Short Communication

Enzymatic synthesis of sialic acid derivative by immobilized lipase from Candida antarctica

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1. Introduction

Sialic acids (N-acetylneuraminic acids, Neu5Ac) are negatively charged 9-carbon sugars predominantly found in vertebrates, a few higher invertebrates, and certain types of bacteria (Schauer, 2000). In vertebrates, sialic acids are primarily expressed at the outermost ends of the carbohydrate structures on cell surface glycoproteins and glycolipids or as homopolymers of N-acetylneuraminic acid in α-2,8 linkages in mammalian brain tissue (Angata and Varki, 2002). Sialic acid-containing structures in eukaryotic systems play important roles in various physiological and pathological processes, including cell–cell adhesion, viral infection, and cell growth regulation (Schauer, 2000; Angata and Varki, 2002). Therefore, sialic acid and its derivatives have broad applications in health food and the pharmaceutical industry (Roy et al., 1992). In the investigation of the receptor recognition of sialic acid for rational drug development, structurally modified sialic acids are invaluable tools for understanding the important biological and physiological properties of sialylated structures.

Esterification may be a suitable method for increasing the lipophilicity and stability of N-acetyl neuraminic acid methyl ester, because the ester residue is well-characterized as a nontoxic carrier moiety with a high affinity for cell membranes and great hydrophobicity to prevent degradation. However, the traditional chemical synthesis of sugar or polyol esters requires acidic and metal catalysts at high pressures and temperatures (Ress and Linhardt, 2004; Yang et al., 2011), resulting, in most cases, in complex mixtures of monoester and di- or triester isomers and numerous by-products (Zhou et al., 2011). Enzymatic reactions in nonaqueous media by lipases have become increasingly valuable tools for generating sialic acid ester derivatives. By employing enzymatic technology, reactive processes can be carried out under mild conditions with a broad range of substrates in an environmentally friendly manner. Thus, this has become one of the most practical and efficient methods for the production of complex sialates and their derivatives. In the present work, it is found that sialic acid methyl ester monononanoate (SAMEMN) can be efficiently synthesized from N-acetyl neuraminic acid methyl ester and nonanoic anhydride using Novozym 435 in acetonitrile. The influences of several parameters on the esterification reaction are investigated.

2. Methods

2.1. Chemicals and enzymes

The following 5 commercial lipases were used: Pseudomonas cepacia lipase (Amano PS) and Candida rugosa lipase (Amano AY) were purchased from Amano International Enzyme Co. (Nagoya, Japan); Rhizomucor miehei lipase (Lipozyme RM IM) and Candida

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2.2. Optimization of SAMEMN synthesis in organic solvent

SAMEMN was synthesized in a 10-mI reaction vessel via the esterification of N-acetyl neuraminic acid methyl ester (20 mM) with nonanoic anhydride (60 mM) in acetonitrile (Liu and Shaw, 1995). The reaction mixture (1.5 ml) was stirred at 200 rpm. The reaction rate was adjusted to 1 ml/min, and peaks were monitored at 200 nm. Percent molar yields were determined using the standard curve of SAMEMN. The effects of parameters on SAMEMN yield were studied keeping all other conditions constant.

2.3. Product and lipase activity analysis

The analysis and quantization of SAMEMN were performed on a Hitachi L-7100 HPLC system equipped with a UV–VIS detector and a system controller. Samples (5 μl) were run using the gradient mode with a 15-min gradient from solvent A (CH₃CN:H₂O = 26:74) to solvent B (CH₃CN = 100) on a reverse-phase Mighty Sil RP-18 GP column (250 × 4.6 mm ID, 5 μm, particle size) at 40 °C. The flow rate was adjusted to 1 ml/min, and peaks were monitored at 200 nm. Percent molar yields were determined using the standard curve of SAMEMN. Triple samples were each analyzed twice. Lipase activity was measured according to the method described by Rúa et al. (1993) using p-nitrophenyl butyrate as the substrate. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per minute.

2.4. Statistical analysis

A variance analysis of the results was carried out using the General Linear Model Procedure from the SAS Statistical Software, Version 6.11 (1995). Lipase source, reaction time, temperature, acyl donor, and organic solvent were each tested in triplicate. Multiple comparisons of means were carried out by Duncan’s multiple range test at p < 0.05.

3. Results and discussion

3.1. Screening of biocatalysts

The esterification of N-acetyl neuraminic acid methyl ester has previously been carried out with lipases from various sources, in either free or immobilized form; however, because of the relative low stability of free lipases against high pressure and temperature (Knez and Habulin, 2002), immobilized lipases have become the focus of current research and industrial applications. Three commercial free lipases (Lipases AY, PS, and Candida rugosa lipase Type VII) and 2 immobilized lipases (Novozym 435 and Lipozyme RM IM) were tested in acetonitrile at 60 °C to compare their effects on the esterification producing SAMEMN. As shown in Table 1, 36.3 U/mg protein of immobilized C. antarctica lipase, Novozym 435, showed remarkable catalysis and specificity in the enzymatic synthesis of SAMEMN. When synthesis was catalyzed by Lipozyme RM IM, the yield after 6 h was 62.7% lower than that after synthesis catalyzed by Novozym 435. Higher activity of Novozym 435 was also found in the kinetic resolution of secondary alcohols in monothio–functionalized ionic liquids (Zhou et al., 2011). Thus, Novozym 435 was observed to efficiently catalyze the esterification of N-acetyl neuraminic acid methyl ester with nonanoic anhydride and appeared to be suitable for SAMEMN production.

3.2. Effects of reaction time and temperature on SAMEMN production

A time course was produced to monitor reaction progress and possibly minimize process costs. After 6 h of incubation with Novozym 435 at 2% (w/w of reactants), a yield of 87.7% was observed for SAMEMN. The amount of product increased with increasing reaction time up to 6 h and then declined (12–48 h) significantly because of a possible reverse hydrolysis reaction (Mutua and Akoh, 1993). Similar results have been reported for the synthesis of methyl acrylate where the maximum ester yield was obtained in 6 h by the hydrogel-bound lipase of Pseudomonas aeruginosa MTCC-4713 and gradually declined thereafter (Kanwar et al., 2007). In addition, Yang et al. (2011) reported that the quality of poly(oleic diacid-co-glycerol) was high when the reaction catalyzed by Novozym 435 in a vacuum (10 mmHg) system for 6 h. Therefore, after considering these findings, a reaction time of 6 h appeared to be optimal and was used in the following experiments.

In lipase-catalyzed reactions, temperature significantly influences both initial reaction rate and enzyme stability. In most cases, the reaction rate increases with temperature while the stability of enzymes declines (Ward et al., 1997; Foresti and Ferreira, 2007). In order to investigate the effect of temperature on the activity of Novozym 435 in acetonitrile, 4 different temperatures were employed, ranging from 30 to 60 °C. Fig. 1 (Supplementary data) shows the effect of temperature on the catalytic activity of Novozym 435. Nag (1988) have demonstrated that high temperatures can change the conformation of enzymes that can alter the free energy of the system, potentially affecting substrate binding capacity and reducing the yield of the reaction. Because Novozym 435 is quite thermostable, it was possible to run the reaction at temperatures as high as 60 °C, which allowed a maximum yield of 87.7% SAMEMN to be obtained. This result is in agreement with results obtained by De Diego et al. (2011) in the production of biodiesel. Thus, 60 °C was chosen as an optimal temperature for further reactions.

3.3. Selection of acyl donor

Acyl donors with different chain lengths (nonanoic acid, nonanoic anhydride, methyl nonanoate, and trimethanol) were used
Table 2

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Log P</th>
<th>Yield of SAMEMN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane</td>
<td>3.50</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td>Isopropyl ether</td>
<td>2.20</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td>Chloroform</td>
<td>2.00</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td>t-Butanol</td>
<td>0.80</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>0.49</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>−0.33</td>
<td>87.7 ± 0.8 b</td>
</tr>
</tbody>
</table>

Novozym 435 (50 mg) was added to a reaction mixture (1.5 ml) containing 20 mM N-acetyl neuraminic acid methyl ester and 60 mM nonanoic anhydride. The reaction was carried out during 6 h in various organic solvent at 60 °C.

3.4. Effect of different solvents

Sialic acid fatty acid esters consist of a long chain fatty acid, which is soluble in organic solvents, and a sugar, which is insoluble in most organic solvents. A suitable organic solvent had to be identified in which both substrates would dissolve and react as required. The effect of different organic solvents on the lipase-catalyzed synthesis of SAMEMN at 60 °C was studied (Table 2). It was important to select an organic solvent should not affect lipase activity and selectivity and has to be permitted for general use in the manufacture of pharmaceuticals (Liu and Shaw, 1995). Esterification was performed in n-hexane, isopropyl ether, chloroform, t-butanol, tetrahydrofuran, and acetonitrile. The highest yield, namely, 87.7%, was obtained in acetonitrile after a 6 h reaction period at 60 °C. The final yield after a 6-h reaction period at 60 °C was 0% in all other solvents tested (Table 2). In this case, solvents with log P > 3 values are not suitable because the solubility of the glycoside in this medium is very poor or null. These results are a good indication that the nature of the solvent can significantly affect the enzymatic synthesis of SAMEMN.

4. Conclusions

The lipase-catalyzed esterification of N-acetyl neuraminic acid methyl ester with nonanoic anhydride in a small amount of organic solvent—adjuvant was performed at atmospheric pressure. The influence of different types of lipase on reaction rate was studied, with the immobilized lipase Novozym 435 from C. antarctica giving the best results. The highest conversion, 87.7% after 6 h of reaction, was achieved in acetonitrile, which is biocompatible for the production of health foods. These results are of general interest for developing industrial processes for the preparation of SAMEMN, which is used in pharmaceuticals and in the synthesis of other sialic acid derivatives.

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Appendix A. Supplementary data


References


