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Quantification of the Bioactive Compound Eritadenine in Selected Strains of Shiitake Mushroom (*Lentinus edodes*)

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Cardiovascular disease is one of the most common causes of death in the Western world, and a high level of blood cholesterol is considered a risk factor. The edible fungus, shiitake mushroom (*Lentinus edodes*), contains the hypocholesterolemic agent eritadenine, 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid. This study was conducted to quantify the amount of the cholesterol reducing agent eritadenine in shiitake mushrooms, in search of a potential natural medicine against blood cholesterol. The amounts of eritadenine in the fruit bodies of four different shiitake mushrooms, Le-1, Le-2, Le-A, and Le-B, were investigated in this study. To achieve this goal, methanol extraction was used to recover as much as possible of the hypocholesterolemic agent from the fungal cells. In addition, enzymes that degrade the fungal cell walls were also used to elucidate if the extraction could be further enhanced. To analyze the target compound, a reliable and reproducible HPLC method for separation, identification, and quantification of eritadenine was developed. The shiitake strains under investigation exhibit up to 10 times higher levels of eritadenine than previously reported for other shiitake strains. Further, pretreating the mushrooms with hydrolytic enzymes before methanol extraction resulted in an insignificant increase in the amount of eritadenine released. These results indicate the potential for delivery of therapeutic amounts of eritadenine from the ingestion of extracts or dried concentrates of shiitake mushroom strains.

KEYWORDS: Eritadenine; *Lentinus edodes*; bioactive compounds; HPLC

INTRODUCTION

The shiitake mushroom is widely cultivated and consumed not only as food but also as a natural medicine because of its medical properties. This mushroom has a high nutritional value and contains several substances with additional positive effects on health, such as the anti-tumor agent lentinan (*1*). One of the health benefits, which this mushroom possesses, is the ability to reduce blood cholesterol as shown in both animal and human studies (*2*, *3*). The cholesterol reducing agent in shiitake mushrooms is a purine alkaloid (**Figure 1**) designated as eritadenine (lentinacin), 2(*R*),3(*R*)-dihydroxy-4-(9-adenyl)-butyric acid (*4*).

Competitive inhibitors of HMG-CoA reductase, the statins, are produced in a large scale as cholesterol reducing pharmaceuticals. Unlike the statins, eritadenine does not inhibit the biosynthesis of cholesterol in the liver but enhances removal of blood cholesterol (*5*). The exact mechanism by which eritadenine elicits its hypocholesterolemic action is not yet fully understood. However, the hypocholesterolemic action of eritadenine has been investigated in several studies on rats. It has been shown that total plasma cholesterol levels are decreased in rats fed eritadenine in their diets and that the hypocholesterolemic action is caused by a decrease of the phosphatidylcholine (PC)/phophatidylethanolamine (PE) ratio (*6*−*10*). Eritadenine is a very potent inhibitor of the enzyme *S*-adenosyl-L-homocysteine hydrolase in rat liver cells (*11*), thereby causing an increase in the *S*-adenosylhomocysteine concentration (*12*). The increase in the *S*-adenosylhomocysteine concentration in turn inhibits the PE N-methylation, thus increasing the PE content in liver microsomes (*9*). Further studies on rats suggest that eritadenine may increase the uptake of plasma lipoprotein cholesterol by the liver and thus reduce the plasma cholesterol

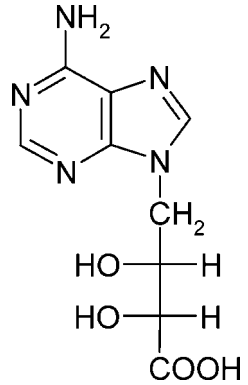

Figure 1. Chemical structure of eritadenine (M_r = 253 g/mol).

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(*10*). There is a possibility that the change in composition of the membrane phospholipids may activate lipoprotein receptors in liver cell membranes, thus regulating the uptake of plasma lipoprotein lipids (*6*).

The amounts of eritadenine in shiitake mushrooms quantified so far are in the range of 50−70 mg/100 g dry weight (dw) in the caps, 30−40 mg/100 g in the stems (*13*, *14*), and 73.7 mg/100 g in shiitake mycelium (*15*). In rats, a diet containing 0.005% eritadenine was shown to lower the serum total cholesterol level with 25% (*4*). Similar studies on humans have not been found in the literature. Previous work indicates the efficacy of shiitake mushrooms in cholesterol reduction; however, the concentrations reported indicate that large quantities may have to be consumed to achieve therapeutic effects (*3*). To establish the dose−response effect of shiitake mushrooms as a cholesterol reducing product, strains producing considerable amounts of eritadenine are favorable, as is a reliable analytical procedure for this substance. To achieve an accurate quantification of eritadenine, losses in the extraction procedure should be minimized and the amount released from the mushrooms maximized.

The goal of the present work was to evaluate the eritadenine content of four commercially cultivated shiitake mushrooms, Le-1, Le-2, Le-A, and Le-B, to determine if they could be used as a viable source of eritadenine, either as a dried product or as an extract. To achieve this objective, a reliable analytical HPLC procedure quantifying eritadenine in shiitake mushrooms was developed.

In an attempt to further increase the amount of eritadenine released, enzymes involved in the breaking of bonds between the polymers in fungal cell walls were used in this study. The cell walls of shiitake mushrooms are mainly composed of the polysaccharides chitin (β-1,4-*N*-acetylglucosamine) and glucans (β-1,3 and β-1,6), which can be degraded by hydrolytic enzymes possessing chitinase or glucanase activity (*16*). Hence, enzymes with these properties were used to enhance the extraction procedure by macerating the fungal cells.

MATERIALS AND METHODS

Fungal Material. Four different commercial shiitake mushrooms were used in this study. The fruit bodies of the *Lentinus edodes*-1 (Le-1) and *Lentinus edodes*-2 (Le-2) strains were kindly supplied by Dr. Gary L. Mills, Diversified Natural Products, Inc., Scottville, MI. The fruit bodies of the two other shiitake mushrooms were bought at local stores and denoted here as *Lentinus edodes*-A (Le-A) and *Lentinus edodes*-B (Le-B). The supplier of Le-A was Limax, Horst, The Netherlands, and the supplier of Le-B was Mykora Oy, Kiukainen, Finland. The fruit bodies were dried in a mushroom dryer. To eliminate individual differences among the mushrooms, dried fruit bodies were subsequently homogenized in a blender, making one batch of 50 g of homogeneous mushroom powder for each strain. The extraction procedures were all repeated 3 times, using 3 g of the homogeneous batches for each extraction.

Preparation of Eritadenine Standard. Since eritadenine is not commercially available, it was synthesized. In the first step, methyl 2,3-*O*-isopropylidene-β-D-ribofuranoside was synthesized (*18*). This product was further processed to give the compound methyl 2,3-*O*-isopropylidene-5-*O*-*p*-toluenesulfonyl-β-D-ribofuranoside (*19*). The third step was a reaction of sodium salt of adenine with methyl 2,3-*O*-isopropylidene-5-*O*-*p*-toluenesulfonyl-β-D-ribofuranoside. This reaction gave the product methyl 5-(6-aminopurin-9*H*-9-yl)-2,3-*O*-isopropylidene-5-deoxy-β-D-ribofuranoside. Hydrolysis of this product resulted in 5-(6-aminopurin-9*H*-9-yl)-5-deoxy-D-ribofuranose. The final step was an air oxidation of the previous compound to obtain the product 2(*R*),3-(*R*)-dihydroxy-4-(9-adenyl)-butyric acid (i.e., D-eritadenine (*20*)). All chemicals were of analytical grade. To verify the correct product and its purity, NMR analysis was conducted for each step of the synthesis and compared with the literature. An LC/MS run further confirmed the final product. A stock solution (1.98 mg/mL) of the standard was prepared by dissolving synthesized eritadenine in distilled water.

Methanol Extraction and Isolation of Eritadenine. The extraction was a modified version of the method developed by Tokita et al. (*17*). Powder from dried fruit bodies was weighed and extracted with 80% (v/v) methanol for about 3 h under reflux, with a solid−liquid ratio of 1:20. The extract was filtered through Whatman No. 5 filter paper, evaporated to dryness in vacuo at 50−60 °C, and diluted in 50 mL distilled water. The sample was further extracted with 50 mL of diethyl ether 3 times. To the aqueous layer, 4 volumes of 99.5% ethanol was added and incubated at −20 °C overnight. The precipitate was removed by filtration through Whatman OOH filter paper, and the filtrate was evaporated to dryness in vacuo at 50−60 °C. The extract was diluted in 50 mL of distilled water and applied to a column of Amberlite IR-120 (H^+) ion-exchange resin. The substance was eluted with 2% ammonia, showing a high absorbance at 260 nm. The volume collected was evaporated to dryness in vacuo at 50−60 °C, diluted in 50 mL of distilled water, and applied to an Amberlite IRA-67 (OH^-) ion-exchange resin. The substance was eluted with 0.1 M acetic acid, and fractions showing high absorbance at 260 nm were collected. After evaporation to dryness in vacuo at 50−60 °C, the mushroom sample was dissolved in 100 mL of distilled water. The completely isolated eritadenine was confirmed with LC/MS.

Enzymatic Pretreatment. The enzyme preparation, NS 33075, was kindly supplied by Novozymes, Bagsvaerd, Denmark. NS 33075 is a multicomponent carbohydrase preparation originating from *Trichoderma harzianum*. The main components of this enzyme mixture are various α- and β-glucanases, but it also contains some side activities like chitinases and proteases. According to the supplier, this enzyme mixture shows a fairly high activity at pH 4.8 and 50 °C. The enzyme powder was dissolved in water to a stock solution of 2% (w/v). A volume of the stock solution corresponding to 0.2 mg of enzyme per gram of mushrooms was added to either distilled water (pH 6.0) or 0.1 M acetate buffer (pH 4.8). The volume of the water and the buffer used was 10 mL per gram of mushrooms. The enzyme−water or enzyme−buffer mixture was added to the weighed mushroom powder, and the reaction was incubated at 50 °C for 3 h with gentle stirring. Following the enzymatic treatment, a methanol extraction was preformed as previously described.

HPLC Analysis. The eritadenine concentration in shiitake fruit bodies was analyzed by HPLC (Series 200 Quaternary LC pump and UV−vis detector, TotalChrom software, Perkin-Elmer, Wellesley, MA) and separated over a C18 column (RESTEK Ultra Aqueous, 5 μm, 150 mm × 4.6 mm). Prior to analysis, the samples from the extractions were diluted twice with the initial mobile phase and filtered through a 0.2 μm syringe filter. The HPLC analysis was conducted at 23 °C, with a flow rate of 1 mL/min and UV detection at 260 nm. The initial mobile phase was 0.05% TFA in aqueous solution/0.05% TFA in MeCN, in the proportions of 98:2 followed by a linear change to 40:60 over 10 min, and then returned to the initial condition for 15 min. All data were collected and processed using Perkin-Elmer's TotalChrom analytical software. Peak areas from the chromatograms were evaluated on the basis of a reference curve prepared from standard samples of eritadenine diluted in the initial mobile phase to concentrations in the range of 0.0124−0.198 mg/mL.

RESULTS AND DISCUSSION

The usage of shiitake mushroom extracts as food additives against blood cholesterol may have some benefits. Since the active substance eritadenine is water soluble, no excessive fat has to be ingested as is the case with phytosterols used for the same purpose. Eritadenine consumed in combination with cholesterol reducing statins might reduce severe side effects since the mechanism of action for eritadenine differs from the corresponding one for statins. Using shiitake mushroom as a cholesterol reducing natural medicine requires a reliable method

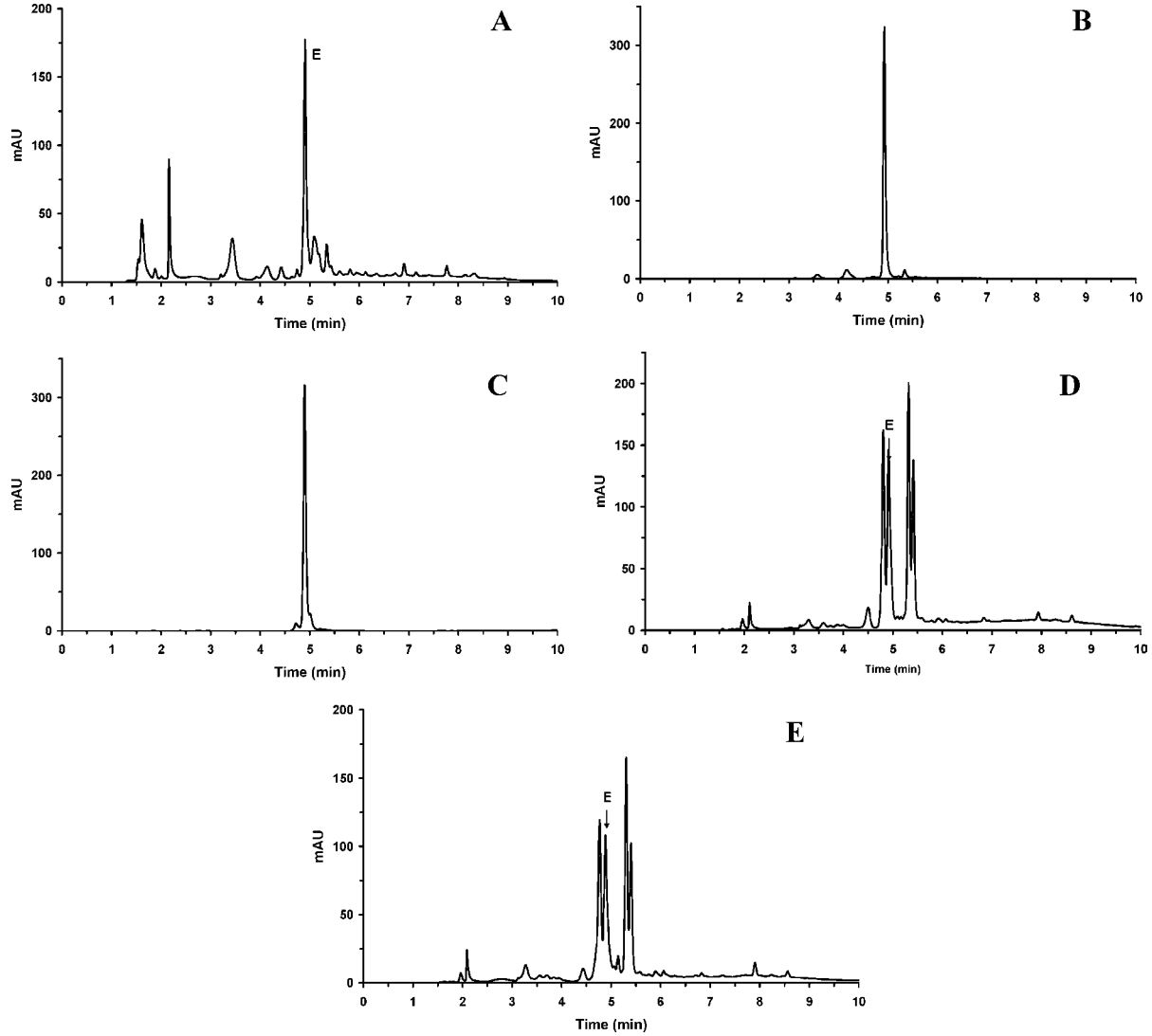

Figure 2. HPLC chromatograms at 260 nm of a methanol extract from shiitake mushrooms (**A**), completely isolated eritadenine from shiitake mushrooms (**B**), synthesized eritadenine (**C**), methanol extract from shiitake mushrooms treated with hydrolytic enzymes in buffer (pH 4.8) (**D**), and methanol extract from shiitake mushrooms treated with hydrolytic enzymes in water (pH 6.0) (**E**). Eritadenine is eluted at a retention time of 4.9 min and is represented by E in the chromatograms of methanol extracts.

of quantifying eritadenine amounts and careful dose−response studies on humans.

To make the quantification of eritadenine as accurate as possible, the losses from the extraction procedure should be minimized. By comparing the HPLC chromatogram from simple methanol extraction (**Figure 2A**) with the HPLC chromatogram of isolated eritadenine (**Figure 2B**), it is clearly shown that methanol extraction, without further purification, is reliable enough for quantification. Further, the chromatogram of the synthesized standard (**Figure 2C**) coincides with the chromatogram of isolated eritadenine (**Figure 2B**). The chromatogram resulting from methanol extraction preceded by enzymatic hydrolysis in either buffer (**Figure 2D**) or water (**Figure 2E**) is not as clean as that from pure methanol extraction but acceptable for quantification. From recovery studies of isolated eritadenine, the accuracy values were about 50%. Since the chromatographic separation was acceptable, methanol extracts were used for quantification, and further isolations were omitted. Samples from the extraction procedures stored in a refrigerator were stable for at least 1 week.

The HPLC analyses of eritadenine content in the fruit bodies of the four different shiitake mushrooms (**Table 1**) used in this study show a statistically significant difference between Le-B and the other shiitake varieties ($p < 0.05$). Each extraction was repeated 3 times, and the spread of the measurements by means of standard deviations was within a reasonable range. There were no statistically significant differences between Le-1, Le-2, and Le-A ($p > 0.05$). These results indicate the importance of the source for high eritadenine content, which can be due to both strain specific properties and cultivation conditions.

Table 1. Eritadenine Content Measured in the Fruit Bodies of Four Different Shiitake Mushrooms from Various Treatments by HPLC Analysis

	eritadenine content (mg/g mushrooms (dw))[a]			
treatment	Le-1	Le-2	Le-A	Le-B
methanol extraction	3.50 ± 0.26	3.17 ± 0.07	3.24 ± 0.27	6.33 ± 0.03
enzymatic pretreatment in acetate buffer pH 4.8	3.82 ± 0.30	NA[b]	NA	NA
enzymatic pretreatment in water pH 6.0	3.60 ± 0.11	NA	NA	NA

[a] All values are mean ± SD from triplicate analyses. [b] NA: not analyzed.

Methanol extraction preceded by enzyme hydrolysis in acetate buffer (pH 4.8) gave the highest amounts of eritadenine for Le-1, followed by enzymatic hydrolysis in water (pH 6.0) with a slightly lower yield of eritadenine, which is reasonable since the pH for the reaction was not optimized in this case. The results indicate that methanol extraction preceded by enzyme hydrolysis may, to some extent, improve the extraction of eritadenine from shiitake mushrooms. However, the difference between methanol extraction preceded by enzymatic hydrolysis in either buffer or water and pure methanol extraction was not statistically significant ($p > 0.05$), and hence, the efficiency of pretreating the mushrooms with cell wall degrading enzymes can be considered unimportant. There is also a possibility that a maximum yield was reached in this case (i.e., there is no more eritadenine to be released from the fungal cells).

In comparison to other studies (*13*, *14*), the amounts of eritadenine in shiitake mushrooms are significantly higher in this study, up to 10 times. There is no information in the previous studies on what specific strains were used, and the eritadenine content might be strain dependent. Another factor that might contribute to the fairly high difference between the amount of eritadenine found in the present study and previous ones is the extraction procedure. In all cases, quantification was preceded by methanol extraction, but there is either no information on how the extraction procedure was performed (*14*) or the temperature, time, and solid−liquid ratio obviously differ from previous studies (*13*). Also, in this study, the mushrooms were thoroughly crushed into fine particles to make a homogeneous fungal material, highly accessible for the subsequent extraction procedure. Finally, the analytical procedures for quantification differ between the studies. The amount of eritadenine has been determined by column chromatographic fractionation without any reference samples (*14*) or by GC (*13*). No data have been found in the literature pertaining to HPLC quantification of eritadenine. Since eritadenine is a nonvolatile compound, it has to undergo derivatization prior to GC analysis; no such modification has to be done to the target compound for HPLC analysis.

To validate the reliability of the proposed HPLC method, a reference curve was obtained by triplicate measurements of five different concentration levels in the range of 0.0124−0.1980 mg/mL. This method showed a linear response, r^2, of >0.999 and a degree of reproducibility expressed as a relative standard deviation (RSD%) of <2.1%. Furthermore, the retention peak obtained for eritadenine in this study indicates a high column efficiency, signifying sufficient resolution for quantification.

In this study, it is clearly shown that HPLC analysis of eritadenine is highly applicable, and it offers a simple and sensitive method for separation, identification, and quantification of this compound.

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