dodecyl ether; Penta-EGME: penta-ethylene glycol mono *n*-dodecyl ether; Sod DC: sodium deoxycholate; CTAB: Cetyltrimethylammonium bromide.

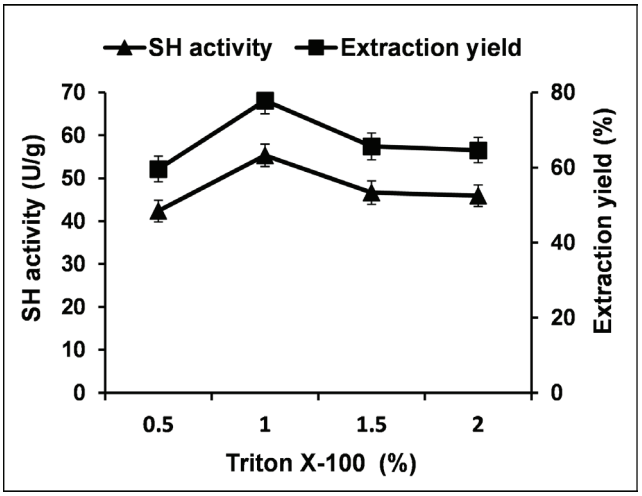

Figure 4. Effect of different Triton X-100 concentrations on SH activity and extraction from *A. flavus* cells. The experiments were performed in triplicate and the standard deviations were lower than ± 4.5%.

Detergent molecules consist of two parts: a hydrophobic hydrocarbon moiety, and a polar or charged head group. Detergents are classified into four types based on their head-groups; namely nonionic, anionic, cationic or zwitter ionic detergents (Lin and Guidotti 2009). Detergent screening must be carried out for each membrane protein as not only one detergent works for all membrane proteins. Figure 3 shows the effect of different detergent types; nonionic (Tween 20, Tween 80, polyethylene glycol 20000, Triton X-100, Triton X-114, tetra-ethylene glycol mono *n*-dodecyl ether, and penta-ethylene glycol mono *n*-dodecyl ether), anionic (sodium deoxycholate), and cationic (Cetyltrimethylammonium bromide, CTAB) on SH extraction from *A. flavus* when evaluated experimentally. Among all detergents, Triton X-100 proved to be the best one followed by sodium deoxycholate showing extraction results of 77.87 and 70.86% of total SH activity, respectively. Consequently, different concentrations of Triton X-100 (0.5-2%) were used in lysis Tris buffer, pH 8 in an attempt to improve the extraction process of the enzyme part associated to the cell wall. Results in Figure 4 revealed that 1% Triton X-100 is the most favorable concentration for enzyme extraction, as it resulted in about 77.87% of enzyme activity of the total extract. Ahmad and Goswami (2013) used the same concentration of Triton X-100 (1%) for extraction of cell-bound cholesterol oxidase from *Rhodococcus* sp. (NCIM 2891).

Regarding the effect of extraction time, the extraction ability of the lysis buffer increased gradually by time to reach a maximum (85.83%) after 24 h incubation (Fig. 5). Thereafter, there was no significant changes in SH extraction yields with further incubation. Additionally, data in Table 3 show

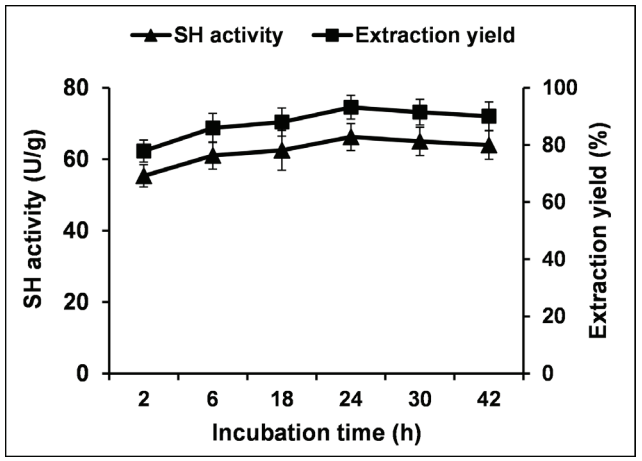

Figure 5. Effect of incubation time with 1% Triton X-100 on SH activity and extraction from *A. flavus* cells. The experiments were performed in triplicate and the standard deviations were lower than ± 6%.

Table 3. Effect of incubation temperature with 1% Triton X-100 on SH activity and extraction from *A. flavus* cells.

Temperature (°C)	SH activity (U/g)	Extraction yield (%)
4	66.20 ± 3.5	93.18 ± 5.2
25	67.01 ± 3.2	94.31 ± 4.8
40	49.43 ± 2.8	69.57 ± 3.1

the effect of the incubation temperature on SH activity and extraction from *A. flavus*. There was almost stability in SH activity and the extraction yield in a temperature range from

Table 4. Partial purification of SH extracted from *A. flavus.*

Purification step	Total activity (U)	Total protein (mg/ml)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Cell extract	1166	12993	0.09	100	1
Precipitation with acetone	420	1576	0.27	36.0	2.97
After lyophilisation	416	1468	0.28	35.6	3.15
After dialysis	370	1381	0.27	31.7	2.98
After DEAE column	142	218	0.65	12.2	7.27

4 to 25 °C. However, increasing the temperature to 40 °C resulted in decreased SH activity and extraction yield probably due to the denaturation effect of temperature on SH.

Partial purification of SH

The SH enzyme extracted from *A. flavus* cells, under the optimized extraction conditions was precipitated by 75% acetone followed by lyophilisation, dialysis and DEAE column purification. These purification steps enhanced the overall enzyme activity as shown in Table 4. It is noticed that, the SH specific activity of 0.27 U/mg protein was obtained using 75% acetone precipitation, which represents about 3-fold purification. In addition, the obtained yield after precipitation with acetone was 36% of the initial crude enzyme activity. There are no significant changes in the specific activity after both lyophilisation and dialysis steps. However, the yield was decreases to 12.2% after DEAE column. Interestingly, the purification of *A. flavus* SH that has been done by DEAE Fractogel column, led to increase the purification fold to 7.27 with a specific activity of 0.65 U/mg protein compared to the crude extract. Further purification and characterization are being carried out and will be separately published elsewhere.

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