Enzymatic esterification of $\beta$-methylglucoside with acrylic/methacrylic acid in organic solvents

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Abstract

The enzymatic esterifications of $\beta$-methylglucoside with acrylic acid/methacrylic acid were carried out using Novozym 435. $\tau$-Butanol indicating the highest conversion value was determined as an optimal solvent. The molar ratio ($\beta$-methylglucoside:acids) of 1:15 was most favorable to the esterification. The enzyme concentration of 5% (w/v), and the temperature ($50\,^\circ\text{C}$ for $\beta$-methylglucoside:acrylic acid, $45\,^\circ\text{C}$ for $\beta$-methylglucoside:methacrylic acid) resulted in the highest final conversion. $\beta$-Methylglucoside of 60g l$^{-1}$ was found to be most effective in terms of short reaction time as well as product concentrations. Under these conditions, the maximum conversions for the esterification of $\beta$-methylglucoside with acrylic acid and $\beta$-methylglucoside with methacrylic acid were 59.3% after 12h and 71.3% after 72h, respectively. The structural analysis of the products was performed by FT-IR spectroscopy and $^1$H NMR.

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1. Introduction

There has been an increasing interest in the production of esters containing sugar molecules because of their wide employment as surfactants in detergent and pharmaceutical industries. Moreover, these esters are biodegradable, biocompatible and essentially non-toxic (Torres and Otero, 2001; Naoe et al., 2001).

Previously, sugar esters were synthesized mostly by esterification in aqueous media causing hydrolytic side reactions. In order to prevent these side reactions, polar/aprotic solvents such as pyridine and dimethylformamide were used as reaction media (Ferrer et al., 1999). However, the solubility of sugars and the activity of enzyme were decreased due to the increased hydrophobicity by these organic solvents in the reaction system. In addition, the use of sugar esters as food additives and pharmaceuticals was incompatible with the use of these toxic solvents.

The synthesis of sugar esters have been studied extensively by both chemical and enzymatic methods.
The regioselective synthesis of sugar esters using chemical methods, however, is complicated by the requirement for several protection and deprotection steps resulting from the presence of multiple hydroxyl groups having similar reactivity in sugar molecules (Maugard et al., 1997). On the contrary, enzymatic methods are being applied to the regioselective transformations of mono- and disaccharides without causing any complication. Therefore, various sugar esters can now be prepared by a single reaction step employing enzymes—lipase—as a biocatalyst (Roy and Chawla, 2001).

For the past few years, several researchers have investigated the lipase-catalyzed synthesis of sugar-containing acrylic esters for their biomedical applicability (Staples et al., 2000; Park and Chang, 2000). Acrylic acid has a high hydrophilicity due to its carboxylic group capable of esterifying with the hydroxyl group of sugars. Moreover, it has a vinyl group that can be polymerized. Therefore, acrylic esters are expected to be efficient monomers for sugar polymers having high hydrophilicity and biocompatibility.

In this work, enzymatic esterifications of β-methylglucoside with acrylic acid/methacrylic acid were carried out by Novozym 435, lipase from *Candida antarctica*, as a biocatalyst. Reaction conditions such as reaction media, enzyme amount, molar ratio of substrates, initial substrate concentration and reaction temperature for the esterification of β-MG with AA, and for 120 h for the esterification of β-MG with MAA.

2.2.1. Effect of solvents
β-MG (400 mg, 2.06 mmol) was added in screw capped test tubes and mixed with either AA (4.12 mmol) or MAA (4.12 mmol). Solvents including acetone, acetonitrile, t-amyl alcohol, t-butanol, and 1,4-dioxane were then added to the mixtures to achieve the total volume of 10 ml. Reactions were initiated by adding 5% (w/v) of Novozym 435 at 50 °C with magnetic stirring. Reactions were performed for 48 h for the esterification of β-MG with AA, and for 120 h for the esterification of β-MG with MAA.

2.2.2. Effect of molar ratio
β-MG (500 mg, 2.57 mmol) was added in screw capped test tubes, and subsequently either AA or MAA was added to meet the molar ratios (β-MG:acids) of 1:3, 1:5, 1:10 and 1:15. t-Butanol was then added to the mixtures to achieve the total volume of 10 ml. Reactions were initiated by adding 3% (w/v) of Novozym 435 at 50 °C.

2.2.3. Effect of molecular sieves
β-MG (500 mg, 2.57 mmol) was mixed with either AA (38.6 mmol) or MAA (38.6 mmol) to meet the molar ratio (β-MG:acids) of 1:15. t-Butanol was then added to the mixtures to achieve the total volume of 10 ml. The molecular sieves were added in the mixtures with the amounts of 0, 0.5, 1.0, 1.5 and 2.0% (w/v). Reactions were initiated by adding 5% (w/v) of Novozym 435 at 50 °C. Then reactions were performed for 48 h for the esterification of β-MG with AA, and for 120 h for the esterification of β-MG with MAA.

2.2.4. Effect of enzyme concentration
β-MG (500 mg, 2.57 mmol) was mixed with either AA (38.6 mmol) or MAA (38.6 mmol), and then t-butanol was added to the mixtures to achieve the total volume of 10 ml. Different amounts, 1, 3 and 5%...
(w/v), of Novozym 435 were used, and reactions were initiated by adding each amount of Novozym 435 at 50 °C.

2.2.5. Effect of reaction temperature

β-MG (500 mg, 2.57 mmol) was mixed with either AA (38.6 mmol) or MAA (38.6 mmol). t-Butanol was then added to the mixtures to achieve the total volume of 10 ml. Reactions were initiated by adding 5% (w/v) of Novozym 435 at different temperatures (45, 50, 55 and 60 °C). Reactions were performed for 48 h for the esterification of β-MG with AA, and for 120 h for the esterification of β-MG with MAA.

2.2.6. Effect of initial concentration of β-MG

Each β-MG of 30, 40, 50 and 60 g l\(^{-1}\) was mixed with either AA or MAA with the molar ratio (β-MG:acids) of 1:15, and then t-butanol was added to the mixtures to achieve the total volume of 10 ml. Reactions were initiated by adding 5% (w/v) of Novozym 435 at 50 °C for the esterification of β-MG with AA, and at 45 °C for the esterification of β-MG with MAA.

2.3. Purification

Novozym 435 was removed at the end of the reaction. Equi-volume of hexane was added to the reaction mixture; vigorously mixed for 1 h; and the upper layer was recovered, then it was vaporized at 60 °C under the reduced pressure. Hexane of 10 ml was added to the syrup; the organic phase was recovered after mixing for 1 h intensively; and vaporized at 40 °C under the reduced pressure.

2.4. Analysis

The degree of esterifications were estimated by the measurements of the conversion of β-MG by HPCL. with carbohydrate column (Nova-Pak, 250 mm × 4.6 mm, Waters) kept constant at 35 °C. A mixture of acetonitrile/methanol/water (80:15.5, v/v) was used as an eluent at flow rate of 1.2 ml min\(^{-1}\). 0.2 ml of sample was taken from the reaction mixture at specific time intervals; filtered to remove enzymes; and then 20 μl of sample was injected. Detection was performed by a differential refractometer (RI), and β-MG was used as an inner standard for calibrations. For the measurement of products, β-methylglucoside acrylate (MGA) and β-methylglucoside methacrylate (MGMA), ODS2 column (Spherisorb, 250 mm × 4.6 mm, Waters) was used with an equal component of mobile phase and the same detector at flow rate of 1 ml min\(^{-1}\).

2.5. FT-IR spectroscopy/\(^1\)H NMR

FT-IR (Tensor 27, Bruker) analysis of MGA and MGMA was performed to observe the presence of specific groups of β-MG esters. FT-IR spectra were obtained at 4 cm\(^{-1}\) resolution with 32 scans on Bomem spectra. The spectrometer was equipped with a 6 mm DTGS detector. The solid samples used for the FT-IR studies were compression-molded with KBr powders and the liquid sample was spread over the KBr window.

\(^1\)H NMR analysis of MGA and MGMA was performed to observe the existence of specific groups of β-MG esters. \(^1\)H NMR spectra were recorded on a Varian 300 MHz instrument using CDCl\(_3\) as solvents.

3. Results and discussion

3.1. Effect of solvents

In organic solvents, enzymes have been reported to catalyze reactions that are not the case in water, and their selectivities are different from those in water (Klibanov, 1997). Although enzymes were proven to work efficiently in organic solvents, the more appropriate solvent should be determined for a specific case since it significantly depends on the type of enzyme, substrate and product (Akoh and Yee, 1998; Yan et al., 1999). The partition coefficient of solvents between water and octanol, log \(P\), can be used as a good indicator for enzymatic synthesis (Yahya et al., 1998). Table 1 shows the effect of organic solvents on the esterification of β-MG with AA and MAA. The highest conversion (21.4% with AA and 24.6% with MAA) was observed when using t-butanol, and solvents with lower log \(P\) (less than or equal to −0.23) resulted in a lower conversion. In general, it was reported that the biocatalytic activity of enzymes was reduced as the log \(P\) of solvents lowered (Akoh and Yee, 1998; Athawale and Manjrekar, 2000).
Table 1

<table>
<thead>
<tr>
<th>Solvent</th>
<th>log P</th>
<th>Conversion of β-MG (%) With AA</th>
<th>Conversion of β-MG (%) With MAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-Dioxane</td>
<td>−1.10</td>
<td>10.5</td>
<td>5.3</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>−0.33</td>
<td>3.88</td>
<td>0</td>
</tr>
<tr>
<td>Acetone</td>
<td>−0.23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>t-Butanol</td>
<td>0.80</td>
<td>21.4</td>
<td>24.6</td>
</tr>
<tr>
<td>t-Amyl alcohol</td>
<td>1.30</td>
<td>19.8</td>
<td>23.2</td>
</tr>
</tbody>
</table>

Reaction conditions: β-MG (400 mg, 2.06 mmol), 5% (w/v) Novozym 435, molar ratio (β-MG:acids) of 1:2, 48 h for β-MG:AA, 120 h for β-MG:MAA, 50°C.

was selected for use in subsequent experiments for its ability to produce the highest conversion, and it was known as a solvent that did not affect enzyme activity as well as stability (Cao et al., 1999).

3.2. Effect of molar ratio

The effects of molar ratios of β-MG to AA and β-MG to MAA on the synthesis of MGA and MGMA were investigated (Fig. 1). The degree of esterification increased with the increasing molar ratio in both cases. The conversion for the esterification of β-MG with AA (Fig. 1A) at the molar ratios of 1:3, 1:5, 1:10 and 1:15 reached 26.9, 36.3, 47.6 and 57.0% after 48 h, respectively. For the esterification of β-MG with MAA (Fig. 1B) at the molar ratios of 1:3, 1:5, 1:10 and 1:15, the conversions were 22.3, 34.2, 55.9 and 67.4% after 120 h, respectively. Although, the use of the molar ratio of β-MG:acids over 1:15 might increase the reaction rate, the excess use of acids would lead to the additional steps for removing non-reacted acids in the purification of products (Bousquet et al., 1999a). Therefore, the molar ratio was determined from the viewpoint of the easy separation of products and acids. Thus the molar ratio of 1:15 was used for all subsequent experiments.

3.3. Effect of molecular sieves

The amount of water not only present in non-aqueous media but also bound to enzymes influenced the dynamic and catalytic properties of enzymes (Bell et al., 1995). Molecular sieves were often introduced to remove water accumulated by esterifications, and many research groups reported that the use of molecular sieves enhanced the reaction rate (Tsuzuki et al., 1999; Torres et al., 2000). In Fig. 2, however, the addition of molecular sieves resulted in a reverse effect on the esterification. In general, lipase-catalyzed esterification in organic solvent required a minimal amount of water for the enzyme to maintain its optimal conformation and activity (Colombié et al., 1998). It is believed that the use of molecular sieves, in this work, caused a minimal amount of water to be reduced, and acted as an inhibitor for forming enzyme-substrate complexes. Table 2 indicates the effect of molecular sieves on both the initial rates and the final conversions. Reduction
Fig. 2. Effect of molecular sieves on the esterification of β-MG with (A) AA and (B) MAA. Reaction conditions: β-MG (500 mg, 2.57 mmol), 5% (w/v) Novozym 435, molar ratio (β-MG:acids) of 1:15, 50 °C, 48 h for β-MG:AA, 120 h for β-MG:MAA.

in both the final conversions (from 72.3 to 60.0%) and the initial rates (from 118 to 102 μmol l⁻¹ min⁻¹) in the esterification of β-MG with MAA were observed with the increasing amount of molecular sieves. The kinetic parameters in the esterification of β-MG with AA, however, showed different trends. Even at the higher initial rates, the final conversions had lower values, especially at the molecular sieves amounts of 0, 1.0 and 1.5%. The use of molecular sieves is believed to play an inhibitive role in these reactions, even when they have higher initial rates. Therefore, the molecular sieves were not used in the subsequent experiments.

Table 2
Effect of molecular sieves on the esterification of β-MG with AA/MAA

<table>
<thead>
<tr>
<th>Molecular sieves (%, w/v)</th>
<th>Initial rates (μmol l⁻¹ min⁻¹)</th>
<th>Final conversions of β-MG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With AA</td>
<td>With MAA</td>
</tr>
<tr>
<td>0</td>
<td>397 ± 12.9</td>
<td>118 ± 5.5</td>
</tr>
<tr>
<td>0.5</td>
<td>410 ± 13.5</td>
<td>112 ± 4.2</td>
</tr>
<tr>
<td>1.0</td>
<td>420 ± 16.8</td>
<td>111 ± 4.8</td>
</tr>
<tr>
<td>1.5</td>
<td>408 ± 12.2</td>
<td>108 ± 5.1</td>
</tr>
<tr>
<td>2.0</td>
<td>388 ± 7.7</td>
<td>102 ± 4.3</td>
</tr>
</tbody>
</table>

Reaction conditions: β-MG (500 mg, 2.57 mmol), 5% (w/v) Novozym 435, molar ratio (β-MG:acids) of 1:15, 50 °C, 48 h for β-MG:AA, 120 h for β-MG:MAA, 50 °C.

* Initial rates were defined as the amount of the converted β-MG (μmol l⁻¹) within 30 min.
3.4. Effect of enzyme concentration

Although enzymes have regioselectivity to simplify reaction steps, a compromise between productivity and enzyme concentration should be achieved. Fig. 3 indicates the effect of enzyme concentrations on the esterification of β-MG with AA (Fig. 3A) and β-MG with MAA (Fig. 3B), respectively. The conversion of β-MG obviously depends on the amount of enzyme. The final conversions for the esterification of β-MG with AA after 48 h and β-MG with MAA after 120 h were 57.8 and 70.4%, respectively, with the enzyme concentration of 5% (w/v). In Fig. 3A, however, there was not much difference in the final conversions after 48 h between the enzyme concentrations of 3 and 5% (w/v).

Table 3

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Initial rates (μmol·min⁻¹·mL⁻¹)</th>
<th>Water activity (a_w)</th>
<th>Final conversions of β-MG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With AA</td>
<td>With MAA</td>
<td>With AA</td>
</tr>
<tr>
<td>45</td>
<td>420 ± 17.6</td>
<td>118 ± 6.2</td>
<td>0.275</td>
</tr>
<tr>
<td>50</td>
<td>468 ± 16.4</td>
<td>111 ± 5.7</td>
<td>0.330</td>
</tr>
<tr>
<td>55</td>
<td>498 ± 16.9</td>
<td>109 ± 4.8</td>
<td>0.397</td>
</tr>
<tr>
<td>60</td>
<td>506 ± 17.7</td>
<td>112 ± 5.6</td>
<td>0.475</td>
</tr>
</tbody>
</table>

Reaction conditions: β-MG (500 mg, 2.57 mmol), 5% (w/v) Novozym 435, molar ratio (β-MG:acids) of 1:15, 48 h for β-MG:AA, 120 h for β-MG:MAA.

* Mean values from five independent measurements.
It can be explained by the fact that the excess use of enzyme not only prevents the active sites of the enzyme molecules from exposing to the substrates but also leads to internal diffusional limitations within the heterogeneous catalyst (Krishna et al., 2001; Bousquet et al., 1999b). While higher enzyme concentrations (>5%) seemed to reduce reaction times, the final conversion of β-MG would be independent of the enzyme concentrations. Therefore, the use of enzyme concentration over 5% (w/v) was not considered in this study.

3.5. Effect of reaction temperature

The reaction temperature had a great influence on the rate of esterification. The effect of temperature on the esterification is shown in Fig. 4. The optimal temperature in the esterification of β-MG with AA and β-MG with MAA was observed at 50 and 45 °C, respectively. Subsequently the final conversion was decreased over 50 °C from 57.9 to 43.0% for the esterification of β-MG with AA. For the esterification of β-MG with MAA, the decrease in the final conversion from 72.3 to 62.1% was observed over 45 °C. The esterifications below 45 °C were not carried out due to the low solubility of β-MG in reaction system. C. antarctica lipase had a good thermoresistance in t-butanol, and Novozym 435 was known as heat-tolerant enzyme which maintains its activity even at 90 °C (Bousquet et al., 1999b). Therefore, it is considered that the reduction in the final conversion is caused not by raised temperature but by increased water activity ($a_w$) at higher temperature. $a_w$ may influence the reaction rate as well as the enzyme activity in organic media. The water transfer is determined by Fick’s first law that is proportional to the diffusion coefficient for water and used to describe the effects of $a_w$ control in esterification (Wehtje et al., 1997). The diffusion coefficient and water transfer increase at elevated temperature, and consequently it caused $a_w$ in reaction system to enhance. Therefore, higher $a_w$ at elevated temperature is considered to be the major factor for the decrease in the final conversion.

![Fig. 6. FT-IR spectra of β-MG, MGA and MGMA.](image)
shows the effect of temperature on the esterification of β-MG with acids. In the esterification with AA at 45, 55 and 60 °C, the final conversions showed lower values at high temperature regardless of higher initial rates. Moreover, a similar trend was observed in the reaction with MAA at 60 °C. Therefore, the decrease in the final conversion at higher temperature may be caused by the elevating activity of water formed during the esterification at high temperature. In order to verify these phenomena, $a_w$ at different temperature was measured by Novasina AW LAB Set H (Switzerland). Since the esterification was influenced by the water formed during the reaction as a by-product, $a_w$ was measured after 2 h for the esterification of β-MG with AA and 8 h for the esterification of β-MG with MAA. The range of $a_w$ for the esterification of β-MG with AA was 0.275–0.475, and the final conversion was decreased over 50 °C ($a_w > 0.330$). The slight increase in the final conversion at 50 °C in spite of the higher $a_w$ than that at 45 °C ($a_w = 0.275$) was a good indication that an optimal amount of water (or $a_w$) was required to maintain enzymatic activity (Chand et al., 1997; Wehtje et al., 1997; Tweddell et al., 1998). For the esterification of β-MG with MAA, the range of $a_w$ was 0.154–0.405, and the final conversion was decreased at $a_w > 0.154$. Thus, the decrease in the final conversion was mainly influenced by the increase in $a_w$ at higher temperature.

![Fig. 7. Structures and $^1$H NMR spectra of MGA and MGMA.](image-url)
3.6. Effect of initial concentration of β-MG

Fig. 5 shows the effect of the initial concentration of β-MG on the esterification of AA (Fig. 5A) and MAA (Fig. 5B). As shown in Fig. 5A, β-MG of 60 g l\(^{-1}\) reached a maximum (59.3%) after 12 h, and it was continually decreased to 49.1% after 48 h. The fast reaction rate in early stage of reaction might lead to the formation of more amounts of water that caused shifting the equilibrium of reaction to the hydrolysis of the product. β-MG of 40 and 50 g l\(^{-1}\) reached the final conversion of 58.1 and 57.4%, respectively, after 48 h without any reduction in conversions. Although the conversion was decreased after the maximum point, 60 g l\(^{-1}\) with the conversion of 59.3% after 12 h seemed to be more effective than both 40 and 50 g l\(^{-1}\), with 58.1% and 57.4% after 48 h since it could produce more amounts of product in shorter reaction time. Likewise, β-MG of 60 g l\(^{-1}\) in Fig. 5B reached a maximum (71.3%) after 72 h, and it was decreased to 66.5% after 120 h. However, no decrease in the conversion during the reaction was observed when β-MG of 30, 40 and 50 g l\(^{-1}\) were used. Although 50 g l\(^{-1}\) reached the highest final conversion (72.8%) after 120 h, 60 g l\(^{-1}\) with the conversion of 71.3% after 72 h seemed most effective for the esterification of β-MG with MAA.

3.7. Structure analysis

FT-IR spectra of MGA and MGMA are shown in Fig. 6. The successful incorporation of the acrylate and methacrylate group into β-MG is demonstrated by the presence of an ester (C=O) FT-IR band (1724 cm\(^{-1}\)) of MGA/MGMA was confirmed by the FT-IR bands at 1637/1636 cm\(^{-1}\) (C=C) and 812/810 cm\(^{-1}\) (C=C-H). The band for C–O stretching of secondary alcohols appeared at 1200–1300 cm\(^{-1}\). The δCH\(_2–\text{C}–\text{CH} = \text{CH}_{2}\) peaks for MGA and MGMA were at 3.6 and 3.7 ppm. The assignments of the peaks were: 6.11, 6.12 (1, CH\(_2–\text{C}–\text{CH} = \text{CH}_{2}\)) and 6.3, 6.43 (3, CH\(_3–\text{C}–\text{CH} = \text{CH}_{2}\)) for MGA, 6.24 (3, CH\(_3–\text{C}–\text{CH} = \text{CH}_{2}\)) for MGMA. The 1H NMR analysis of the MGA and MGMA was carried out in CDCl\(_3\) (Fig. 7). The band for C–O stretching produced a strong as well as a broad band at 2800–3500 cm\(^{-1}\). These are due to the presence of β-MG moiety.

4. Conclusion

The esterifications of β-MG with AA and MAA catalyzed by Novozym 435 were investigated. Solvents with a higher log\(_P\) revealed better degree of esterification, and t-butanol was determined as an optimal solvent for all subsequent experiments. The molar ratio (β-MG:acid) of 1:1.5 resulted in the highest conversion, and the use of molar ratios over 1:1.5 was limited for reducing remnant acids and an easy separation. The decrease in the final conversion of β-MG at higher temperature was caused by the elevating \(a_w\) due to increasing temperature. With the initial β-MG of 60 g l\(^{-1}\) and 5% (w/v) Novozym 435, the maximum conversions of β-MG for the esterification with AA and MAA were 59.3% after 12 h at 50 °C and 71.3% after 72 h at 45 °C, respectively.

Acknowledgements

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References


