Preparative-Scale Enzymatic Synthesis of rac-Glycerol-1-phosphate from Crude Glycerol Using Acid Phosphatases and Phosphate

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Glycerol is a byproduct of biodiesel production and is generated in large amounts, which has resulted in an increased interest in its valorization. In addition to its use as an energy source directly, the chemical modification of glycerol may result in value-added derivatives. Herein, acid phosphatases employed in the synthetic mode were evaluated for the enzymatic phosphorylation of glycerol. Nonspecific acid phosphatases could tolerate glycerol concentrations up to 80 wt% and pyrophosphate concentrations up to 20 wt% and led to product titers up to 167 g L⁻¹ in a kinetic approach. In the complementary thermodynamic approach, phytases were able to condense glycerol and inorganic monophosphate directly. This unexpected behavior enabled the simple and cost-effective production of rac-glycerol-1-phosphate from crude glycerol obtained from a biodiesel plant. A preparative-scale synthesis on a 100 mL-scale resulted in the production of 16.6 g of rac-glycerol-1-phosphate with a reasonable purity (≈ 75%).

Introduction

The worldwide production of biodiesel results in an enormous surplus of approximately 4 Mt/a of crude glycerol as a byproduct. Consequently, the valorization of glycerol has gained increasing attention.[1, 2] Current applications involve the use of glycerol as an energy source[3] and as a green solvent (e.g., as a component of deep eutectic solvents).[4] The main obstacle for the use of glycerol as a platform chemical[5] is connected to the high input of energy required for its purification from the highly concentrated aqueous solutions obtained from biodiesel.

For instance, Pradines et al. employed calf intestine alkaline phosphatase and pyrophosphate for the phosphorylation of a phosphate donor (P-donor), such as pyrophosphate (PPᵢ)
during glycolysis, is a key building block of glycerolphospholipids, the main components of cellular membranes. In contrast, archaea utilize the opposite enantiomer (l-glycerol-1-phosphate) to form ether-type lipids in isoprenoid side-chains.[6] In synthesis, o-glycerol-1-phosphate is employed commonly as a precursor to DHAP that serves as a nucleophile (donor) in asymmetric C–C coupling reactions catalyzed by aldolases.[7–9] As DHAP is an expensive and unstable metabolite, multi-enzymatic cascades that involve the phosphorylation of glycerol followed by oxidation to DHAP have been applied to the aldolase-mediated production of non-natural sugar derivatives.[10–12]

The phosphorylation of glycerol can be catalyzed by kinases or phosphatases that work in the synthesis mode. Kinases, such as glycerol kinase,[10, 13] employ adenosine triphosphate (ATP) as the phosphate donor. This is a costly cofactor that requires recycling.[14, 15] In contrast, phosphatases may be employed in transphosphorylation reactions by using inexpensive phosphate donors (P-donors), such as pyrophosphate (PPᵢ).[16] For instance, Pradines et al. employed calf intestine alkaline phosphatase and pyrophosphate for the phosphorylation of a number of alcohols, which included glycerol.[17] Interestingly, calf intestine phosphatase was able to utilize monophosphate and, thereby, catalyzed the direct condensation of glycerol and phosphate.[18]

We report here the exploitation of acid phosphatases for the preparative-scale enzymatic synthesis of rac-glycerol-1-phosp-
and NaH) and crude glycerol (Scheme 1).

Results and Discussion

A common drawback of phosphatase-catalyzed transphosphorylation reactions lies in the undesired enzyme-catalyzed hydrolysis of the newly formed products. In addition to the use of high loadings of both alcohol substrate and P-donor,[19-22] various techniques have been developed to minimize product hydrolysis, such as continuous-flow synthesis,[23,24] enzyme engineering,[25,26] and reaction engineering.[27] They could enable the mild and selective enzymatic synthesis of phosphate monoesters of functionalized alcohols and sugars.

Encouraged by these previous results, we initiated our study by testing several nonspecific acid phosphatases (NSAPs), which included PhoN-Sf from Shigella flexneri, PhoN-Se from Salmonella typhimurium LT2, PIACP from Prevotella intermedia, Lw from Leptotrichia wadei, and PhoC-Mm from Morganella morganii, as well as a histidine acid phosphatase and a phytase from Aspergillus niger [27] for the regioselective synthesis of glycerol-1-phosphate. High concentrations of glycerol (pure glycerol [1a] and crude glycerol [1b]) and PP, as the P-donor were employed in overnight reactions at 30 °C in water. Importantly, no pH adjustment was necessary as the use of a high concentration of PP, (used as the disodium pyrophosphate salt, Na2HPO4) resulted in a buffered acidic pH of approximately 3.5, a value that was beneficial in a previous study to reduce unwanted product hydrolysis in transphosphorylation reactions.[27]

Pure 1a and crude 1b (obtained from the base-catalyzed transesterification of fats with methanol) were well tolerated as substrates by most enzymes in up to 80 wt% by using 5 wt% PP (Figures S1 and S2). The best results were obtained by using PhoC-Mm with 60 wt% 1a over 24 h, which led to a high product formation (14 g L⁻¹ product titer, and a 0.88 product/P, ratio (Figure S1), which corresponds to 94% transphosphorylation efficiency.[28] The corresponding values obtained with 70 wt% 1b were 94%, 41 g L⁻¹, and 0.91, respectively (Figure S2), which indicates a highly effective transphosphorylation of crude glycerol (96% efficiency). Although 10 wt% PP, was well tolerated by PhoN-Sf, PhoN-Se, and PIACP and yielded high product titers and higher product/P, ratios (Figure S3), strongly diminished activities were obtained at 20 wt% PP, concentration (maximum 20% PP, consumption within 2 d; data not shown). An increase of the amount of water, which was achieved by lowering the consumption of 1a and PP, (55 and 18 wt%, respectively), had a beneficial effect on enzyme activity and allowed access to higher product titers (up to 101 g L⁻¹), although PP, consumptions were incomplete (Figure S3). A similar trend was observed with 1b, and surprisingly, product titers (up to 118 g L⁻¹) and PP, consumption levels were generally superior (Figure S4). The highest product/P, ratio was achieved with PhoC-Mm (up to 0.88), which indicates the most effective transphosphorylation, however, both product titer and PP, consumption were lower than those obtained with PIACP and PhoN-Sf. A product/P, ratio of approximately 0.70 could be maintained with PIACP and PhoN-Sf regardless of the enzyme amount or reaction time. Acid phosphatase Lw showed a performance very similar to that of PhoC-Mm, whereas PhoN-Se was the most active enzyme, however, its strong hydrolytic activity resulted in low product/P, ratios. Overall, PIACP could deliver 101 g L⁻¹ of 2 within 24 h from 1a and up to 121 g L⁻¹ after 4 d (Table 1, entries 1 and 2). With 1b, PhoN-Sf afforded 118 g L⁻¹ of 2 in 24 h and reached an impressive level of 167 g L⁻¹ within 4 d (entries 4 and 5). The use of double the amount of enzyme (8 U mL⁻¹) allowed a significantly higher PP, consumption (82% with both enzymes) and product titer (133 g L⁻¹) for PIACP with 1a and 151 g L⁻¹ for PhoN-Sf with 1b) already after 24 h (entries 3 and 6).

The use of phytase from Aspergillus niger (phytase cc; see Supporting Information), which has been reported to convert 1a non-stereoselectively to rac-glycerol-1-phosphate,[31] resulted in a nearly full consumption of PP, in 24 h and a low product/P, ratio (up to = 0.2) even at 20 wt% PP, with 1a (Table S2). An increased concentration of 1a allowed a slightly better product/P, ratio (∼ 0.3; Table S2, entries 8 and 9). In the case that 1b was used, a similar trend was observed (Table S3). Interestingly, product formation took place even upon full PP, consumption (Table S2, entries 2–4 and Table S3, entries 2–7). A detailed time study indicated that PP, was hydrolyzed fully in less than 10 min regardless of the concentration used (5–
20 wt% and that product formation only happened afterwards, which was the result of the presence of monophosphate only (Figure S5). Inspired by this observation, we studied different monophosphate salts (NaH$_2$PO$_4$ and KH$_2$PO$_4$) as the P-donor by using 64 wt% glycerol and 20 wt% P. Similar to the use of PP, the use of monobasic phosphate salts allowed a simple reaction set-up without pH adjustment (final pH ≈ 3.5). Two types of phytase were tested at three different concentrations, Natuphos and phytase cc (Natuphos E). Both enzymes are produced by BASF on a > 1000 t/a scale as an additive for animal feed to improve phosphate availability. Although Natuphos is a 3-phytase from a fungal organism (Aspergillus), phytase cc is a 6-phytase, which is a hybrid of various bacterial phytases developed for increased stability towards proteolytic digestion and elevated temperatures. Interestingly, KH$_2$PO$_4$ was a poor donor compared to the sodium salt (2 was obtained at a maximum of 17 g L$^{-1}$; data not shown); the latter delivered in contrast conversion levels up to approximately 20% and product titers up to 75 g L$^{-1}$ in 29 h and 24% and 104 g L$^{-1}$, respectively, after 4 d (Figure S6, Table 2 entries 1, 2, 4, and 5). The main difference between the two types of phytase lies in the initial velocity of the reaction (Figures S6 and S7). The conversion using Natuphos reached a plateau faster than that with phytase cc, however, maximum product levels were comparable over longer incubation times, and 20 U mL$^{-1}$ enzyme appeared optimal for increased product formation. Given the successful use of inorganic monophosphate as the P-donor by phytase, it appears that the enzyme catalyzes a reversed hydrolytic reaction (Scheme 1) instead of transphosphorylation (according to the known mechanism$^{[16]}$). The reaction likely reaches an equilibrium that depends on the reaction parameters (most notably, substrate and phosphate concentration, temperature, and water content). In particular, 1b delivered slightly lower conversions (up to 21%) and product concentrations (up to 75 g L$^{-1}$) compared to 1a, which presumably is the result of the presence of residual H$_2$O (≈ 10% content) in 1b (Figure S7, Table 1 entries 7, 8, 12, and 13), which impacts the reaction equilibrium. Interestingly, class A acid phosphatases PhoN-Sf, PhoN-Sc, PiACP, and Lw tested under similar conditions also showed product formation, although to a lower extent compared to the two phytases (maximum 15 g L$^{-1}$ product concentration within 2 d; Table S4). Furthermore, these enzymes prefer KH$_2$PO$_4$ over NaH$_2$PO$_4$. This observation suggests that the ability of acid phosphatases to reverse the hydrolysis of phosphate esters is a general property, which has not been considered so far. However, the extent of ester formation with an inorganic monophosphate as a donor is highly enzyme-dependent. The good performance of phytases under these conditions lies in (so far) unknown mechanistic details and provides a catalytic system with a high atom economy of interest for synthetic applications as no byproduct is generated, except water. The high space–time yield achieved (up to 20 g L$^{-1}$) by 1b appears particularly attractive for the synthesis of glycerol monophosphate under industrial conditions.

Next, the effect of temperature on the equilibrium of the reaction between glycerol and monophosphate was studied. Both types of phytase as well as 1a and 1b were subjected to screening conditions (Figures S8 and S9, respectively; results obtained at 30 °C are given for comparison). Both enzymes were active at 60 °C, although only phytase cc is considered thermostable. The high concentration of glycerol, a known enzyme-stabilizing agent,$^{[31]}$ may have a beneficial effect on the thermal stability of Natuphos. The elevated temperature significantly enhanced the rate of product formation and thereby improved maximum product titers by approximately 30–39% (e.g., from 75 to 104 g L$^{-1}$ with 1a and 67 to 87 g L$^{-1}$ with 1b in 24 h, at 30 and 60 °C, respectively; Table 2, entries 3, 6, 9, and 14). As noted earlier, lower conversions (maximum ≈ 22%) and product concentrations (maximum 87 g L$^{-1}$) were obtained with 1b. The difference in the initial activity of the enzymes remained unchanged at 60 °C, and conversion with Natuphos again reached a plateau faster than that with phytase cc.

Finally, 1a and 1b were mixed with NaH$_2$PO$_4$ without added H$_2$O, followed by the addition of phytases. The reaction was catalyzed successfully by phytase cc at 60 °C and reached approximately 28% conversion with 1b within 24 h (vs. ≈ 22% in the presence of added H$_2$O) to attain approximately 33% after 48 h (Table 2, entries 10 and 11). Natuphos was inactive in the absence of added water. Neither of the enzymes was active in 1a.

The activity of phytase cc in 1b without added water simplifies the reaction set-up significantly and also allows the recycling of unreacted P, which contributes positively to the sustainability and the commercial attractiveness of the process overall. Therefore, we investigated the scalability of this system along with a suitable isolation protocol for the prod-

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**Table 2.** Selected results obtained with two phytases in phosphorylation reactions with monophosphate salt as the donor.

| Entry | Enzyme | Enzyme amnt$^{[a]}$ | T | t | Conv. | [Product]/[P] |
|-------|--------|-----------------|---|---|-------|----------------|
| | | [μg mL$^{-1}$] | °C | [h] | [%] | [g L$^{-1}$] |
| 1 | 1a | phytase cc | 201 (30) | 30 | 29 | 15 | 56 | 0.21 |
| 2 | 1a | phytase cc | 201 (30) | 30 | 101 | 22 | 80 | 0.24 |
| 3 | 1a | phytase cc | 201 (30) | 60 | 24 | 25 | 101 | 0.33 |
| 4 | 1a | Natuphos | 588 (30) | 30 | 29 | 20 | 75 | 0.29 |
| 5 | 1a | Natuphos | 588 (30) | 30 | 101 | 24 | 86 | 0.26 |
| 6 | 1a | Natuphos | 588 (30) | 60 | 24 | 26 | 104 | 0.34 |
| 7 | 1b | phytase cc | 201 (30) | 30 | 29 | 14 | 50 | 0.17 |
| 8 | 1b | phytase cc | 201 (30) | 30 | 101 | 18 | 67 | 0.20 |
| 9 | 1b | phytase cc | 201 (30) | 60 | 24 | 22 | 85 | 0.28 |
| 10$^{[b]}$ | 1a | phytase cc | 201 (30) | 60 | 24 | 28 | 139 | 0.39 |
| 11$^{[b]}$ | 1a | phytase cc | 201 (30) | 60 | 48 | 33 | 165 | 0.50 |
| 12 | 1b | Natuphos | 588 (30) | 30 | 29 | 18 | 67 | 0.23 |
| 13 | 1b | Natuphos | 588 (30) | 30 | 101 | 21 | 75 | 0.23 |
| 14 | 1b | Natuphos | 588 (30) | 60 | 24 | 22 | 87 | 0.29 |

[a] Enzyme amount expressed in μg mL$^{-1}$ in brackets; [b] reaction contained 1 mL 1b and 350 mg NaH$_2$PO$_4$. Reaction conditions: 64 wt% 1a/b, 20 wt% NaH$_2$PO$_4$, 1 mL reaction volume, pH = 3.5, 1% DMSO as internal standard, 1400 rpm shaking. Conversions with respect to the limiting reactant (NaH$_2$PO$_4$). Product titer was calculated from the molecular weight of the monosodium phosphate ester salt.
uct. A phosphorylation reaction on a preparative scale (i.e., 100 mL of 1b and 40.0 g NaH₂PO₄) was performed at 60 °C and led to 33% conversion within 19 h. Unreacted Pᵢ was first removed by precipitation as a magnesium ammonium salt (struvite).³⁰-³² Product 2 was finally isolated as the magnesium salt in 26% yield (16.6 g) and approximately 75% purity (measured by NMR spectroscopy), which is an acceptable value given the simplicity of the overall set-up and the crude nature of the starting material. The detected impurities consisted of approximately 5% Pᵢ, 10% glycerol-2-phosphate, and 10% glycerol. Finally, an enzymatic assay coupled with³³³³ P NMR spectroscopy indicated that 2 obtained from the preparative-scale synthesis was racemic (Figures S14 and S15).

Conclusions

Several acid phosphatases were investigated at high substrate loadings for the catalyzed (trans)phosphorylation of glycerol to produce rac-glycerol-1-phosphate. The effect of the type of phosphate donor (pyrophosphate or monophosphate salt) and of the type (crude or pure) and concentration of glycerol as well as the reaction temperature were studied. Nonspecific acid phosphatases retained their activity in the presence of 18 wt% pyrophosphate as the phosphate donor and 55 wt% glycerol to result in the production of up to 167 g L⁻¹ glycerol-1-phosphate. In contrast, phytases could utilize 20 wt% NaH₂PO₄ as the monophosphate salt for the direct condensation in 64 wt% glycerol to furnish 104 g L⁻¹ product. In comparison, calf intestine alkaline phosphatase, which also delivers approximately 10% of glycerol-2-phosphate in the phosphorylation of glycerol, could deliver 71 g L⁻¹ product at best, whereas the product titer with nonspecific acid phosphatases reached only approximately 17 g L⁻¹ from pyrophosphate under standard conditions.³⁴-³⁶ Importantly, several enzymes could operate efficiently in crude glycerol obtained from biodiesel production. Moreover, phytase cc was able to work under neat conditions, which enabled the development of a preparative-scale synthesis on a 100 mL scale and the isolation of rac-glycerol-1-phosphate by fractional crystallization as a magnesium salt in 26% yield (16.6 g).

Experimental Section

Standard conditions for small-scale transformations with added water

A solution was prepared that contained 1a or 1b and P-donor (Na₃P₂O₇, NaH₂PO₄ or KH₂PO₄) in H₂O at the concentrations (wt%) indicated in the footnotes of tables and captions of figures with 1 vol% DMSO as an internal standard. The P-donors were used as fine powders. The resulting solution had a pH of ≈3.5; no pH adjustment was applied. The suspension was shaken thoroughly, and 1 mL was transferred into a 1.5 mL screw-capped glass vial followed by the addition of the corresponding amount of enzyme (27–588 µg mL⁻¹; Table S1). The mixture was shaken vigorously in an Eppendorf thermoshaker at 1400 rpm at 30 or 60 °C for 1–4 d. Samples (25 µL) were diluted with aq. H₂SO₄ (475 µL of 8 mm for NSAPs or 140 mm for phytases) and analyzed by HPLC with refractive index (RI) detection. Experiments were performed in duplicate. Product titer (g L⁻¹) was calculated from the molecular weight of the monosodium phosphate ester salt.

Standard conditions for small-scale transformations without added water

To a 1.5 mL screw-capped glass vial were added NaH₂PO₄ (350 mg) and 1a or 1b (1 mL) followed by phytase cc (201 µg mL⁻¹) or Natypo (588 µg mL⁻¹). The mixture was shaken vigorously by using an Eppendorf thermoshaker at 1400 rpm at 60 °C for 2 d. Samples (25 µL) were diluted with aq. H₂SO₄ (475 µL, 140 mm) and analyzed by HPLC–RI. Experiments were performed in duplicate.

Preparative-scale phosphorylation of 1b

Compound 1b (100 mL), NaH₂PO₄ (40.0 g, 333 mmol), and phytase cc (100 mg) were stirred at 60 °C in a 250 mL round-bottomed flask (Figure S10). The reaction was quenched after 19 h by adding the mixture to NaOH (500 mL, 0.5 m). The resulting solution (~620 mL) had a pH of ≈7.5. Then, DMSO (final 1 vol%) was added, and the concentrations of unreacted Pᵢ and 2 were measured by using HPLC–RI, which indicated 168 mm of product and 345 mm of Pᵢ, which corresponds to ≈33% conversion with respect to Pᵢ. Next, NH₄Cl (1 equiv., 17.8 g, 333 mmol) was added, and the pH was adjusted to 7.5–8.0. Then, MgCl₂·6H₂O (1 equiv., 67.8 g, 333 mmol) was added portionwise, and the pH was kept between 7.0 and 8.0 by adding NaOH (10 m). The resulting suspension was stirred for 5 min followed by filtration (glass frit porosity 3). The solid residue was washed with H₂O (≈20 mL). Finally, the pH of the filtrate was adjusted to ≈8.0, and EtOH (4 volumes) was added to the mixture. The product was allowed to precipitate at 4 °C overnight. Product stability during the work-up procedure was checked by taking a sample before treatment with EtOH. The precipitation resulted in a sticky solid, which was impossible to filter. Instead, the supernatant was decanted, and the product was dissolved in water followed by lyophilization overnight. This step gave a light brownish powder (16.6 g, yield 26%). The product was analyzed by NMR spectroscopy, which showed ≈75% (molar) purity (Figures S11–S13). The impurities consisted of ≈5% Pᵢ, 10% glycerol-2-phosphate, 10% glycerol, and traces of EtOH.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: alcohols · biocatalysis · enzyme catalysis · phosphorylation · synthesis design

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