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Improving Enzymatic Production of Ginsenoside Rh_2 from Rg_3 by using Nonionic Surfactant

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Abstract In this study, several nonionic surfactants were tried to improve the enzymatic hydrolysis of ginsenoside Rg_3 into Rh_2 which was catalyzed at 50 °C and pH 5.0 by a crude glucosidase extracted from *Fusarium* sp. ECU2042. Among the biocompatible nonionic surfactants, polyethylene glycol 350 monomethyl ether was shown to be the best. After optimizing some influencing factors on the reaction, the conversion of Rg_3 (5 g/l) with 10 g/l crude enzyme reached almost 100% in the presence of the nonionic surfactant (7.5%, *w*/*v*), which was 25% higher than that in buffer without any surfactant. Furthermore, the enzyme stability was affected faintly by the surfactant.

Keywords Nonionic surfactant · *Fusarium* sp. ECU2042 · Biohydrolysis · Ginsenoside Rg_3 · Ginsenoside Rh_2

Introduction

Ginseng is an important traditional Chinese medicinal herb and has got various uses in traditional Asian medicine for thousands of years. Ginsenosides are generally believed to be the main bioactive compounds in ginseng and are classified into three categories: protopanaxadiol (PPD), protopanaxatriol, and oleanolic acid, according to their chemical constitutions. These compounds are reported to have a wide variety of physiological and pharmacological effects [1]. Among them, ginsenoside Rh_2 has been suggested to have a measurable effect on various cancer cells [2] Ginsenoside Rh_2 is a rare ginsenoside that is found only in red ginseng so many efforts have been made to manufacture ginsenoside Rh_2 from other available ginsenosides, such as ginsenoside Rg_3, which belongs to PPD-type ginsenosides as the same as ginsenoside Rh_2 and just has one more glucose molecule at C-3

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position (Fig. 1). Among reported attempts, biocatalytic approaches have remarkable predominance due to their high selectivity, mild reaction conditions, and environmental compatibility. In a previous report from our laboratory, ginsenoside Rh_2 was obtained by enzymatic hydrolysis of ginsenoside Rg_3 using a newly isolated fugus strain, *Fusarium proliferatum* ECU2042 [3]. To obtain a higher percent conversion, more efforts need to be made to improve the efficiency of the enzymatic reaction.

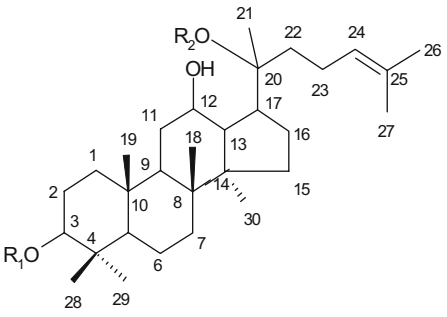
Recently, interesting studies have been reported on the effects of several surfactants on biodegradation and biohydrolysis of compounds that have low aqueous solubility [4–14]. Bioconversions of these hydrophobic compounds often meet with two serious obstacles: limited substrate accessibility to the biocatalyst as a result of the low aqueous solubility; inhibition, or toxicity of both substrate and product exerting upon the enzyme or the whole-cell biocatalyst. Surfactant molecules, which consist of a hydrophilic part and a hydrophobic part, aggregate into micelles when the concentrations in aqueous solution are above its critical micelle concentration. Chemicals may be partitioned into the hydrophobic center of surfactant micelles, so that the substrate/product concentration in aqueous solution drops below the inhibitory concentration. This partitioning also results in increased pseudo-water solubilities of hydrophobic compounds hereby increasing the concentration gradient and mass-transfer rates. Results of studies on the effect of surfactants were very diverse, and nonionic surfactants were more preferable for free of any electrostatic interaction [15]. Moreover, some nonionic surfactants were reported to increase the catalytic activity or selectivity of enzymes [16–19].

In this paper, we examined the influence of several nonionic surfactants on the biohydrolysis of ginsenoside Rg_3, which was slightly soluble in water and exhibited some inhibition on the crude enzyme extracted from *Fusarium* sp. ECU2042. Factors affecting the biohydrolysis were examined experimentally, and enzyme stability in the micro-emulsion system was also investigated.

Materials and Methods

Materials

Fusarium sp. ECU2042 is screened from soil by our laboratory and is currently deposited at China General Microbiological Culture Collection Center with an accession number of CGMCC 1495.



Ginsenoside	R_1	R_2
Rb_1	-Glc-Glc	-Glc-Glc
Rb_2	-Glc-Glc	-Glc-Ara(pyr)
Rc	-Glc-Glc	-Glc-Ara(fur)
Rd	-Glc-Glc	-Glc
F_2	-Glc	-Glc
Rg_3	-Glc-Glc	-H
Rh_2	-Glc	-H
Compound K	-H	-Glc

Fig. 1 The chemical structures of protopanaxadiol-type ginsenoside. *Glc* β-D-glucose; *Ara*(*pyr*) α-L-arabinose (pyranose); *Ara*(*fur*) α-L-arabinose (furanose)

Twelve kinds of nonionic surfactants were chosen for this study. The symbols and main components of them were shown in Table 1. Synperonic PE/F68, Synperonic PE/L61, PEGDME 250, PEGMME 350, PEGDME 400, Synperonic NP 10, Triton X-45, Lgepal CA 630, and Brij 92V were obtained from Fluka Chemical Co., USA. Trion X-100, Tween 80 and OP-10 were from Shanghai Reagent Co. Ltd, China. Ginsenoside Rg_3 was purchased from Hongjiu Biotech Co. Ltd, Jilin, China. All other chemicals were obtained from local suppliers and of reagent grade.

Microorganism Culture and Crude Enzyme Preparation

Fusarium sp. ECU2042 was shaken aerobically at 180 rpm and 30 °C for 72 h in 500-ml Erlenmeyer flasks with 100 ml of Czapek medium, which was inoculated with an 18-h preculture.

The cells harvested from the culture broth were suspended in NaAc/HAc buffer (20 mM, pH 5.0), then quartz sand was added, and the cells were grinded. After centrifugation, the supernatant was precipitated with an equal volume of ice-cold acetone, and the precipitate achieved through centrifugation was used as crude enzyme of *Fusarium* sp. ECU2042 after vacuum freeze drying.

Enzymatic Hydrolysis

The standard reaction mixture consisted of 0.2 ml NaAc/HAc buffer (0.2 M, pH 5.0), 2 mg lyophilized crude enzyme from *Fusarium* sp. ECU2042, and 1 mg ginsenoside Rg_3, giving a final substrate concentration of 5 g/l. A surfactant was added to the system when required. After incubation at 50 °C and 1,100 rpm conducted in a thermomixer compact (Eppendorf, Germany), the reaction mixture was extracted by 0.2 ml of *n*-butanol. After centrifugation, the supernatant was directly subjected to HPLC analysis, as described previously [3], to determine the quantity of ginsenoside Rh_2 generated. When investigating one of the influencing factors, the others were kept unaltered.

One unit of β-glucosidase activity (U) was defined as the amount of enzyme catalyzing the formation of 1.0 nmol ginsenoside Rh_2 per hour under above conditions.

Table 1 Symbols and main components of selected nonionic surfactants.

Surfactant	Main component
Synperonic PE/F68	Poly(ethylene glycol)-*block*-poly(propylene glycol) -*block*-poly(ethylene glycol)
Synperonic PE/L61	Poly(ethylene glycol)-*block*-poly(propylene glycol) -*block*-poly(ethylene glycol)
Synperonic NP 10	Polyethylene glycol nonylphenyl ether
PEGDME 250	Polyethylene glycol 250 dimethyl ether
PEGMME 350	Polyethylene glycol 350 monomethyl ether
PEGDME 400	Polyethylene glycol 400 dimethyl ether
Triton X-45	Polyethylene glycol 4-*tert*-octylphenyl ether
Trion X-100	Polyoxyethelene (10) octylphenyl ether
Tween 80	Polyoxyethylene (80) sorbitan monooleate
Lgepal CA 630	[Octylphenoxy]polyethoxyethanol
OP-10	Alkylphenol ethoxylates
Brij 92V	Diethylene glycol oleyl ether

Enzyme Stability Against Surfactant

Several samples of β-glucosidase in 0.2 M NaAc/HAc buffer (pH 5.0) were preincubated at 50 °C and 1,100 rpm in the presence of surfactant. At regular intervals, ginsenoside Rg_3 was added to two of the incubated enzyme samples, and the hydrolysis was carried out to assay the residual activity as described above. Control experiments were performed similarly by omitting the surfactant, and the initial activity assayed in the absence of surfactant was taken as 100%.

Results and Discussion

Screening of Nonionic Surfactants

The biocompatibility or potential toxicity of surfactants to an enzyme is perhaps the most important issue to be considered at first. Sequential studies were designed to examine the effect of various surfactants. The first was made with 12 different nonionic surfactants (as described in Table 1) at 0.5% (*w*/*v*), and samples were taken to determine the quantity of ginsenoside Rh_2 generated at 3 and 24 h. The second run was made with six surfactants (Synperonic PE/F68, Synperonic PE/L61, PEGDME 250, PEGMME 350, PEGDME 400 and Brij 92V) at two concentrations, i.e., 0.5% (*w*/*v*) and 5% (*w*/*v*). These surfactants were known to be relatively compatible with this system according to the first round of tests (data not shown). It was designed to find the most effective surfactant among the studied, and the initial activity assayed in the absence of surfactant was taken as 100%. As shown in Fig. 2, most of the nonionic surfactants stimulated the β-glucosidase activity, which agrees with the results obtained in some literatures. The explanation given by them was that the interface of some nonionic micelles could be activators of bioactive enzymes [16, 20]. Another behavior to note was the stimulative function of higher concentration of the surfactants, which could be probably attributed to more micelles formed at higher concentration, resulting in more substrate being partitioned into the micellar pseudophase and reducing the effective concentration of substrate in water so that the inhibition on the enzyme was decreased. The PEGME surfactants (250, 350, and 400) appeared to be slightly

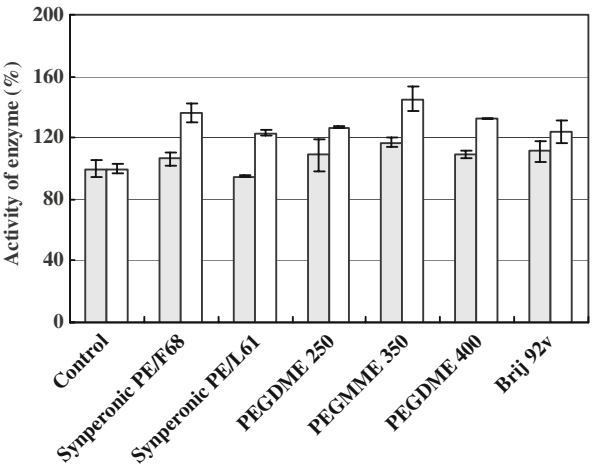


Fig. 2 Effect of surfactants on the β-glucosidase catalyzed hydrolysis reaction. The enzyme activity with no surfactant was expressed as 100%. *Filled bar* content of surfactant was 0.5% (*w*/*v*); *Open bar* content of surfactant was 5% (*w*/*v*)

better than the others, and the PEGMME 350 performed better than 250 and 400; therefore, this surfactant was chosen for further studies.

Optimum Concentration of the Surfactant

Figure 3 shows the enzyme activity of β-glucosidase, which was determined by measuring the amount of ginsenoside Rh_2 formed at 3 h, as a function of PEGMME 350 concentration. The hydrolysis rate increased with an increasing concentration of the nonionic surfactant before reaching 7.5% (*w*/*v*), but a further increase of the concentration beyond that point would make the β-glucosidase activity decrease. This phenomenon was possibly due to toxicity of the surfactant to the enzyme at a high concentration and another possible interpretation was the bioavailability of substrate in each micelle decreased with the increase of the surfactant concentration due to too many and too compacted micelles formed [21]. Therefore, the optimal concentration of PEGMME 350 was 7.5% (*w*/*v*) in terms of the initial rate, where the activity of β-glucosidase could be enhanced by more than 60%.

Effect of Enzyme Concentration

Concentration of enzyme plays a vital role in determining the reaction rate, since the overall rate of reaction must be limited by the amount of enzyme available. Figure 4 shows the effect of enzyme concentration on the production of ginsenoside Rh_2 after 72 h. There was no much difference in the productions of ginsenoside Rh_2 between the enzyme concentrations of 10~40 g/l, where ginsenoside Rh_2 production remained around 3.7 g/l. Probably because 10 g/l of the crude enzyme was enough for the total substrate dissolved in aqueous solution to form enzyme-substrate complex; therefore, 10 g/l of the crude enzyme was taken as the most suitable enzyme concentration for further studies.

Effect of Shaking Speed on Biohydrolysis

The rotation speed of the shaker used for the bioreaction is another important factor that could influence the amount of ginsenoside Rh_2 generated. The concentrations of

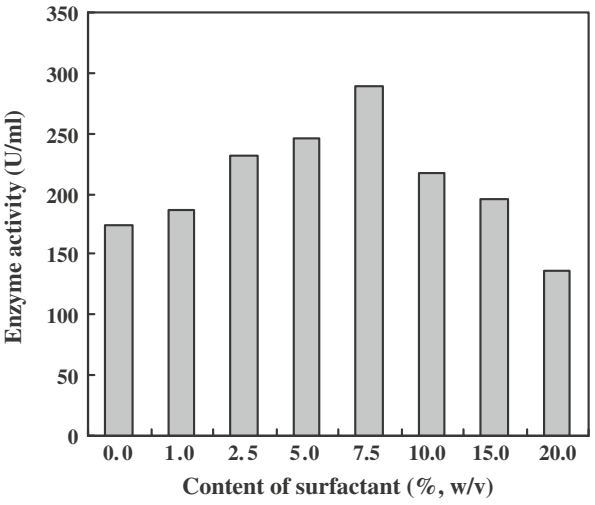


Fig. 3 Influence of surfactant concentration on ginsenoside Rg_3 hydrolysis. The reaction mixture was composed of 0.2 ml NaAc/HAc buffer (pH 5.0), 2 mg lyophilized crude enzyme from *Fusarium* sp. ECU2042 and 1 mg ginsenoside Rg_3

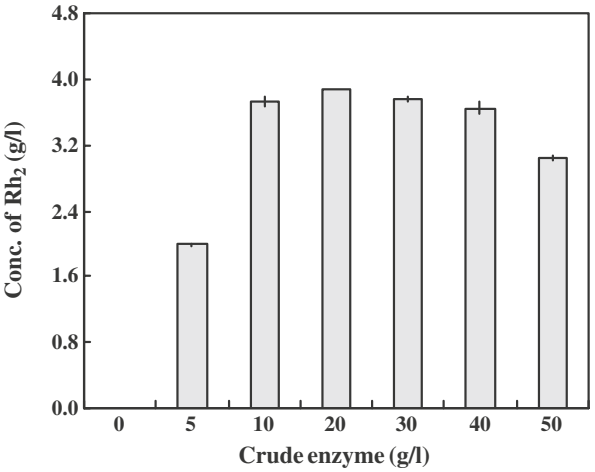


Fig. 4 Effect of enzyme concentration on ginsenoside Rh_2 production. Conditions: reaction time, 72 h; temperature, 50 °C

ginsenoside Rh_2 formed at different rotation rates were shown in Fig. 5. At lower rotation speed, increasing shaking rate made little improvement of production, and a significant improvement was observed when the rotation speed reached 1,100 rpm. Abundant rotation could improve the mixing of the system components, accelerate the mass transfer of substrate to the enzyme, and accordingly enhance the hydrolysis reaction. Moreover, too high rotation speed would be harmful to the enzyme and not conducive to the product generated as exhibited in Fig. 5. Therefore, a rotation speed of 1,100 rpm was preferred.

Time Course of the Biohydrolysis Reaction

The time course of ginsenoside Rg_3 hydrolysis by *Fusarium* sp. ECU2042 was studied at a substrate concentration of 5 g/l with (or without) the surfactant (7.5%, *w*/*v*). Figure 6

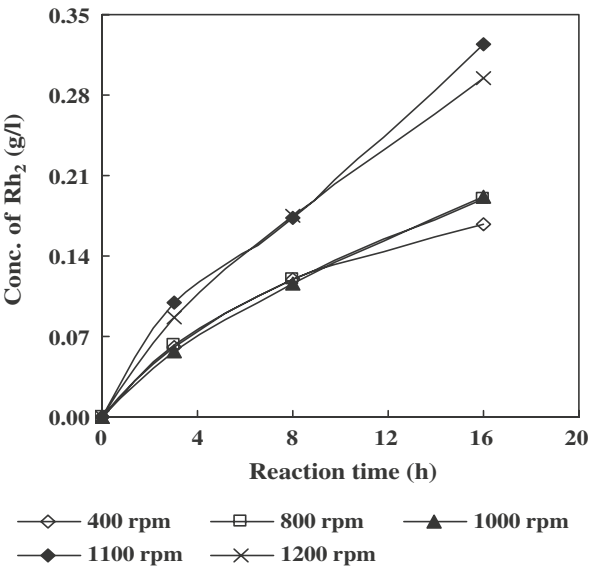


Fig. 5 Influence of agitation speed on ginsenoside Rg_3 hydrolysis with surfactant by *Fusarium* sp. ECU2042. All results are the means of two independent experiments

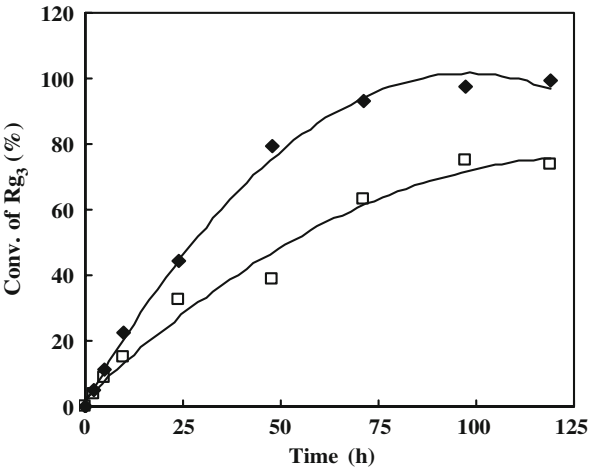


Fig. 6 Time course of ginsenoside Rg_3 hydrolysis by *Fusarium* sp. ECU2042 at 50 °C. *Filled diamonds* with surfactant; *Open squares* without surfactant. All experiments were performed in duplicate

indicates that the hydrolysis reaction reached an endpoint at 120 h, and ginsenoside Rg_3 were almost completely transformed into ginsenoside Rh_2 in the system with surfactant, while the percent conversion of ginsenoside Rg_3 in surfactant-free system only reached 74%. It is obvious that the micellar pseudophase partitions not only the substrate so as to reduce its inhibition to a certain extent but also the product in the same way. Just because of the multiple roles that the surfactant plays in this system, the catalytic efficiency of the enzyme could be fully displayed.

Enzyme Stability

Figure 7 shows the inactivation profiles of β-glucosidase in the presence of 7.5% (*w*/*v*) PEGMME 350 compared with that without surfactant, where *r* represents the residual activity at various time intervals and is expressed as a percent decrease. For the first 48 h, no much decrease in enzyme activity in the presence of surfactant was noticed, after which a noticeable amount of decrease was recorded. But still, from the starting point to 120 h, around 90% of the enzyme activity was left, which is 30% higher than that without

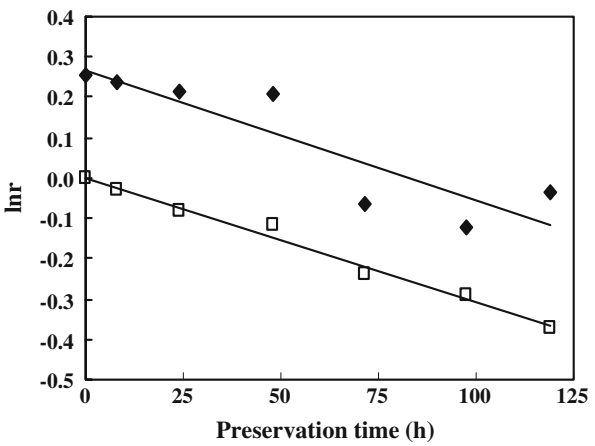


Fig. 7 Deactivation profiles of *Fusarium* sp. ECU2042 β-glucosidase under pseudo-reaction conditions. *Filled diamonds* with surfactant; *Open squares* without surfactant

surfactant. This indicates that the reason for the enzyme inactivation in this system should not be attributed to the introduction of the nonionic surfactant.

Conclusions

In recent years, biocatalysis has attracted more and more attention because of their many advantages, and the biocatalytic efficiency of an enzyme is the key to decide if the enzymatic process can be put into practical application or not. Many efforts were dedicated to this, and adding surfactants is one of the best choices.

Twelve nonionic surfactants were tested in this work as additives to the enzymatic reaction to enhance the productivity. From them, we selected PEGMME 350, which had the best biocompatibility for the subsequent studies. After investigating the effects of surfactant concentration and agitation speed on the reaction, the percent conversion of ginsenoside Rg_3 reached almost 100% under the optimal conditions, which was 25% higher than that in neat buffer. The enzyme stability was affected faintly by the surfactant, and the half life of the enzyme was 216 h with the surfactant, which was only 7 h shorter than that in neat buffer. Therefore, the PEGMME 350 is considered to be potentially useful for the hydrolysis of ginsenoside Rg_3 with *Fusarium* sp. ECU2042. This work proves that some biocompatible nonionic surfactants can certainly be used in enzymatic hydrolysis reactions, especially in biohydrolysis of natural products.

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