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Journal of Molecular Catalysis B: Enzymatic 11 (2001) 307–314

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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# An enzymatic process for the production of the pharmacologically active glycoside desglucodesrhamnoruscin from *Ruscus aculeatus* L.

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**Abstract**

The pharmacological properties of the extract of *Ruscus aculeatus* L. have been well established for many years now. The compounds which possess these properties are the steroid glycosides ruscin and ruscoside and their hydrolysis products desglucoruscin, desglucodesrhamnoruscin and desglucoruscoside. As the pharmacological action increases with the decrease of the amount of the sugar molecules, the plant extracts must be submitted to chemical or enzyme hydrolysis in order to obtain the most active compounds.

In our laboratory, a bioconversion process to produce the monoglycoside desglucodesrhamnoruscin from dry extracts of the rhizome of *R. aculeatus* has been developed using enzyme preparations containing a β-glucopyranosidase and an α-rhamnopyranosidase. Identifying the concentrations of substrate, enzyme and ethanol that are most advantageous for the bioconversion has optimized the process. The developed process gave a final product containing 19% of desglucodesrhamnoruscin. © 2001 Elsevier Science B.V. All rights reserved.

*Keywords:* *Ruscus aculeatus*; *Ruscus* glycosides; Ruscoside; Desglucodesrhamnoruscin; Bioconversion process

## 1. Introduction

*Ruscus aculeatus* L., familiarly known as butcher's broom, is an evergreen shrub typical of the underbrush of the Mediterranean scrub with false thorny leaves called cladophylles. The extracts have considerable pharmacological properties and they are used in the prevention and in the treatment of the venous insufficiency [1–4]. Moreover, they are provided with anti-elastase activity and they are components of drugs administered as anti-inflammatory and vasoconstrictor agents [5–7]. The anti-edematous effect of the sterolic heterosides was demonstrated on some edematous phenomena [8,9].

The pharmacologically active steroid glycosides, whose chemical structures were established 27 years ago, are mainly present in the rhizome of the plant (Fig. 1) [10,11]. Ruscin (1-*O*-[β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabino-

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PII: S1381-1177(00)00152-1

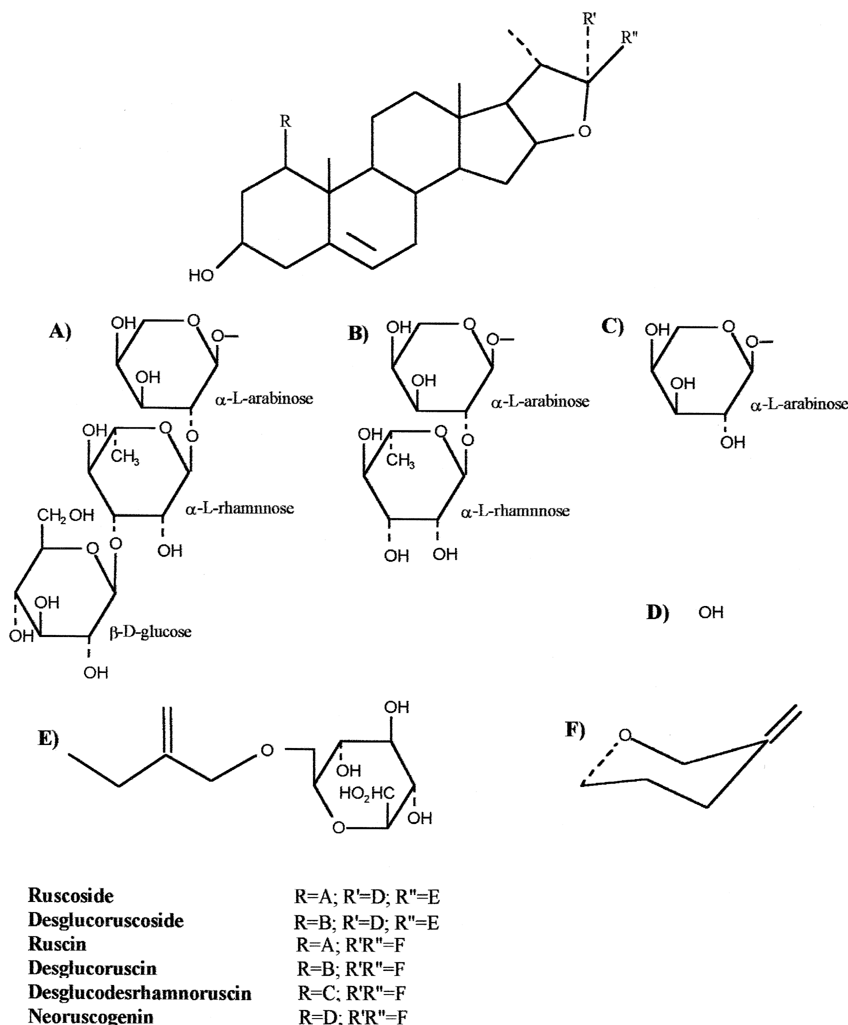


Fig. 1. Chemical structures of the main steroid glycosides present in the rhizome of *R. aculeatus* L.

pyranosyl (1)]-spirosta-5(6), 25(27)-diene-1β, 3β-diol), desglucoruscin (1-*O*-[α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl (1)]-spirosta-5(6), 25(27)-diene-1β, 3β-diol) and desglucodesrhamnoruscin (1-*O*-[α-L-arabinopyranosyl (1)]-spirosta-5(6), 25(27)-diene-1β, 3β-diol) are characterized by a sugar chain linked to the C-1 hydroxy group of the neoruscogenin. Two additional glycosides, belonging to the 22-hydroxy-furostanol saponins group and named ruscoside (1-*O*-[β-D-glucopyranosyl-(1 → 3)-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl (1)]-1β, 3β, 22α, 26-tetrahydroxy-furosta-5(6), 25(27)-dien-26-β-D-glucopyranoside) and desglucoruscoside (1-*O*-[α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl (1)]-1β, 3β, 22α, 26-tetrahydroxy-furosta-5(6), 25(27)-dien-26-β-D-glucopyranoside), can be converted in ruscin and desglucoruscin, respectively, by acid or enzyme removal of the glucose at the C-26 hydroxy group followed by immediate cyclization of the furostane 22–26 diolic system.

The pharmacological importance of these molecules has encouraged the industries to search efficient and feasible procedures for their production. The conversion of the ruscoside into its derivatives can be obtained by following two alternative proce-

dures: a chemical approach, based on acid hydrolysis and a biotechnological approach which involves an enzyme treatment. The first procedure is less selective than the enzyme hydrolysis since it produces undesired compounds and consequently, low yields of the product of interest; on the contrary, the biotechnological process is more suitable from an industrial point of view because it uses glycosidases which remove sugar residues from the glycosides in selective manner and in mild operative conditions.

In our laboratory, a process for the production of the monoglycoside desglucodesrhamnoruscin using a commercial enzyme preparation containing the activities of interest was developed. The ruscosaponins and the desglucoruscin were chosen as starting materials for the following reasons: the ruscosaponins do not need a treatment before use but the bioconversion of the ruscoside needs the action of β-glucopyranosidase and α-rhamnopyranosidase, which could not optimally work at the same conditions; on the contrary, a pre-treatment of the *Ruscus* extract is necessary to obtain the desglucoruscin, but its transformation requires just the action of the α-rhamnopyranosidase.

On these bases, trials were performed using either the desglucoruscin or the ruscosaponins in order to consider advantages and disadvantages of both processes. Moreover, different concentrations of starting materials, enzymes and ethanol were employed to individuate the best conditions for the bioconversion.

## 2. Experimental

### 2.1. Steroid glycosides source

The extracts or *R. aculeatus* L. used for the bioconversion processes were prepared by Indena (Italy). They consisted in a sample of *Ruscus* extract (containing 70% of ruscosaponins) and in a partially purified sample of desglucoruscin, deriving from hydrolysis of the ruscoside.

The ruscosaponins and the desglucoruscin were insoluble in the citrate–phosphate buffer used in the experiments and they were completely solubilized by adding ethanol (17% and 30% final concentration, respectively) to the buffer.

### 2.2. Enzyme sources

The following commercial preparations deriving from cultured broth of *Aspergillus niger* were screened to determine the enzyme activities of interest: Cellulase (Indena, Italy); NovoFermG12 and NovoFerm G76 (Novo Nordisk, Denmark); Cytolase PCL5 and Ar 2000 (Gist Brocades, Netherlands). Cytolase PCL5 was selected for the development of the bioconversion process. In order to remove the stabilizing agents, the preparation was diafiltered with 0.1M citrate–phosphate buffer pH 5.0 in an Amicon apparatus equipped with an XM membrane (cut-off 50 kDa). The obtained preparation resulted to be concentrated 3.7-fold.

### 2.3. Enzyme assay

β-glucopyranosidase and α-rhamnopyranosidase activities were measured using as substrates *p*-nitrophenyl-β-D-glucopyranoside (5.5 mM) and *p*-nitrophenyl-α-L-rhamnopyranoside (1.5 mM) dissolved in 0.1M citrate–phosphate buffer pH 5.0 with increasing concentrations of ethanol (up to 30% v/v). The hydrolysis reaction was followed by monitoring the increase in $A_{400}$ due to the release of *p*-nitrophenol. The standard reaction mixture, containing 450 μl of substrate, 40 μl of buffer and 10 μl of enzyme preparation, was incubated at 25°C for 1–5 min. The reaction was stopped by adding 1 ml of 1M $Na_2CO_3$. One enzyme unit was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate per minute under the described conditions.

### 2.4. Steroid glycosides bioconversion

The incubation mixture was prepared by adding in the following order: substrate (desglucoruscin or ruscosaponins), 0.1M citrate–phosphate buffer pH 5.0, Cytolase PCL5 and slowly, under vigorous stirring, ethanol. The substrate concentration varied from 0.8% to 40% (w/v) and the ethanol concentration from 0 to 30% (v/v). The efficiency of the bioconversion process was also studied in function of Cytolase PCL5 concentration.

### 2.5. TLC and HPLC analyses

TLC — The bioconversion over time of the steroid glycosides was followed by TLC. Silica gel plates (60 F254, Merck) were developed using a mixture of ethylacetate/methanol/water (100/15/10 by vol.) as eluent. The samples for the analysis were withdrawn from the incubation mixture at different times and diluted with methanol to reach a final concentration of substrate of 0.8% (w/v). Then, they were centrifuged in an Eppendorf centrifuge for 5 min at maximum speed, and the clarified supernatants were loaded on the silica gel plates. The plates were sprayed with 10% sulfuric acid in ethanol and the hydrolysis products were revealed drying the pates at 120°C.

HPLC — Quantitative determination of desglucodesrhamnoruscin in the final product was carried out using a Dionex chromatograph equipped with a UV detector operating at 200 nm and a Zorbax-ODS column (5 μm particle size, 250 × 4.6 mm ID). Aliquots of 50 μl of a solution of the final product (0.52 mg $ml^{-1}$ in methanol) diluted 4-, 8- and 12-fold were eluted with a mixture of acetonitrile (solvent A) and water (solvent B) at a flow rate of 1 ml $min^{-1}$. The following gradient was used: $t = 0$ min 40% solvent A; $t = 30$ min 60% solvent A.

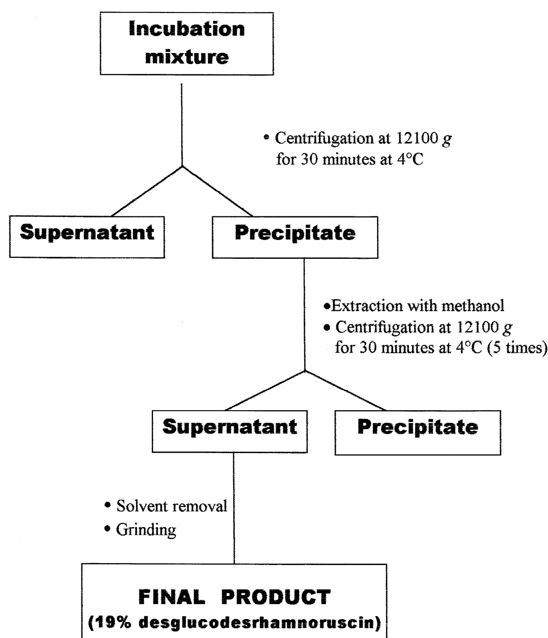


Fig. 2. Procedure for the recovery of the desglucodesrhamnoruscin.

### 2.6. Recovery of desglucodesrhamnoruscin

The desglucodesrhamnoruscin was recovered at the end of the bioconversion process by centrifuging the reaction mixture in a Beckman centrifuge at 12 100 g for 30 min at 4°C. The precipitate was washed with methanol and centrifuged as reported above. This step was repeated five times and the supernatants were pooled. The methanol was removed from the solution by *vacuum* evaporation and the resulting product was ground until a fine yellow powder was obtained (Fig. 2).

## 3. Results and discussion

### 3.1. Enzyme characterization and stability

The enzyme activities contained in the crude preparations were assayed using the synthetic substrates dissolved in a mixture of citrate–phosphate buffer and ethanol since the ruscosaponins and the desglucoruscin were soluble in 17% and 30% ethanol, respectively.

Among the screened preparations, Cytolase PCL5 was chosen for the highest α-rhamnopyranosidase activity and the lowest cost. The behaviour of the β-glucopyranosidase and the α-rhamnopyranosidase contained in the concentrated preparation of Cytolase PCL5 was differently influenced by ethanol (Fig. 3). An increase of the β-glucopyranosidase activity of 35% was detected in presence of 30% ethanol (from 70.1 to 94.6 U $ml^{-1}$), while the α-rhamnopyranosidase activity was decreased reaching 38% of its initial activity when measured in 30% ethanol (from 14 to 5.3 U $ml^{-1}$).

In order to establish if the presence of the organic solvent could cause a reduction of the enzyme activities over time, we measured the stability of β-glucopyranosidase and α-rhamnopyranosidase at 25°C in 0.1M citrate–phosphate buffer pH 5.0 either in absence or in presence of 17% ethanol. During 7 days we did not observe in the samples with ethanol a

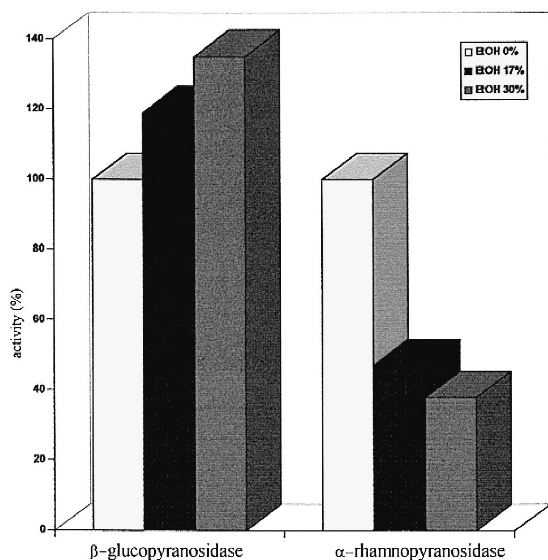


Fig. 3. Influence of ethanol on β-glucopyranosidase and α-rhamnopyranosidase activities. The assay was performed at 25°C using the synthetic substrates dissolved in 0.1M citrate–phosphate buffer pH 5.0/17% or 30% ethanol (v/v). 100% enzyme activity is defined as the activity measured in absence of ethanol.

decrease of the α-rhamnopyranosidase while the residual activity of the β-glucopyranosidase was 91% (Fig. 4). On the contrary, the enzyme stabilities in absence of ethanol appeared inferior, probably because of microbiological contamination which could rise in absence of the organic solvent in the concentrate culture broth of *A. niger*.

### 3.2. Bioconversion process

Preliminary trials of bioconversion were performed using desglucoruscin as starting material to verify if this substrate, already lacking one sugar on the carbohydrate chain, could be converted in desglucodesrhamnoruscin in less time respect to the ruscoside. Different amounts of Cytolase PCL5 (containing from 0.3 to 2.3 units of α-rhamnopyranosidase per ml of incubation mixture) and various concentrations of desglucoruscin (from 0.8% to 5% w/v) were tested. The best results were achieved using 2.3 units of α-rhamnopyranosidase per ml of incubation mixture with 5% (w/v) desglucoruscin in 0.1M citrate–phosphate buffer pH 5.0/30% ethanol. Desglucodesrhamnoruscin was obtained after 3 days of incubation at 25°C in a stirred bioreactor (data not shown).

The transformation of the ruscoside into the corresponding monoglycoside was carried out incubating a quantity of Cytolase PCL5, containing 38 units of β-glucopyranosidase and 2.3 units of α-rhamnopyranosidase per ml of incubation mixture, with variable amounts of ruscosaponins (from 5% to 40% w/v) in 0.1M citrate–phosphate buffer pH 5.0/17% ethanol. As expected, the reaction time needed for the complete transformation of the ruscoside in desglucodesrhamnoruscin depends on the initial substrate concentration. 2 and 7 days at 25°C were necessary to achieve the complete conversion of the ruscoside starting from 5% and 20% ruscosaponins, respectively, whereas traces of desglucoruscin, the intermediate reaction product, were still present after 13 days in the process performed with 40% ruscosaponins (Fig. 5). The time course of a bioconversion of 10% ruscosaponins is shown in Fig. 6. The disappearance of the ruscoside could be observed after 1 day, while the desglucoruscin was completely converted into desglucodesrhamnoruscin after 4 days.

These experimental findings showed us that both starting materials were completely converted into

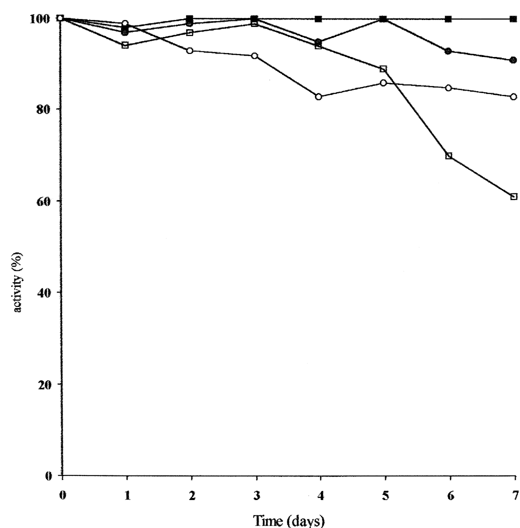


Fig. 4. β-glucopyranosidase and α-rhamnopyranosidase stabilities. Samples were withdrawn every 24 h and the enzyme activities were assayed at 25°C using the corresponding synthetic substrates dissolved in 0.1M citrate–phosphate buffer pH 5.0/17% ethanol (v/v), (β-glucopyranosidase: 0% ethanol, ○; 17% ethanol, ●) (α-rhamnopyranosidase: 0% ethanol, □; 17% ethanol, ■).

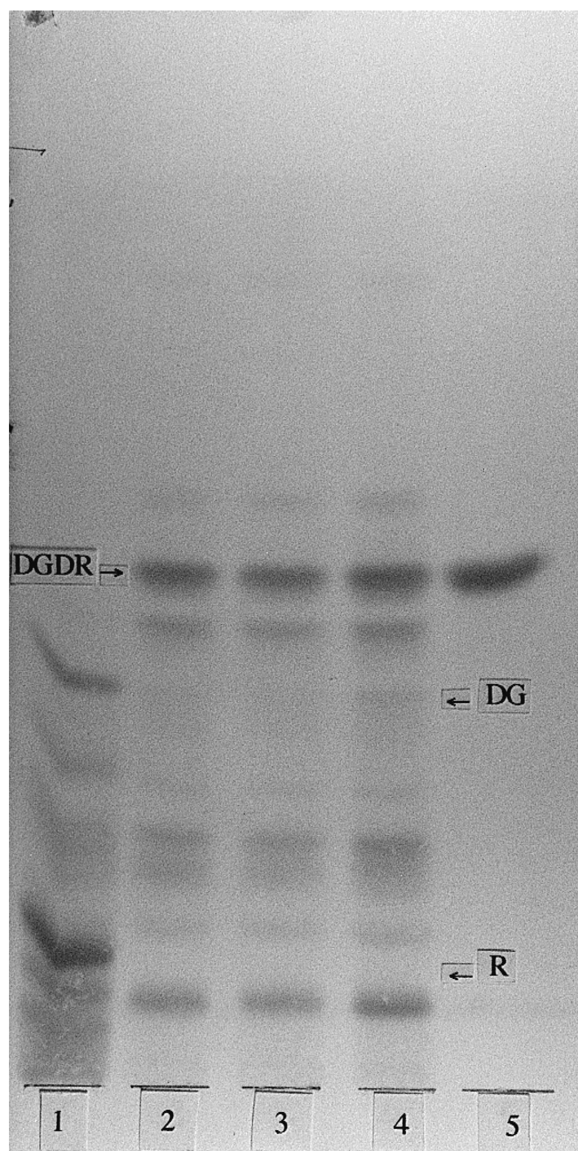


Fig. 5. Ruscoside bioconversion. The experiments were performed incubating different amounts of ruscosaponins at 25°C in 0.1M citrate–phosphate buffer pH 5.0/17% ethanol (v/v) with 38 units of β-glucopyranosidase and 2.3 units of α-rhamnopyranosidase per ml of incubation mixture (Lane 1: blank: 40% ruscosaponins at 0 h without enzyme preparation; lane 2: 5% ruscosaponins after 2 days; lane 3: 20% ruscosaponins after 7 days; lane 4: 40% ruscosaponins after 13 days; lane 5: desglucodesrhamnoruscin. R = ruscoside; DG = desglucoruscin; DGDR = desglucodesrhamnoruscin).

desglucodesrhamnoruscin but the use of the ruscosaponins as substrate can be more advantageous for the production of the monoglycoside for some reasons. They do not need a treatment before use, they require a lower percentage of ethanol for the complete solubilization respect to the desglucoruscin and consequently, they allow using less amount of Cytolase PCL5 since the α-rhamnopyranosidase activity increases with the decrease of the percentage of the organic solvent. Moreover, the β-glucopyranosidase and the α-rhamnopyranosidase can work with great efficiency at the same operative conditions since their pH optima are 5.0 and 4.5, respectively [12,13]. Basing on these considerations, we decided to use the ruscosaponins for the future trials.

To test the reproducibility of the process and basing on the experimental results, 50 g of ruscosaponins were transformed in desglucodesrhamnoruscin using the following selected conditions of bioconversion: 20% ruscosaponins in 0.1M citrate–phoshate buffer, pH 5.0/17% ethanol, Cytolase PCL5 (38 units of β-glucopyranosidase and 2.3 units of α-rhamnopyranosidase per ml of incubation mixture), 25°C and 7 days of incubation. The insoluble product formed in the reaction environment, containing the desglucodesrhamnoruscin, was separated from the aqueous phase by centrifugation. Then, it was extracted several times with methanol which was subsequently vacuum evaporated. The resulting product was dried and ground and its desglucodesrhamnoruscin content was determined by reversed-phase HPLC. The amount of the monoglycoside recovered was 5 g, corresponding to the 19% of the final product.

In order to investigate the possibility to reuse the Cytolase PCL5 in new cycles of biotransformation, the activities of the β-glucopyranosidase and the α-rhamnopyranosidase were measured in the aqueous supernatant. The residual activity of β-glucopyranosidase was 95% whereas a decrease of 25% was observed for the α-rhamnopyranosidase although the result of the stability test showed that the enzyme maintained its activity during 7 days in the mixture buffer/17% ethanol. Our opinion is that the decrease of the α-rhamnopyranosidase activity was due to a denaturation caused by molecules with surface-active action present in the reaction mixture since this effect was only observed in presence of the substrate. A dialysis of the supernatant in order to remove possible inhibitors did not lead to an increase

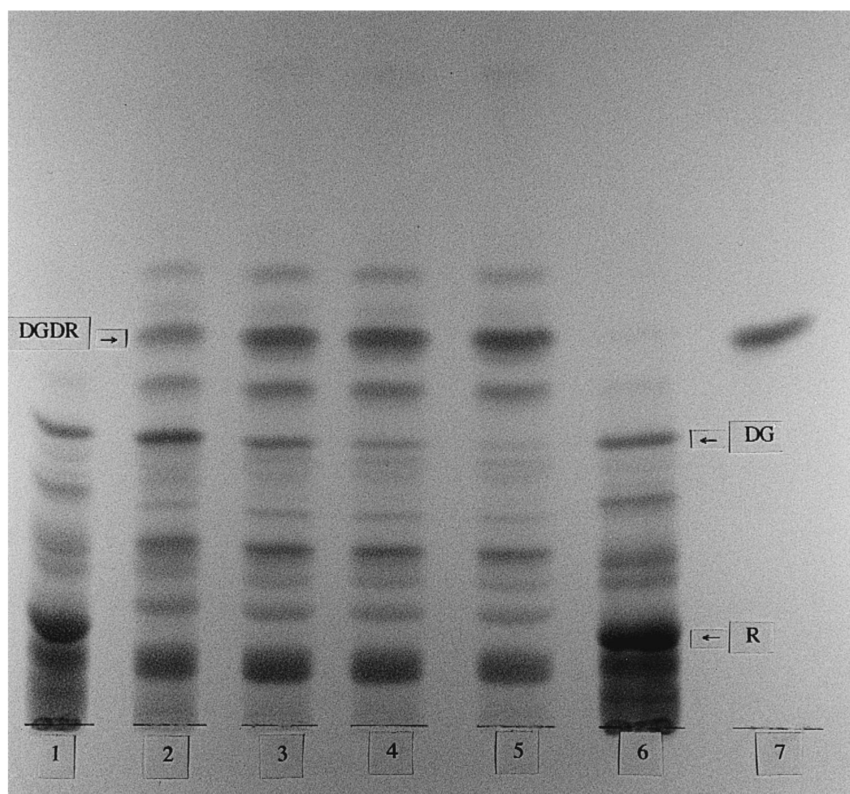


Fig. 6. Ruscoside bioconversion. The experiment was performed incubating 10% (w/v) ruscosaponins. (Lane 1: 10% ruscosaponins at 0 h with enzyme preparation; lane 2: 10% ruscosaponins after 24 h; lane 3: 10% ruscosaponins after 48 h; lane 4: 10% ruscosaponins after 72 h; lane 5: 10% ruscosaponins after 96 h; lane 6: blank: 10% ruscosaponins at 0 h without enzyme preparation; lane 7: desglucodesrhamnoruscin. R = ruscoside; DG = desglucoruscin; DGDR = desglucodesrhamnoruscin). For enzyme units and incubation conditions see the legend of Fig. 5.

of the enzyme activity, thus confirming our hypothesis.

## 4. Conclusions

The bioconversion process for the production of the desglucodesrhamnoruscin was planned considering the possibility to apply the developed methodology to industrial level. For this reason, the search for the most suitable enzymes to produce the monoglycoside was directed towards crude enzyme preparations, currently employed in the food industry, since they are easily available and have low costs. The developed process does not require specific and expensive equipment and it seems to be compatible with a large-scale production. Moreover, the simple procedure used to obtain the final product allows to easily recover with the aqueous supernatant the enzymes which could be used in new biotransformation cycles. At present, this opportunity is under investigation.

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