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Biocatalytic Asymmetric Phosphorylation catalyzed by recombinant Glycerate-2-kinase

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Abstract: The efficient synthesis of pure D-Glycerate-2-phosphate is of much interest due to its importance as enzyme substrate and metabolite. A straightforward one-step biocatalytic phosphorylation of glyceric acid has been investigated. Glycerate 2-kinase from *Thermotoga maritima* has been expressed in *Escherichia coli* allowing easy purification. The selective glycerate 2-kinase-catalyzed phosphorylation has been followed by ^{31}P-NMR and showed excellent enantioselectivity towards the phosphorylation of the D-enantiomer of glyceric acid. This straightforward phosphorylation reaction and the subsequent product isolation has enabled the preparation of enantiomerically pure D-Glycerate 2-phosphate. This phosphorylation reaction using recombinant glycerate 2-kinase yields D-Glycerate 2-phosphate in less reaction steps and with higher purity than chemical routes.

Introduction

D-Glyceric acid 2-phosphate (scheme 1) is a key metabolite in central carbon metabolism, glycolysis/gluconeogenesis, pentose phosphate pathway, glycine, serine and threonine metabolism, methane metabolism, biosynthesis of plant secondary metabolites, phenylpropanoids, terpenoids, steroids, alkaloids derived from the shikimate pathway, antibiotics and amino acids.

Scheme 1. D-Glyceric acid 2-phosphate

The glycolytic intermediate between D-glycerate-3-phosphate and phosphoenolpyruvate was first isolated from yeast and characterized as a crystalline D-glycerate-2-phosphate barium salt by Meyerhof and Kiessling.[1] An efficient, robust and scalable route for the preparation of a soluble form of pure D-glycerate-2-phosphate has been of much interest since its discovery. It continues to be required both as a metabolite reference standard as well as an enzyme substrate for measuring activities of enolases and their inhibitors, e.g. enolase activities in a number of human diseases and disease-causing human pathogens such as *Streptococcus mutans* and the inhibiting action of fluoride or phosphate in caries prevention, *Staphylococcus aureus*, *Streptococcus pyogenes* or the blood parasites *Schistosoma mansoni* causing schistosomiasis and *Trypanosoma brucei* causing sleeping sickness.[2] The first definitive synthesis of D-glycerate-2-phosphate has been described by Ballou and Fischer.[3] The identity and purity of the final product D-glycerate-2-phosphate, which was obtained as the crystalline trisodium salt pentahydrate, was proven, but nine reaction steps were required to get from the starting compound D-galactose to the final product. Although a shorter synthesis starting from D-gluconolactone has been described,[4] the bottlenecks in chemical synthesis and the number of reaction steps using protection-deprotection and purification steps have created interest in an attractive direct enzymatic phosphorylation of glyceric acid.[5] The enzymatic phosphorylation of glyceric acid is also of clinical relevance for patients with D-glyceric aciduria, a rare inborn error of serine and fructose metabolism, as mutations in the *GLYCTK* gene have been revealed as the cause of D-glycerate kinase deficiency and D-glyceric aciduria.[6]

Site- and stereoselective kinases have been very powerful and versatile catalysts for biocatalytic phosphorylations of small molecules, e.g. in straightforward syntheses of phosphorylated metabolites.[7] As class II glycerate kinases catalyze the formation of D-glyceric acid 2-phosphate in nature, a stable and highly selective glycerate kinase from this class (EC 2.7.1.165) has been selected for the biocatalytic phosphorylation of glycerate in the 2-position.

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Supporting information for this article is given via a link at the end of the document.

Scheme 2. Biocatalytic phosphorylation of D-glyceric acid by glycerate 2-kinase using the phosphoenolpyruvate(PEP)/pyruvate kinase-system for the regeneration of ATP

While a number of glycerate kinases from rat liver,[8] *Escherichia coli*,[9] *Pyrococcus horikoshii*,[10] *Picrophilus torridus*,[11] *Thermoplasma acidophilum*,[12] *Sulfolobus tokodaii*[13] and *Hyphomicrobium methylovorum*,[14] which are able to catalyze the formation of glycerate-2-phosphate, were investigated, detailed enzymatic information on the key enzyme glycerate 2-kinase was reported on the glycerate kinase of the hyperthermophilic archaeon *Thermoproteus tenax*.[15] From the comparison of the kinetic and biochemical properties of the characterized class II glycerate kinases of *T. tenax*, *P. torridus*, *T. acidophilum* and *H. methylovorum*, the glycerate 2-kinase from *Thermotoga maritima* was selected due to its favorable biocatalytic properties and structure-function characterization.[16-17]

The 1.25 kb glycerate-2-kinase gene from *Thermotoga maritima* was expressed heterologously in *E. coli* as a fusion protein with the maltose-binding-protein (MBP) and was purified by affinity chromatography. The active form of the protein was obtained by cleaving off the MBP. We have investigated biocatalytic phosphorylations catalyzed by recombinant glycerate-2-kinase with quantitative ^{31}P-NMR spectroscopy using phosphoenolpyruvate/pyruvatekinase-system for ATP-regeneration, starting with racemic and the enantiopure D- and L-glycerate as substrate. Nearly 100% conversion of D-glycerate to D-glycerate-2-phosphate was found.

With pure L-glycerate as substrate, no L-glycerate-2-phosphate was formed, while DL-glycerate gave 50% conversion with excellent enantioselectivity.

Results and Discussion

The glycerate-2-kinase gene from *Thermotoga maritima* was cloned into pMAL-C2X vector and expressed in *E. coli* as soluble fusion protein with MBP (87 kDa). Highest protein yield was obtained when expressed at a temperature of 17 °C. After harvesting and purification using affinity chromatography with amylose resins, the active glycerate 2-kinase (52 kDa) has been obtained by cleaving off the MBP with factor Xa protease maintaining its maximum activity without any tag.

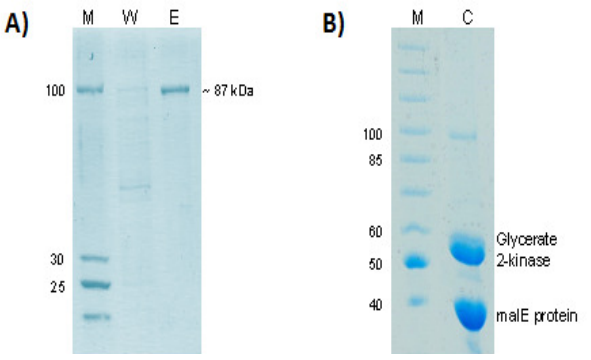


Figure 1. SDS-PAGE analysis of expression and purification of glycerate 2-kinase. Wash and elution fraction of purified fusion protein with MBP (A) and cleaved protein after using factor Xa protease (B). Approximately 1.5 (A) and 35 µg (B) total proteins were loaded, respectively. M: marker, W: wash, E: eluate, C: cleaved protein.

Enantioselectivity of the glycerate 2-kinase-catalyzed phosphorylation of glycerate

The enantioselectivity was determined by analyzing the kinetics of the glycerate 2-kinase-catalyzed conversion of equal amounts of DL-glyceric acid, D-glyceric acid and L-glyceric acid. Every 15 minutes a ^{1}H-NMR spectrum (left) and a ^{31}P-NMR spectrum (right) has been measured. T=0 (red, bottom) is measured app. 2 minutes after addition of the enzymes. The conversion can be tracked by the decrease of the PEP signals (^{1}H: 5.0-5.4 ppm; ^{31}P: -0.9 ppm) and the increase of the product signals of D-glyceric acid 2-phosphate and pyruvate. (^{1}H: 4.4 ppm and 2.3 ppm; ^{31}P: 2.4 ppm), as shown in figures 2 and 3.

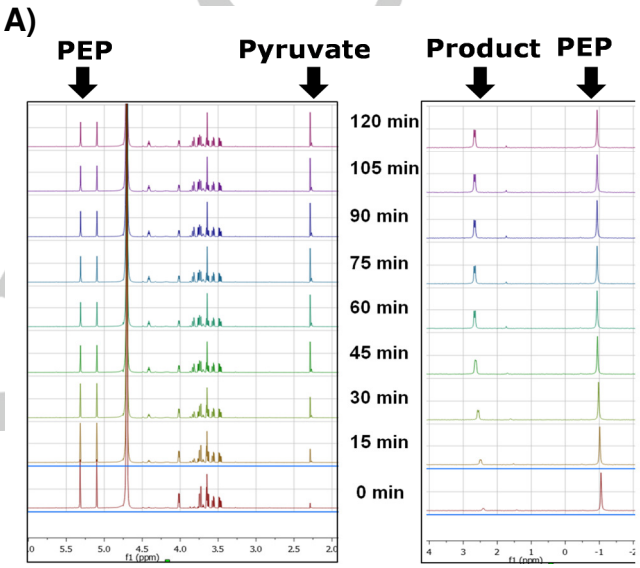
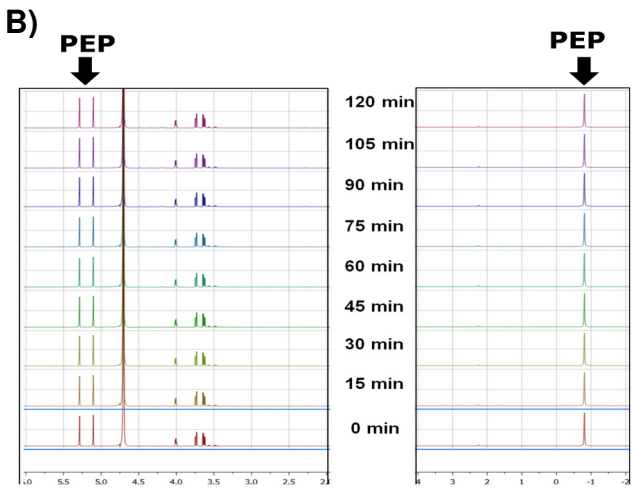


Figure 2. (A) NMR-Kinetics of the enzymatic conversion of DL-Glyceric acid. 10 mg DL-Glyceric acid (94 µmol, 1.0 eq), 2.6 mg ATP (5 µmol, 0.05 eq) and 17.5 mg PEP (85 µmol, 0.9 eq) were dissolved in 1.7 ml D_2O with 10mM $MgCl_2$. pH was adjusted to 7. Subsequently 50 Units pyruvate kinase (in 50 µl) and 250 µl of the prepared recombinant glycerate 2-kinase (*Thermotoga maritima*) were added. The enzymatic reaction was observed via NMR. (B) NMR-Kinetics of enzymatic conversion of L-Glyceric acid, whereby the same procedure as in (A) was followed except that instead of DL-Glyceric acid as substrate 10 mg L-Glyceric acid (94 µmol, 1.0 eq) was used.



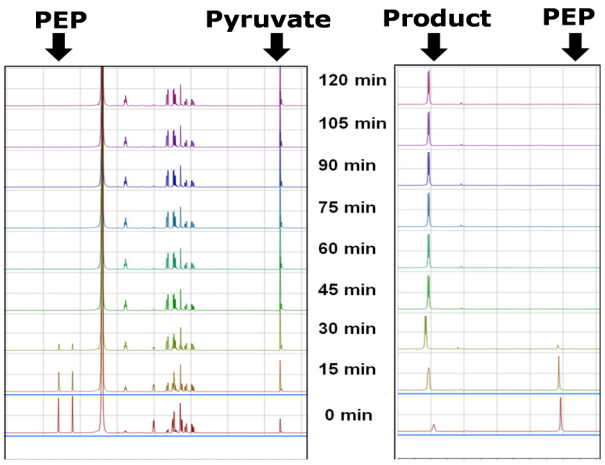


Figure 3. NMR-Kinetics of enzymatic conversion of D-Glyceric acid. The same procedure as in figure 2 was applied, but 10 mg D-Glyceric acid (94 µmol, 1.0 eq) was used as starting material.

The NMR-experiments in figures 2 and 3 clearly demonstrate the conversion of only the D-glyceric acid and no conversion of the L-glyceric acid to the corresponding product phosphorylated in the 2-position.

Kinetic measurements of glycerate 2-kinase from *Thermotoga maritima*

A K_m value of (0.13 +/- 0.03) mM to D-glyceric acid, a catalytic turnover number k_{cat} of (5.4 +/- 0.7) 10^{-2} s^{-1} and a k_{cat}/K_m of (4.0 +/- 0.05) 10^4 $M^{-1}s^{-1}$ has been determined at 37°C for this glycerate 2-kinase (see experimental section). The K_m value for our glycerate 2-kinase agrees well with the wild-type TM1585. While the k_{cat} for the D-glycerate was not determined in the wild-type TM1585, the temperature dependence of the specific activity showed a large increase from 37°C to 80°C, levelling off at 90°C.[17]

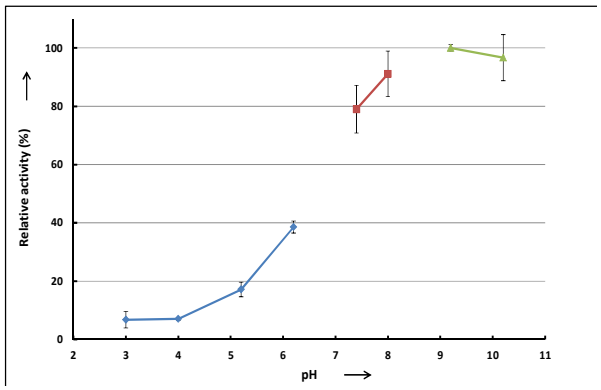


Figure 4. Glycerate 2-kinase activity as a function of pH. 50 mM citrate buffer pH 3.0, 4.0 5.2 and 6.2 (◆), 50 mM Tris-HCl buffer pH 7.4 and 8.0 (■) and 50 mM sodium carbonate-bicarbonate buffer pH 9.2 and 10.2 (▲).

As shown in figure 4, glycerate 2-kinase from *Thermotoga maritima* is most active at neutral and alkaline pH and its maximum activity was recorded at pH 9.2. The enzyme also maintained its full activity after storage at 4°C for several months with 0.02% NaN_3 (w/v). Its excellent stability (both reaction and storage) as well as activity profile, thus allowing a direct coupling to the ATP regeneration system for glycerate 2-phosphate synthesis, is an advantage of this glycerate 2-kinase from *Thermotoga maritima*.

An optimized reaction system for the biocatalytic phosphorylation of D-glyceric acid acid is however not only dependent on the optimum properties of the glycerate 2-kinase, but also on the optimum parameters for ATP-regeneration and the stability profile of D-glyceric acid 2-phosphate. The stability of D-glyceric acid 2-phosphate has been examined in the pH range of 6.0 to 11.0. A 20 mM solution of the lithium salt of D-glyceric acid 2-phosphate in citrate buffer at pH 6.0, in Tris buffer at pH 7.0 and 8.0 as well as in carbonate buffer at pH 9.0, 10.0 and 11.0 was observed by ^{1}H- and ^{31}P-NMR. No decomposition was detected during the time period of observation, which was 5 days for pH 10.0 and 11.0, 11 days for pH 7.0, 8.0 and 9.0 and 18 days for pH 6.0. As the pH optimum of the ATP regeneration system is at pH 7.0 to 7.5, the biocatalytic phosphorylation of D-glyceric acid at a pH around 7 is favoured over a reaction pH at the optimum pH of the glycerate 2-kinase, although the stability profile of the D-glyceric acid 2-phosphate would be compatible.

In order to determine the enantiomeric purity of the product, capillary zone electrophoresis using vancomycin as chiral selector was performed and confirmed an enantiomeric ratio of 98.3:1.7=D:L for the product D-glycerate 2-phosphate (see direct data on capillary zone electrophoresis in the supporting information). The development of suitable reaction conditions for complete conversion in phosphorylations is facilitated by ^{31}P-NMR reaction monitoring,[18] as both product formation as well as phosphoenolpyruvate donor consumption can be measured simultaneously. From the time course of biocatalytic *O*-phosphorylation of D-glyceric acid in the 2-position, followed by ^{31}P-NMR as shown in figure 5, the reaction conditions for complete conversion and optimal process design can be derived

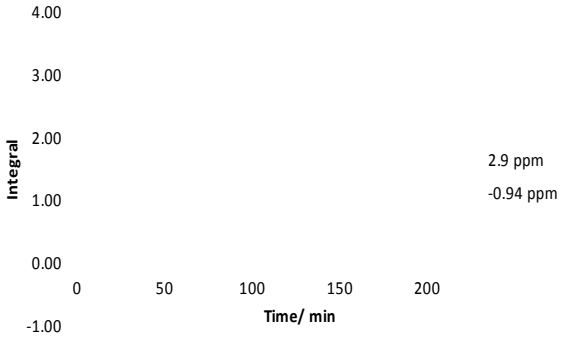


Figure 5. Kinetics of formation of D-glycerate-2-phosphate (blue curve) and consumption of phosphoenolpyruvate (red curve) derived from the ^{31}P-NMR signals.

Conclusions

Glycerate 2-kinase from *Thermotoga maritima* was selected as biocatalyst for the phosphorylation of glyceric acid and the corresponding gene was expressed in *E.coli* as fusion protein with MBP to allow easy affinity purification. After cleaving off MBP by factor Xa protease a highly active and stable glycerate 2-kinase has been obtained. A K_m value of (0.13 +/- 0.03) mM to glyceric acid, a catalytic turnover number k_{cat} of (5.4 +/- 0.7) 10^{-2} s^{-1} and a k_{cat}/K_m of (4.0 +/- 0.05) 10^4 $M^{-1}s^{-1}$ has been determined. The glycerate 2-kinase-catalyzed phosphorylation of the D- and L-enantiomer of glyceric acid as well as its racemic form have been directly investigated by NMR. The experiments clearly demonstrated the power and selectivity of biocatalytic phosphorylation. Only the D-glyceric acid was accepted as a substrate for this recombinant glycerate 2-kinase, while the L-glyceric acid was not phosphorylated at all. This straightforward and highly selective phosphorylation reaction is therefore very valuable for the enzymatic resolution of racemic glyceric acid or for the synthesis of D-glycerate 2-phosphate by phosphorylation of D-glyceric acid in the 2-position.

The glycerate 2-kinase-catalyzed phosphorylation of D-glyceric acid and the subsequent product isolation has enabled the preparation of the key metabolite D-glycerate 2-phosphate in enantiomerically pure form. This biocatalytic phosphorylation reaction using recombinant glycerate 2-kinase is also very sustainable, as it yields D-glycerate 2-phosphate in a one-step reaction and with higher purity than chemical routes.

The excellent enantioselectivity and the first crystal structure[16] of a representative of the glycerate 2-kinase family provide exciting opportunities for extending this work to new phosphorylated compounds which up to now are only accessible though lengthy chemical routes, such as substituted D-glycerate analogues and functional isosteric and negatively charged substrates able to dock properly into the active site. Although highly conserved amino acid residues from the Rossmann-like domain and the C-terminal domain have been identified and contain one highly conserved basic residue, a detailed understanding of the interaction with the negatively charged D-glycerate and the elucidation of the mechanism of the enzyme function require further studies and higher resolution.

Experimental Section

Unless otherwise stated, all chemicals were of analytical grade and were purchased from Sigma-Aldrich.

NMR spectroscopy

NMR-spectra were measured in D_2O at room temperature on a Bruker Avance III 600 MHz spectrometer equipped with a BBO probe head with z-gradient using 600.2 MHz for ^1H, 150.9 MHz for ^{13}C. The NMR-kinetics of enzymatic phosphorylations of DL-glyceric acid, L-glyceric acid and D-glyceric acid was measured by the acquisition each 15 minutes of a ^1H-NMR (left) and a ^{31}P-NMR (right). T=0 (red, bottom) is measured app. 2 minutes after addition of the enzymes. Conversion was tracked by the decrease of the PEP signals (^1H: 5.0-5.4 ppm; ^{31}P: -0.9 ppm) and the increase of product signals (^1H: 4.4 ppm; ^{31}P: 2.4 ppm).

Capillary Electrophoresis

Capillary zone electrophoresis with EOF-reversal (dynamic coating) was performed with a PDA-detector and indirect detection at a wavelength of 233 nm and at 25 C. Noncoated capillaries with 75 µm inner diameter, 120 cm length and 110 cm to the detector were used. Accelerator from CEofix anions 5 Kit was used as electrophoresis buffer, initiator from CEofix anions 5 Kit as conditioner and 40 mM Vancomycin-solution as chiral selector.

Amplification and cloning of glycerate 2-kinase gene

Glycerate kinase gene from *T. maritima* was amplified with PCR using primer pairs G2K-EcoRI_F (5' - gaattcATGTTTGATCCTGAATCCTTG and G2K-HindIII_R (5' - aagcttCTAGACGATGAGGCCTATTATC) and cloned into pDrive cloning vector. The gene was then excised out from the plasmid with endonucleases EcoRI and HindIII and subcloned into pMAL-C2X in the same translational reading frame as the *malE* gene for a N-terminal MBP-fused expression. The clone was verified via sequencing using primers mal2E (5' - AGCTGGCAAAAGAGTTCC) and M13 forward (5' – TGTAAAACGACGGCCAGTG) both binding within vector sequence upstream (within *malE* gene) and downstream of the insert. The fusion plasmid clone was finally transformed using heat-shock into chemically competent *Escherichia coli* BL21 (DE3) host cells for expression.

Culture conditions, protein expressions and purification

E. coli BL21 (DE3) expression host cells carrying the right fusion clone were grown overnight at 37 °C in rich medium (10 g tryptone, 5 g yeast extract and 5 g NaCl) with 2% glucose and 100 µg/ml Ampicillin. Three per cent of the overnight grown cells were transferred into same fresh medium; further incubated until OD_{600} is 0.6 and induced with 0.3 mM isopropyl ß-D-1-thiogalactopyranoside (IPTG) at 17 °C for 18 hrs for maximum soluble protein expression. Cells were harvested using centrifugation and resuspended in column buffer (20 mM Tris-Cl pH 7.4, 0.2 Mm NaCl, 1 mM EDTA, 20 mM 2-ME and 1 mM NaN_3). French press was used to breakup cells and the supernatant after centrifugation was loaded onto pre-equilibrated column packed with amylose resins. After washing with 12 volume of column buffer, the fusion protein was eluted with 10 mM maltose in same buffer.

In order to get tag-free glycerate 2-kinase protein, the MBP was cleaved off from the fusion protein with Factor Xa protease (New England Biolabs) overnight on ice. The protein was further concentrated and simultaneously dialyzed using Vivaspin columns (Sartorius) and then quantified with Roti®-Quant solution (Carl Roth) according to manufacturer's protocol.

Kinetic measurements of glycerate 2-kinase from *Thermotoga maritima*

Kinetic determinations of the glycerate 2-kinase were performed using a continuous coupled assay based on the oxidation of NADH, where the

decrease in absorbance at 340 nm was monitored using a Synergy™ HT multi-mode microplate reader with Gen5 software. The assay mixture contained 0.3 mM NADH, 1.5 mM ATP, 10 mM $MgSO_4$, 1.5 mM phosphoenolpyruvate, 1.5 U of pyruvate kinase, 1.5 U of L-lactate dehydrogenase and 0.1 to 1.4 mM varying D-glyceric acid concenatrions varying from 0.1 to 1.4 mM in 50 mM Tris buffer pH 7.4 to a volume of 200 µl. The reactions were carried out in triplicates at 37°C after adding 0.2 µg purified glycerate 2-kinase. Apparent values of k_{cat} and K_m were determined applying the Michaelis-Menten kinetic model. A discontinuous assay was however used for measuring the enzyme activity over varying pH conditions from pH 3.0 to 10.2. The phosphorylation reaction took place separately at 37°C for 10 minutes and put immediately on ice. The pH of assay reactions was adjusted by adding predetermined volumes of NaOH or HCl just before coupling to the indicator reaction which had 5 times excess pyruvate kinase and lactate dehydrogenase. The ADP formed was then measured by monitoring oxidation of NADH same as above.

Enzyme reactions

Enzymatic syntheses of DL-, D- and L-Glyceric acid 2-phosphate. 10 mg of respectively DL-, D- and L-Glyceric acid (94 µmol, 1.0 eq), 2.6 mg ATP (5 µmol, 0.05 eq) and 17.5 mg PEP (85 µmol, 0.9 eq) were dissolved in 1.7 ml D_2O with 10mM $MgCl_2$. pH was adjusted to 7. Subsequently 50 Units pyruvate kinase (in 50 µl) and 250 µl of the recombinant glycerate 2-kinase were added. The enzymatic reaction was observed via NMR. ^{1}H-NMR and ^{31}P-NMR were measured every 15 min.

Gram-scale preparation of D-glycerate-2-phosphate catalyzed by recombinant glycerate 2-kinase

1.0 g D-Glyceric acid (9.4 mmol, 1.0 eq), 260 mg ATP (0.5 mmol, 0.05 eq) and 1.75 g PEP (8.5 mmol, 0.9 eq) were dissolved in 200 ml 10mM $MgCl_2$ solution. The pH value was adjusted to 7 and subsequently 1000 Units pyruvate kinase (in 1000 µl) and 1100 µl glycerate 2-kinase (*Thermotoga maritima*) were added and stirred gently. The conversion of the enzymatic reaction was observed by NMR and reached completion after 24h. Subsequently the enzyme was filtered off using a 10000 MW Zentricon filter membrane. To the resulting reaction solution containing D-glycerate 2-phosphate 3.3 g calcium acetate monohydrate (28.2 mmol, 3 eq) was added to precipitate the D-glycerate 2-phosphate as its calcium salt and harvest it by filtration. This calcium salt was finally converted into the corresponding lithium salt using 150 ml lithium conditioned Dowex 50W X8 H+ ion exchange resin. The solution of the D-glycerate 2-phosphate lithium salt was evaporated to give 1.255 g white solid product in 72% yield and excellent purity.

^{1}H NMR (D_2O, 600 MHz): δ 4.48 (ddd, J = 8.6, 5.4, 3.1 Hz, 1H), 3.89 (dd, J = 11.9, 3.1, 1H), 3.82 (dd, J = 11.9, 5.4 Hz, 1H).
^{13}C NMR (D_2O, 151 MHz): δ 177.41 (d, J = 5.6 Hz), 76.66 (d, J = 5.5 Hz), 64.24 (d, J = 3.6 Hz).
^{31}P NMR (D_2O, 162 MHz): δ 2.15 (s, 1P).
MS: found: 185.0; calculated: 185.0 ($M\text{-}H^+$, $C_3H_7O_7P^-$);
Capillary Electrophoresis: Enantiomeric Ratio D:L = 98.3:1.7

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Keywords: D-Glycerate-2-phosphate • Glycerate-2-kinase • asymmetric biocatalysis • enzymatic phosphorylation • chiral metabolite

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Recombinant glycerate 2-kinase from *Thermotoga maritima* showed an excellent selectivity towards the phosphorylation of the D-enantiomer of glyceric acid, while the L-enantiomer was not phosphorylated at all. This straightforward phosphorylation reaction and the subsequent product isolation has enabled the preparation of the central glycolytic metabolite D-glycerate 2-phosphate in less reaction steps and with higher purity than chemical routes.

*Norman Hardt, Birhanu M. Kinfu, Jennifer Chow, Bernhard Schoenenberger, Wolfgang R. Streit, Markus Obkircher and Roland Wohlgemuth**

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Biocatalytic Asymmetric Phosphorylation catalyzed by recombinant Glycerate-2-kinase