In-vitro and In-silico study on the acylation reaction of (-)-Isopulegol and L-Menthol Mixtures with Lipase from Rhizomucor miehei

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Abstract. In-vitro and In-silico study on the acylation reaction of (-)-isopulegol and l-menthol with lipase from Rhizomucor miehei have been done. In this study, the acylation reaction was conducted by using two kinds of acyl source, acetic anhydride and vinyl acetate. The reaction was performed at 50 °C and mole ratio of (-)-isopulegol:l-menthol:acetic acid anhydride or vinyl acetate was 1:1:3. In-vitro study, shows that lipase from Rhizomucor miehei have catalyzed the acylation reaction in a good way, whereas acetic anhydride was more effectively as acyl source than vinyl acetate. The selectivity product of (-)-isopulegyl acetate and l-menthyl acetate after reaction in 24 h are 34,58% and 21,52% respectively. In-silico study gives the results which correlated with in-vitro study, in case of determination the suitable of acyl source. There are verified by the value of Kd which represent the interaction between (-)-isopulegol, l-menthol or acyl source as ligand with lipase as macromolecule. This work also gives the prediction of mechanism reaction model from in-silico study.

Keywords: (-)-isopulegyl acetate, l-menthyl acetate, acylation, Rhizomucor miehei, in-vitro, in-silico

1. Introduction
Isopulegol is the product of the cyclization of citronellal and as an intermediate compound in the formation of menthol [1]. Isopulegol can be found in scented plants such as Corymbia citriodora Hook, Eucalyptus citriodora Hook, Zanthoxylum schinifolium. Isopulegol belongs to the monoterpene group of p-mentha-3-ol which has a very important physical and component properties in nature. These compounds are useful as perfumes, food additives, soaps, cosmetic ingredients, refrigerants and for pharmaceutical purposes [2].

Menthol (2-isopropyl-5-methylcyclohexanol) is a cyclic monoterpene alcohol which has eight enantiomers and can be found in the leaves of Mentha canadensis L and M. x piperita L [3]. Menthol is used as a component of candy, toothpaste, to reduce irritation and as a cosmetic ingredient [4].

Lipase (EC 3.1.1.3) triacylglicerol hydrolase is an enzyme that plays an important role in modern biotechnology. Lipase is known for its high activity in hydrolysis reaction and in chemical synthesis. It may act as biocatalyst for hydrolysis and esterification reactions [4]. Lipase from Rhizomucor miehei is a soluble and immobilized enzyme to assist the reaction process with high activity and good stability for experimental conditions like on using anhydrous organic solvents, supercritical fluids, etc [5].
Rhizomucor miehei is a type of thermophilic fungus that can develop optimally at 50 °C [6]. The work of the immobilized Rhizomucor miehei lipase on the liposomal nanosphere material lies in the range of 27 - 80 °C. The optimum temperature of the study lies at 37 °C with a yield of 93% [7].

The active site of Rhizomucor miehei enzyme in the acylation reaction is formed by histidine, serine and aspartate so it will do reaction mechanism is between substrate with the active side [8]. However, the mechanisms created are theoretically and cannot be validated, so a more valid method is needed to predict the mechanism of the acylation reaction. The mechanism of interaction between substrate (ligand) and enzyme (macromolecule) and ligand-enzyme interaction affinity can be known through computational chemistry modeling. This modeling will assist in predicting the chemical aspects that can be described in a qualitative or quantitative computational scheme so that a ligand receptor binding model [9].

Therefore, this study will focus on the selectivity of Rhizomucor miehei lipase to the structure of (-)-isopulegol and l-menthol. Acylation was studied using acetic acid anhydride and vinyl acetate as an acyl donor in which the solvent used was n-hexane. This acylation is supported by computational modeling in order to understand how amino acid residues of enzymes interact with isopulegol and menthol ligands.

2. Experimental Section

2.1 Materials

The materials used in the in vitro study are: (-)-isopulegol, l-menthol, lipase enzyme from Rhizomucor miehei on Immobead 100 from Sigma-Aldrich, n-hexane, anhydrous acetic acid, vinyl acetate. The materials that used for modeling ligand interaction with receptors, based on in silico studies are: ligands of (-)-isopulegol, l-menthol, acetic acid anhydrous and vinyl acetate. Ligands obtained from Chemspider Search and Share Chemistry. The receptor is enzyme of Rhizomucor miehei, Triacylglicerolacyl hidrolase with 3TGL code obtained from RCSB Protein Data Bank.

2.2 Procedure

2.2.1 In-vitro Studies. Lipase (0.15 g) was put into a closed container (volume 100 mL). Then, 15 mL of n-hexane and 5 mL acetic acid anhydrous were added. The mixture was stirred using a magnetic stirrer while 5 mL (-)-isopulegol were added slowly (dropwise) into the sealed container. The stirring process was carried out at 50 °C for 24 hours. Then the mixture was centrifuged for 5 minutes. The obtained acylation product was analyzed by GC-MS and FT-IR. The same procedure was performed for the acylation reaction of (-)-isopulegol and 1-menthol mixtures with vinyl acetate as the acyl source.

Reaction mixtures were analyzed by gas chromatography-mass spectroscopy (GC-MS). On the basis of the GC-MS profile of the mixed (-)-isopulegol and 1-menthol acylation products, % conversion as well as % selectivity of lipase toward isopulegyl acetate would be calculated through formula,

\[
\text{% Selectivity} = \frac{\% \text{Isopuleglyl acetate}}{\% \text{initial substrate} - \% \text{final substrate}} \times 100\% \quad \text{(eq.1)}
\]

\[
\text{% Conversion} = \frac{\% \text{initial substrate} - \% \text{final substrate}}{\% \text{initial substrate}} \times 100\% \quad \text{(eq.2)}
\]

\[
\text{% Selectivity} = \frac{\% \text{Menthyl acetate}}{\% \text{initial substrate} - \% \text{final substrate}} \times 100\% \quad \text{(eq.3)}
\]

\[
\text{% Conversion} = \frac{\% \text{initial substrate} - \% \text{final substrate}}{\% \text{initial substrate}} \times 100\% \quad \text{(eq.4)}
\]

2.2.2 In-silico Studies. The reagent used in this study were l-menthol, (-)-isopulegol, acetic acid anhydride, vinyl acetate from ChemSpider Search and Share Chemistry with file.mol type. Optimization of ligand geometry using HyperChem Professional software to obtain the most stable ligand structure.
The resulting optimization results are stored with hin file format which is then converted to file.pdb using Open Babel GUI to be read by iGEMDOCK software.

The macromolecule used in this study is macromolecule of Rhizomucor miehei enzyme Triacylglycerolacyl hidrolase from RCSB Protein Data Bank with 3TGL code with file.pdb type. This macromolecule optimized using Discovery Studio Visualizer 2016.

The results of ligands and macromolecule optimization were docked by iGEMDOCK v2.1. Then the files is saved in .dock extension. Position and energy estimates are seen in view docked poses and post-analyze. To analyze the interaction of ligands with amino acid residues on the macromolecule side is visualized using Discovery Studio 2016.

3. Result and Discussion

3.1 Acetic Anhydride and Vinyl Acetate as Acyl Source in Mixed Acylation Reaction of (-)-isopulegol and l-menthol (In-Vitro Experiment)

The catalytic activity of lipase from R.miehi (RML) was studied using acylation reaction of (-)-isopulegol and l-menthol mixtures in different acyl source at 50 °C for 4-24 hours (figure 1).

![Figure 1. Acylation reaction of (-)-isopulegol and l-menthol mixtures using RML](image)

A summary results obtained in two different acyl source (acetate anhydride and vinyl acetate) and selectivity of product (isopulegyl acetate and menthyl acetate) are shown in Table 1 and 2. The data of these tables shows that the selectivity values of product are strongly dependent on the acyl source nature. It is also evident from Table 1 and 2 that acetic anhydride is more active and selective than vinyl acetate in the acylation reaction. Procentage, thus, after 24 hours of acylation reaction with the acetic acid anhydride gives selectivity products of (-)-isopulegyl acetate and l-menthyl acetate with an area of 21.52% and 34.58% respectively whereas no products are formed when using vinyl acetate. This shows that the acyl group from acetic anhydride can interact more effectively with (-)-isopulegol and l-menthol to give (-)-isopulegyl acetate and l-menthyl acetate.

| entry | Time (hour) | Reactant (%) | Product (%) | Selectivity |
|-------|-------------|--------------|-------------|-------------|
|       |             | (-)-Isopulegol | l-Menthol | Isopulegyl acetate | Menthyl acetate | Isopulegyl acetate | Menthyl acetate |
| 1     | 4           | 28.96 | 30.45 | 16.28 | 18.13 | 23.8 | 27.1 |
| 2     | 8           | 31    | 32.02 | 11.51 | 19.44 | 33.52 | 29.76 |
| 3     | 12          | 37.23 | 37.01 | 11.29 | 13.92 | 18.78 | 23.07 |
| 4     | 16          | 33.07 | 26.25 | 18.10 | 19.50 | 40.84 | 27.43 |
| 5     | 20          | 30.29 | 21.93 | 21.81 | 25.97 | 32.7 | 34.43 |
| 6     | 24          | 22.50 | 16.11 | 26.03 | 33.66 | 21.52 | 34.58 |

*a from % area of GC chromatogram
b using acetate anhydride as acyl source
c using eq.1 and 3
Table 2. Results of lipase catalyzed acylation of (-)-isopulegol and l-menthol mixtures using vinyl acetate in ratio (1:1:3) in n-hexane as a solvent at T= 50 °C

| entry | Time (hour) | Reactant (%) | Product (%) | Selectivity |
|-------|-------------|--------------|-------------|-------------|
|       |             | (-)-Isopulegol | l-Menthol | Isopulegyl acetate | Menthyl acetate | Isopulegyl acetate | Menthyl acetate |
| 1     | 4           | 41.43        | 46.89      | 4.48         | 6.48         | 8           | 12.84          |
| 2     | 8           | 27.91        | 42.48      | 8.83         | 16.43        | 12.72       | 29.94          |
| 3     | 12          | 44.15        | 48.02      | 1.60         | 2.16         | 3           | 4.38           |
| 4     | 16          | 48.26        | 49.59      | 0.59         | 0.90         | 1.88        | 1.88           |
| 5     | 20          | 43.33        | 56.67      | 0            | 0            | 0           | 0              |
| 6     | 24          | 34.23        | 51.45      | 0            | 0            | 0           | 0              |

* from % area of GC chromatogram
* using acetate anhydride as acyl source
* using eq.1 and 3

3.2 Interaction between Ligands (isopulegol, menthol and acetic anhydride or vinyl acetate) with Macromolecules (lipase/receptor) (In-Silico Experiment)

The results of multiple docking which visualizing the interaction between ligand (isopulegol, menthol and acetic anhydride) with macromolecule (lipase), by 3TGL Triacylglicerolacyl hidrolase receptors, in 2D form, are presented in Figure 2, and the summary of energy values of docked product are presented in Table 3.

![Figure 2](image-url)

**Figure 2.** The 2D interaction model between ligands of mixed isopulegol (a), menthol (b), acetic anhydride (c) with 3 TGL Triacylglicerolacylhidrolase.
Figure 2, shows that a green dotted line indicates hydrogen bonding between the lipase’s active site and the oxygen atom at the substrate. Meanwhile, the pink dotted line shows the bond between the active site of the lipase with the alkyl of l-menthol and (-)-isopulegol. Based on Fig 2, it can be seen that glycine (chain no. 81) and arginine (chain no. 80), binds to oxygen atom of (-)-isopulegol. In addition, the alkyl and cyclohexil ring of (-)-isopulegol bind to leusin (chain no.145). Meanwhile, the active site of lipase which binds to l-menthol at oxygen atom are tyrosine (chain no. 99) and glutamine (chain no. 159). Then, the active site of lipase for hydrogen bonding of acetic anhydride are tyrosine (chain no. 195), glutamine (chain no. 176), valine (chain no. 179) and histidine (chain no 217).

Table 3. Energy data of docked product with acetic anhydride as acyl source

| Interaction                  | ΔG\(^0\)  | VDW       | H Bond  | Kd   |
|------------------------------|-----------|-----------|---------|------|
| Acetic Anhydride-lipase      | -57,8457  | -42,1154  | -15,7303| 1,094|
| (-)-isopulegol-lipase        | -55,9209  | -44,5757  | -11,3452| 1,091|
| l-menthol-lipase             | -49,142   | -41,8039  | -7,6081 | 1,079|

Table 3 shows that the value of Kd for acetic anhydride-lipase interaction is greater (1.094) than the both of the interaction (-)-isopulegol-lipase and l-menthol-lipase. This data give evident that acetic anhydride will be easily detached from the active site to form acyl ions and bind to other ligands, that is (-)-isopulegol and l-menthol.

Next, the results of multiple docking for interaction between ligand (isopulegol, menthol and vinyl acetate) with macromolecule (lipase), also represented by 3TGL Triacylglicerolacyl hidrolase receptors, in 2D form are presented in Figure 3, and the summary of energy values of docked product are presented in Table 4.

Figure 3. The 2D interaction model between ligands of mixed (-)-isopulegol (a), l-menthol (b), and vinyl acetate (c) with 3TGL Triacylglycerolacylhydrolase receptors.
Figure 3, shows that glycine (chain no. 81) and arginine (chain no. 80), binds to oxygen atom of (-)-isopulegol. In addition, the alkyl and cyclohexyl ring of (-)-isopulegol bind to leusin (chain no.145). Meanwhile, the active site of lipase which binds to l-menthol at oxygen atom are tyrosine (chain no. 20 and 76), threonine (chain no. 74) and lysin (chain no. 137). Then, the active site of lipase for hydrogen bonding of vinyl acetate is only histidin (chain no. 42).

| Interaction          | ∆G°  | VDW  | Hbond  | Kd    |
|----------------------|------|------|--------|-------|
| Vinyl acetate-lipase | -46,1007 | -33,7114 | -12,3893 | 1.074 |
| (-)-isopulegol-lipase| -55,9328 | -44,432 | -11,5008 | 1.091 |
| l-menthol-lipase    | -48,6571 | -38,0591 | -10,598  | 1.065 |

Table 4 shows that vinyl acetate capable to form vinyl ions with value of Kd is 1.074. But it still lower than acetic anhydride. The data proofs that vinyl acetate is more strongly bound to the lipase. So the binding between macromolecule-vinyl acetate will be more stable. That is why vinyl acetate is uneffective as acyl donor for (-)-isopulegol and l-menthol in the acylation reaction.

3.3 Overview of the acylation reaction mechanism

Based on the results of multiple docking (In-silico experiment), which recommend the kind of lipase’s site active which interacted with ligand (isopulegol, menthol, acetate anhydride or vinyl acetate), Figure 4 shows the proposed mechanism of the acylation reaction by using acetate anhydride as acyl source. Meanwhile, for vinyl acetate as acyl source are presented in Figure 5.

Figure 4 shows that at the beginning of the reaction, glutamine, tyrosine, histidine and valine bind to acetate anhydride using hydrogen bonding. This is possible because glutamine, tyrosine, histidine and valine are active sites that have hydrophilic or polar sites. Then, in the next step isopulegol binds to glycine and arginine. Also Glycine and arginine are polar or hydrophilic active site, able to bind hydrogen with O atom of isopulegol. The released acyl group then binds with O isopulegol, thus forming isopulegyl acetate. The mechanism of menthyl acetate formation is very similar. The only difference lies in the active site of the enzyme binding to l-menthol namely tyrosine and glutamine.

Figure 5 shows that at the beginning of the reaction, histidine binds to vinyl acetate through hydrogen bonding. In the next step, isopulegol binds to glycine and arginine. Histidine, glycine and arginine are all polar or hydrophilic, so they can bind the OH group of isopulegol. The acyl group which have been released (since it previously binds to histidine, arginine and lysine), binds to O isopulegol, thus forming isopulegyl acetate. The mechanism of the formation of l-menthyl acetate formation is similar to the mechanism of formation of isopulegyl acetate formation. The only difference lies in the active side of the enzyme that binds to l-menthol is histidine, tyrosine, threonine and lysine.
Figure 4. Proposed mechanism reaction of acylation isopulegol to isopulegyl acetate by using acetic anhydride as acyl source.
Figure 5. Proposed mechanism reaction of acylation isopulegol to isopulegyl acetate by using vinyl acetate as acyl source.

4. Conclusion
Results obtained from the in vitro test can be easily accounted for by the in silico results. Thus, the invitro test show that acetic acid anhydride is much more effective than vinyl acetate as acylating agent in the synthesis of acetate from isopulegol and l-menthol. Results of the in silico test in particular the Kd values obtained clearly show that when using acetic acid anhydride, the enzyme active site is able to bind not only the C=O group of the donor but also the C-O one. On the contrary when using vinyl acetate, the active site only binds the carbonyl group, thus reducing very much the effectiveness of the acylation process. This is evident by comparing the values of Ki and Kd, that turn influence the Gibbs energy value of the two acyl donor. Therefore, this is a good example of how in silico test can support the in vitro experiments and help to improve the design of a catalytic system.
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