Enzymatic Pre-Treatment of Shiitake Mushroom

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Abstract

Eritadenine is a substance that might help the Western world to overcome one of the biggest cause of death, cardiovascular disease, by lowering the levels of cholesterol in blood by an accelerated excretion of ingested cholesterol.

The aim of this thesis work was to elucidate if enzymatic pre-treatment of shiitake mushrooms increases the yield of the extraction process of the valuable substance eritadenine by increasing the degradation of the fungal cell walls. The enzymes used in this study, had glucanase and chitinase activity and was a gift from Novozymes, Denmark.

The extraction process involves treatment with methanol followed by extraction with ether and finally ion exchange in order to obtain a sample as pure as possible for analysis. In contrast to previously used methods the mushrooms are treated with enzymes before the methanol extraction, which hopefully facilitate the degradation of the cell walls.

The results obtained with enzymatic pre-treatment showed a deterioration compared to extraction without enzyme treatment. This might be explained by an undeveloped method, where a great fraction of eritadenine was lost after the second ion exchanger. However, after this thesis work, an improved method yielded a 6% increase of eritadenine when enzymes were used.
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1 Background

The major cause of death in Western countries is cardiovascular disease where a primary risk factor is hypercholesterolemia (high blood cholesterol) that contributes to hardening of the arteries. Clinical intervention studies have demonstrated the therapeutic importance of correcting hypercholesterolemia. The best-known pharmacological agent for drug therapy is lovastatin (mevinolin) and its analogues. Those pharmaceuticals containing statin have severe side effects on the liver and muscles.

The reason for examine shiitake mushroom is the fact that it contains a substance called eritadenine that has cholesterol reducing capabilities just like statins. It might be possible to combat hypercholesterolemia by developing a functional food product, where eritadenine is the functional component. Functional foods mean that the foods have been changed to give specific and positive health effect beyond ordinary food. These healthy components added influence and support a healthy living to avoid different disease that comes with for example higher levels of cholesterol in the blood.

1.1 Shiitake mushroom as a source for eritadenine

Historically \textit{L. edodes} has been cultivated in China for almost 1000 years because of its good spicy taste and healing effects. In Japan \textit{L. edodes} has become the national fungus and gives a considerable contribution to the export market [1]. In the latest years this fungus has become more present in western kitchens \textit{L. edodes} is cultivated in an extremely controlled environment, because it is very sensitive to changes in humidity and the presence of foreign particles, furthermore is has a production time of 6 months [2].

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{shiitake_mushroom.png}
\caption{Shiitake mushroom}
\end{figure}
For some time it has been known that the Shiitake mushroom has cholesterol lowering capabilities [3]. This reduction of cholesterol have been shown earlier by tests on rats, rabbits and humans [4-6]. When dried and fresh shiitake mushroom was feed to two different groups of ordinary people, the blood serum cholesterol values lowered 12 and 6% for each group within one week. [6].

In 1969 eritadenine was isolated from shiitake mushroom for the first time, and identified as the active cholesterol lowering component. Added to the diet of rats, eritadenine (0.005%) caused a 25% decrease in total cholesterol in as little as one week [7]. Feeding studies with humans have indicated a similar effect, but for a more specific daily dosage clinical trials are needed [6].

Shiitake consists of several other beneficial compounds. For example, it contains all the human essential amino acids, some non-essential amino acids and amides which make it interesting as an amino acid source for vegetarians [8].

Eritadenine (fig. 2) has the molecular formula C_{9}H_{11}N_{5}O_{4} and the name 2(R),3(R)-dihydroxy-4-(9-adenyl)-butyric acid. Unlike the statins, eritadenine does not inhibit the biosynthesis of cholesterol in the liver, but enhances removal of blood cholesterol [9].

In 1972 Tokita et al. developed a method for extraction of eritadenine from shiitake mushroom resulting in a yield of 60 mg eritadenine per 100 g dry weight of mushroom [10]. The first step in this method is extraction with methanol for three hours. When it is done filtration and evaporation of the sample finishes this first part of the process. Next DNA and lipids are removed by extraction with ether and later mixing with ethanol and freezing in -20°C. The final step is ion exchange to increase the purity.

Figure 2. Structure of Eritadenine
The content of eritadenine in the fungi is estimated somewhere between 30-70 mg/100g dry weight (dw.) [11-12]. The fungal cell wall consists of a complex structure of the main components proteins and the polysaccharides chitin, β-1,3-glucan and β-1,6-glucan. These polysaccharides are bonded to each other in a web together with other components of the cell wall [13]. This complex structure provides mechanical strength. By efficiently degrading the cell wall it might be possible to increase the yield of eritadenine during extraction.
1.2 Enzyme hydrolysis

Enzymes are catalytic macromolecules, most often proteins, which speeds up biological reactions by lowering the activation energy for the reaction. Besides their biological role some enzymes are used commercially for other purposes. An advantage of using enzymes in the industry is that certain reactions can be performed with high specificity (few side reactions) at low temperature and moderate pH.

The cell wall of the Shiitake mushroom consists of polysaccharides chitin, β-1,3-glucan and β-1,6-glucan. The enzymes glucanase and chitinase are able to hydrolyse glycosidic bonds in the polysaccharides. The position of cleavage is indexed three, located to the right from the oxygen atom in the picture below (fig 3).

![Figure 3. 1,3-glucan chain](image)

The enzyme 1,3 glucanase will break down the 1,3-glucan chain, 1,6 glucanase will break down the 1,6-glucan chain and chitinase will hydrolyses the chitin molecule.
Novozymes, a Danish biotech-based company, manufactures an enzyme cocktail consisting of a mixture of 1,3- and 1,6-glucanases and chitinase. Enzyme activity of this cocktail as a function of pH and temperature is shown in figure 4 and 5.

![Figure 4. Enzyme activity as a function of pH](image1.png)

![Figure 5. Enzyme activity as a function of temperature](image2.png)

### 1.4 HPLC analysis

HPLC is an abbreviation for high-performance liquid chromatography. HPLC is a method for separation of substances from mixture of compounds depending on the properties of the substances in question, the mobile phase and the column used.

There are different types of HPLC methods, among them are normal and reversed phase chromatography and also size exclusion and ion exchange chromatography.

Normal phase uses a polar stationary phase and a non polar mobile phase in contrast to reversed phase that uses a non polar stationary phase and a polar mobile phase. Normal phase is preferred if the analyte is polar and reversed phase for non polar analyte. Size exclusion chromatography is used when there is need for separation of substances with different size and finally ion exchange chromatography is used when a difference in charge for the compounds can be exploited.

For quantification of eritadenine reversed phase HPLC is used with a C-18 column as stationary phase and gradient elution with the mobile phase (1 mL /
min) starting at 2:98 acetonitril:water and progressing linearly to 60:40 acetonitril:water. Eritadenine is then detected with UV light at 260 nm.

Quantification of a compound is possible by making a standard curve where samples with known concentrations of the compound are eluted from column. From the resulting chromatogram the peak areas of the eluted compound can be integrated by suitable software and a standard curve can be made where a specific concentration corresponds to a certain peak area. This area can then be compared with the area from a sample with unknown concentration.

When later applying the sample with eritadenine and other unknown substances to the column the standard makes it possible to recognize eritadenine by the corresponding retention time of the standard. With known dilution it is possible to calculate the amount of eritadenine in the sample.
2 Objective

Eritadenine is not today commercially accessible at a realistic price level. This fact leads to the start of this thesis.

The aim with this thesis is to elucidate the efficiency of enzymatic pre-treatment of shiitake mushroom with the goal of improving the extraction of eritadenine to get a better process economy. The enzymes used are a mix consisting of glucanases and chitinase because they degrade glucan and chitin in the cell wall, it might improve the extraction process resulting in an increased yield of extracted eritadenine.

3 Experimental

3.1 Enzymatic pre-treatment

The enzyme cocktail, consisting of a mixture of 1,3- and 1,6-glucanases and chitinase, was a gift from Novozymes. The recommendation from the supplier was to use 200 g of enzyme cocktail per 1000 kg of mushroom on a dry weight basis. For 10 g mushroom this corresponds to 100 µL of a 2 % stock solution. The stock solution was freshly made for every trial. Recommended pH and temperature was 4.8 and 50°C respectively (Fig. 4 and 5).

Before the enzyme pre-treatment the whole batch of mushrooms was homogenized to minimize variations in the eritadenine content. Ten grams of homogenized mushrooms was incubated with enzymes (0-2 mg) in 100 mL 0.1 M acetate buffer, pH 4.80. For additional information see table 1 page 11. The mixture was stirred on a Heidolph MR 3001 magnetic stirrer for three hours at 50°C.

Figure 7. Magnetic stirrer
3.2 Methanol extraction

Enzyme treated mushroom was extracted with hot 80% methanol for three hours in a heat jacket with suitable vessel and cooling.

The extract was filtered with a 7 cm Büchner funnel, Schott Duran 500 mL suction flask and Munktell filter paper with paper quality 5 once and paper quality 00H three times.

The filtrate was evaporated in a Büchi Rotavapor R-114 (Fig. 8) and heated in a round flask by a Büchi Water bath B-480, to remove the methanol content.

3.3 Ether extraction

The sample was extracted with ether three times. This treatment removes lipids.

Four volumes of 99.5% ethanol was added to the water phase and stored at -20 °C. This procedure gives a precipitation of DNA at the bottom of the container.

The liquid was filtered three times with paper quality 00H to remove solid remains. The extract was then evaporated to dryness.
3.4 Ion exchange purification

Eritadenine is a zwitter ion, which means that it has a positive and a negative charge. The positive charge at the amino group and the negative charge at the carboxylic end of the ion make ion exchangers perfect for separating this substance from other unwanted substances as ion exchanger separates different species from each other by their different charge distribution.

Amberlite IR-120 is a strong cation exchanger. This ion exchanger resin binds to the amine group of eritadenine and keeps it bonded until another and stronger base is present and releases eritadenine from the ion exchange resin. Amberlite IRA 67. IRA 67 is a weak basic ion exchanger resin that binds to the carboxylic part of eritadenine until another stronger acid is present.

3.4.1 Cation exchange

Cation exchange chromatography was preformed by using Amberlite IR-120 (H\(^+\) form) resin. The ion exchange material was first washed with 400 mL water to clean the resin from possible impurities. After that the dried extract was dissolved in water and added to the column. Unbound compounds were eluted by addition of 600 mL water. Finally, eritadenine was eluted from the column with 4% \(\text{NH}_4\text{OH}\). Fractions of about 5 mL were checked for presence of eritadenine by UV detection at 260 nm. Eluate from the first 30 test tubes was saved and evaporated in order to concentrate the sample.

3.4.2 Anion exchange

Anion exchange chromatography was preformed by using Amberlite IRA 67. After water was added to the concentrated sample from the first ion exchange the sample was applied to the column. The column was washed with 300mL 0.1M acetic acid at pH 2.9 and finally the sample was eluted with 300mL 0.5M acetic acid at pH 2.5. Absorbance check at 260 nm was used to exclude unwanted liquid. The last step was to evaporate the liquid to dryness.
3.5 HPLC analysis

To the dry sample 20 mL of water was added. One millilitre of the solution was filtered through a Whatman 0.2 μm filter with a syringe to get rid of particles that might interfere with the HPLC system.

The concentration of eritadenine was measured by HPLC (Series 200 Quaternary LC pump and UV-VIS detector, PerkinElmer) equipped with a C-18 column (Ultra aqueous, 5μm, 4.6mm x 150mm, Restek) using a linear gradient of acetonitrile:water (from 2:98 to 60:40) at a flow rate of 1 mL / min as the mobile phase. The mobile phase was also spiked with trifluoracetic acid (TFA) to sharpen the peaks of the absorbance curve. The detector used a wavelength of 260 nm. TotalChrome from Perkin Elmer was used for integration of the peak areas of the chromatograms. The concentration of eritadenine was determined by comparing the peak area with standard curves made earlier for eritadenine.
4 Results

Table 1 shows the results of eritadenine extractions from shiitake mushrooms.

Table 1. Results from the different extractions where 2 mg corresponds to the recommended amount of enzymes from the supplier, 200 grams of enzyme to 1000 kg of dried mushroom. Results from different extractions are in the extreme right column.

<table>
<thead>
<tr>
<th>Shiitake [g]</th>
<th>Temp. [°C]</th>
<th>Time [h]</th>
<th>pH</th>
<th>Enzyme [mg]</th>
<th>Eritadenine (mg/100 g dw.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td>4.8</td>
<td>2</td>
<td>26.0</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td>4.8</td>
<td>1</td>
<td>27.3</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td>4.8</td>
<td>0.75</td>
<td>18.0</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td>4.8</td>
<td>0.5</td>
<td>6.5</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td>4.8</td>
<td>0.25</td>
<td>8.2</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td>*</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td>4.8</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>3</td>
<td>*</td>
<td>0</td>
<td>37.1</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>3</td>
<td>*</td>
<td>0</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Figure 11. Amount of extracted eritadenine as a function of increased addition of enzyme mixture.
It can be seen in figure 11 that the points are an approximate saturation curve. When adding more enzymes the amount of extracted substance does not increase.

Table 2. Extraction results quantified by HPLC

<table>
<thead>
<tr>
<th>Date</th>
<th>Enzyme addition</th>
<th>Extracted substance [ng/20 µL]</th>
<th>Mean value</th>
<th>Deviation [±%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005-04-21</td>
<td>0</td>
<td>310,37</td>
<td>309,84</td>
<td>0,2</td>
</tr>
<tr>
<td>2005-04-22</td>
<td>0</td>
<td>287,24</td>
<td></td>
<td>7,2</td>
</tr>
<tr>
<td>2005-05-02</td>
<td>0</td>
<td>331,89</td>
<td></td>
<td>7,1</td>
</tr>
<tr>
<td>2005-04-22</td>
<td>75</td>
<td>720,31</td>
<td>747,87</td>
<td>3,7</td>
</tr>
<tr>
<td>2005-05-02</td>
<td>75</td>
<td>775,42</td>
<td></td>
<td>3,7</td>
</tr>
</tbody>
</table>

Time does not seem to influence the concentration of eritadenine. If a sample from an early run is tested again in the HPLC the result is reasonably close. This indicates that eritadenine is quite stable in water solution. It also indicates reproducibility of the HPLC method.
5 Discussion

The results are both good and bad from my point of view. The good part is that the enzyme seems to work since the yield increase until the solution is saturated. The bad part is that the amount of extracted substance is way under the normal yield for same kind of extraction but without enzymes.

The results from this test series is a deterioration with 30% in comparison without enzymes. Without enzymes I managed to extract 37,1 mg / 100 g dw. when the amount of enzyme was decreased to 1 mg I extracted 27,3 mg / 100 g dw.

Of course good results are always wanted, also in this case. In advance I hoped for good results and started this thesis work with good courage. When time went by the results confused me a lot and forced me to consider the results many times.

The information from the supplier of the enzyme was of great importance. From the graphs shown earlier pH 4.8 and temperature 50 °C was chosen. However there are still some questions to be answered regarding this subject. In this little series of trials there are not enough data to find out if right temperature and pH was chosen, so nothing tells if right conditions are used. The exact composition of the fungus wall was unknown which makes it hard to choose the best adjustment for each parameter. Depending on the composition there should exist optimum for pH and temperature to maximize the enzyme activities and thereby maximize the yield.

To elute the substance from the Amberlite IR 120 ion exchanger 2% NH₄OH was used in the start of the test series. The concentration was later changed from 2% to 4% to reduce time and amount of liquid to evaporate. When the mushrooms were pre-treated with enzymes the volume for eluting eritadenine from the first ion exchanger increased greatly. Needed volume to elute eritadenine increased from about 100 mL to 600 mL of 4% NH₄OH in the first ion exchanger. This might indicate that the treatment resulted in increased release of compounds absorbing at 260 nm.
Later experiments, done by Josefine Enman at LTU, have also shown that eritadenine elutes too early from the second ion exchanger. It is possibly that remains from the buffer used in the extraction process lowers the pH enough to elute eritadenine from the second ion exchanger before the final step with 0.5M acetic acid. This leads to losses of eritadenine and deteriorates the effect of the enzymes. Josefine has also shown that enzymes increase the amount of extracted eritadenine with about 6%. The drawback with enzymes is that it makes the whole analyze process considerably more difficult. This also indicated that a lot more substances that absorbs at 260 nm are freed from the fungus during enzymatic treatment.
6 Conclusions
Enzymes do improve the extraction process of eritadenine from Shiitake mushroom.

7 Future work
In the future more trials should try to improve this method with enzymes. With correct conditions this process might get economical feasible, but today more experiment are needed to find the optimal conditions.
References


