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Analytical Considerations for the Successful Evaluation of Hyoscyamine Biotransformation into 6β-Hydroxyhyoscyamine and Scopolamine

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SUMMARY. Hyoscyamine, 6β-hydroxyhyoscyamine and scopolamine are anticholinergic agents that belong to the tropane alkaloids, a pharmacological important group of secondary metabolites. Hyoscyamine and scopolamine were historically used in medicine. Additionally, potential medical applications for 6β-hydroxyhyoscyamine were described in the last years. Previous works carried out in our lab allowed us the construction of a *Saccharomyces cerevisiae* strain harboring the Hyoscyamine-6β-hydroxylase (H6H) enzyme which is responsible of the conversion of hyoscyamine into 6β-hydroxyhyoscyamine and scopolamine. Several factors influenced and complicated the optimization of the hyoscyamine bioconversion process. The aim of this work was to evaluate the analytical factors that critically affect the performance of the alkaloid extraction and the detection and quantification method of the alkaloids implied in the biocatalytical process. The mechanical breakdown of yeast cells by continuous agitation at 4 °C in 2 mL tubes was the method of choice for an efficient recovery of the functional H6H enzyme. In addition, the different pH assayed for the alkaloid extraction caused significant variations in the recovery of the alkaloids, specifically impacting on scopolamine recovery which decreased a 35 % after the increase of the pH of the extraction. The development of robust and sensitive analytical methods was requisite for the correct monitoring and quantification of the alkaloids produced in order to evaluate the technological and economic feasibility of this process.

INTRODUCTION

Hyoscyamine-6β-hydroxylase (H6H, EC 1.14. 11.11) is the final enzyme of the tropane alkaloid biosynthetic pathway and catalyzes the conversion of hyoscyamine into 6β-hydroxyhyoscyamine (also called anisodamine) and scopolamine by two sequential reactions (Fig. 1).

Hyoscyamine and scopolamine are anticholinergic agents that have been extensively used as pharmaceuticals [1,2]. In addition to the anticholinergic action, further therapeutic properties were described for 6β-hydoxyhyoscymine that includes the treatment of microvascular diseases, glomerulonephritis, rheumatoid arthritis, gastrointestinal colic, hemorrhagic necrotic enteritis, eclampsia as well as the control of toxic shock, septic shock, and organophosphorus poisoning [3]. Additionally, this alkaloid is not as toxic as atropine (dl-hyoscyamine) having lesser negative effects on the Central Nervous System than scopolamine [3].

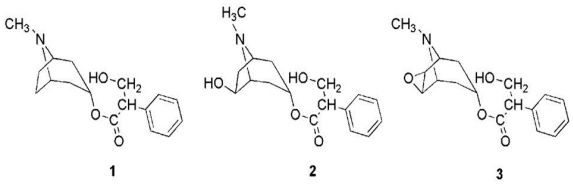

Figure 1. Hyoscyamine (**1**), 6β-hydoxyhyoscyamine (**2**) and scopolamine (**3**) chemical structures.

Nowadays these alkaloids are extracted from producer plants belonging to the Solanaceae family. Also, they can be synthesized chemically [4], however, the chemical synthesis is complicated and time consuming. The biotransformation

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of hyoscyamine is an attractive strategy for 6β-hydroxyhyoscyamine and scopolamine production [5] due to the reason mentioned above, the fact that scopolamine has a 10-times higher commercial demand than that of hyoscyamine 6 and the increased interest in 6β-hydroxyhyoscyamine for medical applications.

However, the success in the conversion of alkaloids was influenced by various factors. For an accurate evaluation of the transformation products, the development of efficient and precise analytical methods was necessary in order to ensure a complete isolation, detection and quantification of the alkaloids.

The aim of this work was to evaluate the analytical factors that critically impact on the monitoring of the conversion, including the influence of the alkaloid extraction and the detection and quantification method of the alkaloids involved in the biotransformation process, among others.

MATERIALS AND METHODS

Strains and genetic constructions for H6H production

The recombinant yeast strain used in this work was previously described [5]. Briefly, the wild type strain *S. cerevisiae* CEN PK2 (Acc. No. 30000D, Eurofan) (Mata/Matα ura3-Δ2/ura3-Δ2 trp1-289/trp1-289 leu2-3, 112/leu2-3, 112 his3Δ1/his3Δ1 mal2-8 C/mal2-8 C suc2/suc2) was kindly supplied by Dr. Susana Silverstein, IFYBINE-FCEN UBA. *S. cerevisiae* wild type strain was grown in YPD medium (10 g yeast extract/l, 20 g peptone/l, 20 g glucose/l) at 30 °C. The *h6h*cDNA [5,7] was inserted into the pYES2.1 vector from Invitrogen in order to produce the H6H enzyme in yeast. The recombinant yeast strain was maintained in the selection medium YNBD-U (yeast nitrogen base without uracil).

Yeast cell lysis

Different methodologies were assayed in order to obtain enough quantities of the recombinant H6H enzyme: mechanical breakdown, sonication, and liquid nitrogen freezing and grinding with mortar and pestle.

Mechanical breakdown

The yeast cell pellet corresponding to an optical density (OD) of 60 was washed twice with phosphate buffer 50 mM, pH 7.8 and resuspended in Breaking Buffer (Phosphate buffer 50 mM, pH 7.8, glycerol 8 % and protease inhibitor cocktail from Roche). The cell suspension was mixed with an equal volume of glass beads (0.4 – 0.6 mm).

Classical method. Yeast cells were lysed in 1.5 mL tubes by vortexing at maximum speed for 30 s followed by 30 s chilling on ice. This procedure was repeated 4 times. The lysate obtained was transferred to a fresh tube.

Scale up. The operating conditions applied in this case were the same that those described above. Particularly in this case, the 1.5 mL tubes were replaced by 15 mL conical tubes scaling the volume of the cell pellet lysed. The lysate obtained was transferred to a fresh tube.

Continuous agitation. The cell lysis was performed by vortexing continuously at maximum speed for 20 min at 4 °C. Additionally, the 1.5 mL tubes were replaced by 2 mL tubes. The lysate obtained was transferred to a fresh tube. The total protein content was measured by the Bradford method [8].

Sonication

Yeast cells were washed and resuspended in breaking buffer as it was previously described. The yeast cell suspensions were sonicated using a Sonics & Materials inc. Sonicator (Danbury, Connecticut, USA). Different power (50W, 80W and 100W) and time (30 s, 1, 2, 5, and 10 min) combinations were applied followed by short cooling periods. After the treatments the samples were centrifuged al 10000 rpm for 10 min and the supernatant assayed for total proteins by the Bradford method [8].

Liquid nitrogen freezing and grinding with mortar and pestle

Yeast cells were placed in mortar and ground with liquid nitrogen. After this treatment the sample was resuspended in breaking buffer and centrifuged as it was described above. The supernatant assayed for total proteins by the Bradford method [8].

Biotransformation assay

The transformation of hyoscyamine by crude preparations of the recombinant H6H was assayed by measuring its conversion into 6β-hydroxyhyoscyamine and scopolamine [5]. The reaction mixture includes 50 mM Tris-HCl buffer (pH 7.8 at 30 °C), 4 mM sodium ascorbate, 0.4 mM $FeSO_4$, 1 mM 2-oxoglutarate, 0.2 mM *l*-hyoscyamine hydrobromide, 500 µl catalase [9,10]. The reaction was started by the addition of the crude extract containing the H6H enzyme and stopped by the alkalinization of the reaction mixture with carbonate buffer pH 10.

Alkaloid extraction

The alkaloid extraction was performed at pH 9 and 12 with 5 mL chloroform (ratio 1:1 v/v) by continuous vortexing for 2 min [11,12]. Samples were extracted three times and the organic phases obtained were pooled and evaporated under gaseous N_2 at room temperature. The residue was dissolved in methanol-water (50:50 v/v) and filtered through a 0.45 µm pore nylon membrane.

Analytical Methods

HPLC analysis of the alkaloids was performed on a Shimadzu LC-20AT system with a LiChro CART column 125–4 Lichrospher 60 RP-select B (5 µm), Merck. The elution was performed at 40 °C, isocratically with Octanesulfonic acid 0.01 M pH3/Metanol (65:35 v/v) at a 1 mL/min flow rate. The detection was performed at 220 nm.

Statistical analysis

Results are means of three independent experiments. Sample variability was given as the standard deviation of the mean. The statistical significance was evaluated by performing a T test. Differences between means were considered significant when $p < 0.05$. Statistical analyses were performed by the InfoStat software.

RESULTS AND DISCUSSION

Since the recombinant *S. cerevisiae* strain used in this work produces the H6H enzyme intracellularly [5] it was necessary performing the yeast cell lysis in order to obtain the enzyme. It is well known that the efficient yeast cell lysis is one of the main issues associated with the utilization of yeast for biotechnological purposes. *S. cerevisiae* is considered one of the most difficult microorganisms to disrupt [13]. Frequently, the cell wall characteristics complicate the downstream processing leading to protein isolation and purification [14]. For this reason, different methodologies were assayed in order to obtain enough quantities of the recombinant H6H enzyme.

Firstly, the mechanical lysis using glass beads was assayed as it is described in Material and Methods section. Under these working conditions (classical method) the total protein content obtained was 81.15 ± 0.81 µg/mL (Fig. 2). Additionally, the scale up of the method (scale up) was assayed using 15 mL conical tubes. The protein content obtained in this case was 118.31 ± 6.07 µg/mL (Fig. 2). These findings show that

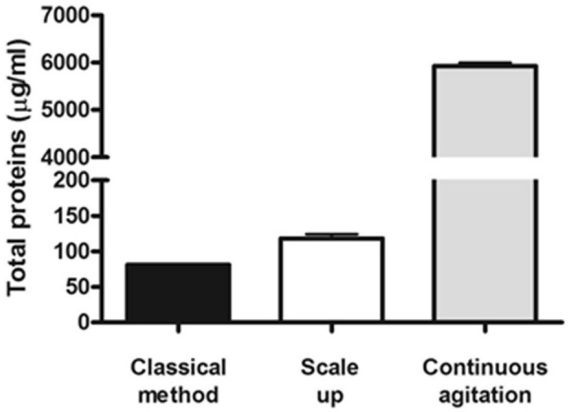

Figure 2. Total proteins measured after the application of the different variants to the mechanical lysis method.

the modifications included allowed us the processing of higher volumes of yeast culture maintaining protein yields. Further modifications of the classical method such us the employment of 2 mL tubes and the continuous agitation at 4 °C (Continuous agitation) increased protein yield up to 5.93 ± 0.067 mg/mL (Fig. 2).

Secondly, the disruption of yeast cells was assayed by sonication. Different treatments were applied varying the potencies and time of exposure as it is described previously. However, the protein yields obtained under these conditions were slightly higher than those obtained for the mechanical lysis using the 15 mL conical tubes (data not shown). Nevertheless, the protein yield was significantly higher when the mechanical method modified by continuous agitation at 4 °C was applied compared to the sonication of samples.

Thirdly, the manual grinding in a mortar using liquid nitrogen was analyzed for the yeast cells lysis. The application of this method was complicated and protein yield was low or not detectable.

The enzymatic lysis of the cell wall is another possibility to obtain large amounts of proteins from yeast. However, enzymatic digestion can be expensive when working with large quantities of samples [14]. Also, an additional step is commonly required to lyse the spheroplasts generated by the lytic enzymes (e.g. lyticase, zymolase) increasing the cost of protein isolation [15].

For the reasons mentioned above, the mechanical breakdown of yeast cells including modifications such as the continuous agitation at 4 °C and the application of 2 mL tubes was the method of choice to obtain enough quanti-

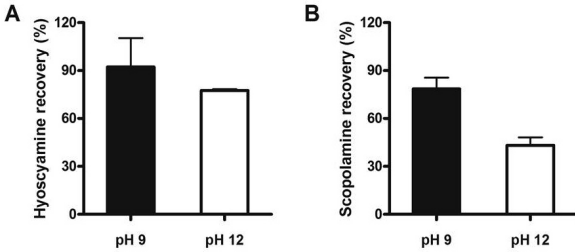

Figure 3. Recovery of hyoscyamine (**A**) and scopolamine (**B**) after the chloroform extraction performed at pH 9 and 12.

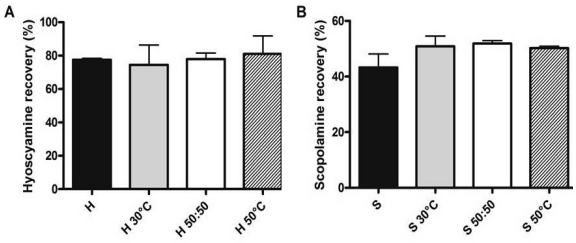

Figure 4. Influence of different factors on the recovery of hyoscyamine (**A**) and scopolamine (**B**). H and S: control condition; H 30 °C and S 30 °C: samples incubated at 30 °C previously to the extraction; H 50:50 and S 50:50: isolation form mixtures; H 50 °C and S 50 °C: samples evaporated at 50 °C.

ties of the H6H enzyme maintaining its functionality.

For evaluation of the hyoscyamine biotransformation using the recombinant H6H enzyme produced in *S. cerevisiae* it was necessary to optimize various factors that were critical in the alkaloid extraction from the samples and the measurement of them.

Regarding to alkaloid extraction, the factors considered were the pH for the organic extraction, the agitation conditions to allow the transfer of the alkaloid molecules to the organic phase and the evaporation conditions of the organic extract among others.

Previous works carried out with tropane alkaloids have reported different working conditions in relation to the pH of the extraction. These values varied between 8 to 12 [16,17].

Figure 3 shows the recovery of hyoscyamine and scopolamine after their isolation at pH 9 and 12. Although hyoscyamine recovery falls from 92.33 ± 18.01 % at pH 9 to 77.56 ± 0.83 % at pH 12, this difference was not statistically significant. However, scopolamine recovery decreased a 35 % after the increase of the pH of the extraction. This difference was statistically significant ($p = 0.001$).

The assays performed have shown the requirement to optimize the methodological conditions in the extraction of alkaloids since different pH assayed caused variations in the recovery of them. This fact is probably related to the hydrolysis of the molecule exposed at alkaline pH values. Specifically considering hyoscyamine, although the decrease in the recovery was not statistically significant it should be careful choosing the working conditions to ensure the correct monitoring of the variations in its content.

The agitation cycles to promote the transfer of the alkaloids to the organic phase had a slightly influence in the recovery of tropane alkaloids. Continuous agitation for 2 min was more effective than the sum of 30 s cycles (data not shown).

Also, other factors that might affect the recovery of tropane alkaloids were analyzed (Fig. 4a and b). Among them, the influence of the incubation of the alkaloids at 30 °C for 2 h as they are exposed during the enzyme activity assays was considered. This condition was analyzed in order to evaluate whether degradation of tropane alkaloids exist during the biotransformation process. Another factor analyzed was the effect of evaporating the organic extracts at 50 °C on the recovery percentage of the alkaloids. Additionally, the recovery of each alkaloid was evaluated when they are isolated from mixtures. The conditions assayed did not significantly affect the recovery of hyoscyamine and scopolamine (Fig. 4).

Regarding to the optimization of the alkaloid detection and quantification method, it was crucial the development of an analytical method that allowed us the detection of small quantities of tropane alkaloids in order to follow the biotransformation processes.

For the detection of the alkaloids present in plants, the method applied previously in our lab [17,18] had enough sensitivity. This method was carried out using a LiChro CART column 250-4 RP-18 (5 µm), Merck. The elution was performed at 40 °C, isocratically with 1 % trietilamine-formic acid/ethanol (9:1 v/v) at a 1 mL/min flow rate. However, for monitoring the conversion of hyoscyamine into its products by the recombinant enzyme it was necessary to optimize an HPLC method in order to achieve reproducibility, sensitivity, and an optimum resolution of the alkaloid peaks. In this way, different modifications were introduced to methods described in bibliography [19].

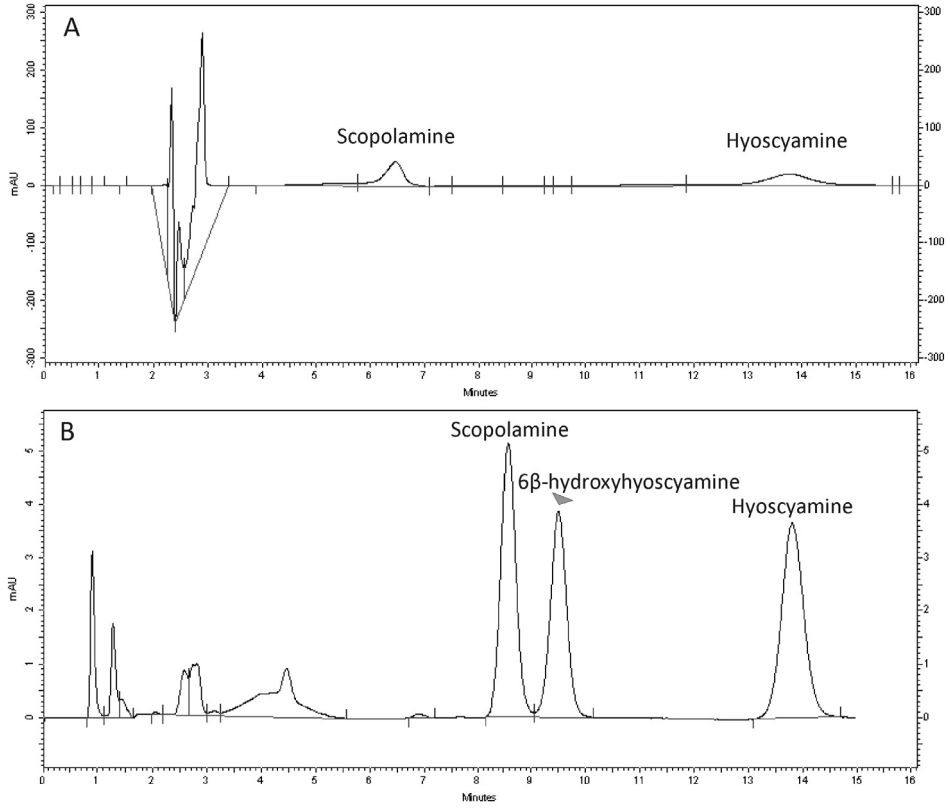

Figure 5. (**A**) HPLC chromatogram obtained by the method described by Mano *et al.* [17] after the injection of a 1000 ppm mixture of hyoscyamine and scopolamine. (**B**) HPLC chromatogram obtained by the optimized method after the injection of 10 ppm mixture of hyoscyamine, 6β-hydoxyhyoscyamine and scopolamine.

In order to avoid the tailing effects on the alkaloid peaks we changed the stationary phase of the HPLC column. A LiChro CART column 125–4 Lichrospher 60 RP-select B (5 µm) was successfully employed for the analysis of tropane alkaloids.

Several works, reviewed by Dräger [20], demonstrated that it is possible to separate and quantify tropane alkaloids by HPLC using different solvent systems. Among them we can mention the potassium phosphate buffer (pH 2.7) with 17 % acetonitrile, methanol-water (40:60, pH 7.25, triethyl ammonium phosphate 0.2 %), tetrahydrofuran containing 1 % ammonia, triethylamine and formic acid pH 3.5, acetonitrile-water-acetic acid-tetrahydrofuran (50:50:5:2) and a buffered triethylamine-phosphoric acid 30 mM, pH 2.2 aqueous acetonitrile mixture (12.5 % acetonitrile). In order to separate, analyze and quantify tropane alkaloids we successfully employed a solvent system conformed by different proportions of octanesulfonic acid 0.01M (pH 3) and methanol [21]. The working conditions selected which permitted the separation of the three alkaloids under analysis are detailed in the Material and Methods section [5,11].

Even though it was possible to separate the alkaloids at room temperature (data not shown), we decided to work under temperature controlled conditions. The analysis was carried out

	6β-hydroxyhyoscyamine (%)		**Scopolamine (%)**	
Time (h)	2	15	2	15
H6H	53.7	83.3	-	7.6
Negative control	-	-	-	-

Table 1. Biotransformation products obtained from hyoscyamine by H6H catalytic activity [5]. The percentage values of 6β-hydroxyhyoscyamine and scopolamine are the means of three independent determinations which differs in no more than 10 %.

at 40 °C in order to accelerate the alkaloid elution, reduce the chromatographic times and to obtain reproducible chromatograms.

Under these experimental conditions it was possible to reach a good resolution between picks maintaining an acceptable running time. The detection limit was 2 ppm of each alkaloid with a resolution factor of 2.6.

Figure 5 shows the typical chromatograms obtained by both HPLC methods. The first method was not sensitive enough for the alkaloid analysis during the biocatalytic processes. The concentrations injected in the present assay were highly superior to those present in the bioconversion assays.

The optimization of all the factors mentioned above allowed us to perform and to study tightly the biotransformation of hyoscyamine by the H6H enzyme [5].

Table 1 shows the percentages of the alkaloids produced in the biotransformation assays. The H6H enzyme was able to transform hyoscyamine into more valuable alkaloids, scopolamine and 6β-hydroxyhyoscyamine.

The results obtained in this work indicated that it is necessary to analyze in depth the different methods for yeast cell lysis for an efficient recovery of the functional H6H enzyme. In this case, the mechanical breakdown of yeast cells including modifications such as the continuous agitation at 4 °C and the application of 2 mL tubes was the method of choice. Also, the analysis of experimental variables that affects the isolation and quantification of hyoscyamine, 6β-hydroxyhyoscyamine and scopolamine was essential in order to follow the hyoscyamine biotransformation by the H6H enzyme. Our studies also highlight the importance of having a reliable method for the alkaloid extraction that did not affect significantly the recovery of them after the isolation from the samples. This was essential to ensure the detection of small amounts of the alkaloids transformed by the enzyme.

The correct monitoring of the quantities produced of each alkaloid during hyoscyamine biotransformation process is essential in order to evaluate the technological and economic feasibility of this process of interest for the pharmaceutical industry. For this reason, the development of a robust and sensitive analytical method was requisite for the detection and quantification of hyoscyamine, 6β-hydoxyhyoscyamine and scopolamine.

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