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Characterization of glycerol phosphate oxidase from *Streptococcus pneumoniae* and its application for ketose synthesis

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ABSTRACT

Glycerol phosphate oxidase from *Streptococcus pneumoniae* ($GPO_{S.pne}$) was purified and characterized. By the actions of $GPO_{S.pne}$ and dihydroxyacetone phosphate (DHAP)-dependent aldolases, various ketoses including rare sugars were synthesized with glyceraldehydes as acceptors in a one-pot four-enzyme system.

The high stereoselectivity of aldolases in carbon–carbon bond formation confers them enormous utility as synthetic biocatalysts.[1–3] Among the aldolase families, dihydroxyacetone phosphate (DHAP)-dependent aldolases are the most investigated and exploited, because aldol reactions catalyzed by this group of aldolases can generate two new stereocenters.[4,5] Moreover, the stereoselectivity of four DHAP-dependent aldolases are complementary, therefore a set of four possible diastereomers of vicinal diols can be synthesized.[4,6] However, the main disadvantage for this group of aldolases is their strict specificity for the donor substrate DHAP, which is a very expensive ($970/250 mg, Sigma–Aldrich) and unstable chemical.[6–8] Therefore, the capability to produce DHAP from cheap sources could eventually expand the scope of aldolase reactions.

Although a number of chemical and enzymatic approaches have been developed for the preparation of DHAP,[9] the yield and purity are not satisfying. Many of the reported enzymatic methods have not advanced beyond small scale and chemical routes usually suffer from the use of toxic and expensive starting materials.[9] In order to improve the practicality of the aldol reaction by avoiding the use of DHAP, one of the most efficient approaches is to provide DHAP in situ from cheaper starting materials. Fessner and co-workers first established a one-pot system in which DHAP was produced by glycerol phosphate oxidase (GPO) with L-glycerol 3-phosphate (L-GP) as the starting material.[10] Employing this strategy, we developed a one-pot four-enzyme approach which can directly produce rare sugars using an even cheaper starting material DL-glycerol 3-phosphate (DL-GP) instead of L-glycerol 3-phosphate with L-rhamnulose-1-phosphate aldolase from *Escherichia coli* ($RhaD_{E.coli}$), L-fuculose-1-phosphate aldolase from *Thermus thermophilus* HB8 ($FucA_{T.HB8}$) and D-fructose 1,6-bisphosphate aldolase from *Staphylococcus carnosus* ($FruA_{S.car}$), respectively[11–13] (Scheme 1). Since DHAP consumption by aldolases could prevent product inhibition of GPO by DHAP, aldolase reactions in situ could also promote the conversion of L-GP into DHAP. Although GPO from *Streptococcus thermophilus* ($GPO_{S.the}$) utilized in our system is commercially available, the cost is still rather expensive ($196.00/500 UN, Sigma–Aldrich) which impedes the application in large-scale synthesis.

GPO (EC 1.1.3.21) is a FAD-dependent α-glycerophosphate oxidase[14] and the coenzyme FAD can be reoxidized by reacting with O_2 to form H_2O_2.[15] It was reported that GPO belonging to *Streptococcus* sp. sources is a dimeric[16] and cytosolic protein.[16,17] Several GPO enzymes, especially from *Streptococcus* sp. sources, have been investigated for their enzymatic activity.[10] However, we noticed that the enzymatic property of GPO from *Streptococcus pneumoniae* ($GPO_{S.pne}$) has not been well studied and exploited in DHAP generation.

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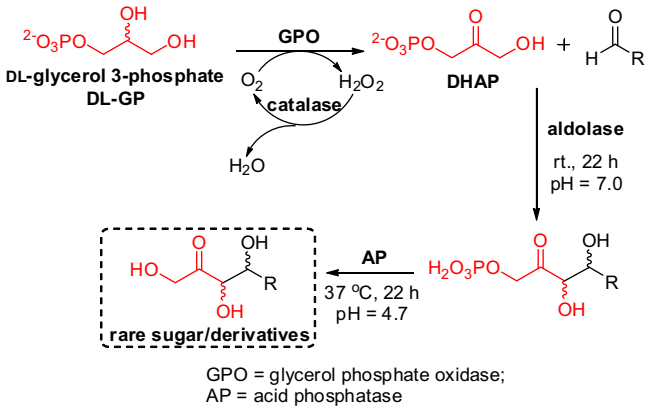


Scheme 1. One-pot four enzyme synthesis of rare sugars.

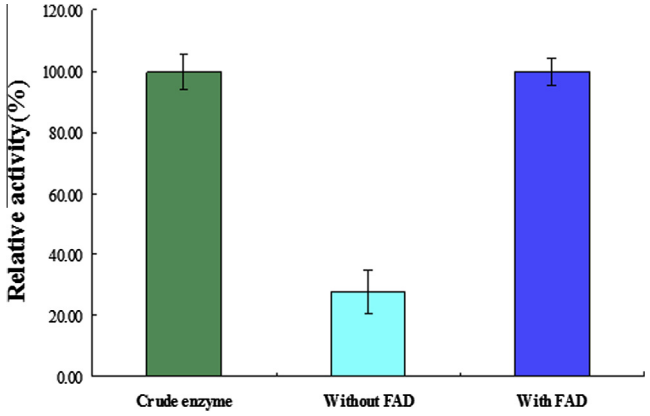


Figure 2. The coenzyme FAD is easily dissociable from $GPO_{S.pne}$. Error bars represent the standard deviation from three parallel experiments.

In order to obtain relatively large amounts of GPO for the one-pot system, we cloned the *glpO* gene encoding $GPO_{S.pne}$ and heterologously overexpressed the target enzyme in *Escherichia coli* Rosetta (DE3). The recombinant cells were disrupted by sonication and purified with Ni^{2+}-NTA column (Fig. 1) (see Supplementary data for detailed procedures of cloning and purification of $GPO_{S.pne}$). The molecular weight of $GPO_{S.pne}$ subunit from SDS–PAGE was consistent with the theoretical value.[18] To our delight, the expression level of $GPO_{S.pne}$ was very high and the yield reached about 50 mg per liter cell culture. As racemic glycerol 3-phosphate (DL-GP) is much cheaper and it has been reported that GPO can exclusively oxidize the L-isomer, the activity of $GPO_{S.pne}$ was thus firstly examined in the presence of DL-GP and oxygen in our research. Unexpectedly, compared with the crude enzyme, the relative activity of pure enzyme was less than 30% (Fig. 2). This low activity can most likely be attributed to the possibility that $GPO_{S.pne}$ requires the coenzyme FAD for activity, which could be easily dissociable during the purification process.[19] To confirm this hypothesis, enough FAD was added into the reaction mixture and the activity of $GPO_{S.pne}$ was detected again. Consistent with our expectation, the activity of $GPO_{S.pne}$ was basically recovered to the same level as the crude enzyme (Fig. 2). Therefore, FAD was supplemented into the reaction mixture in our subsequent experiments to achieve full $GPO_{S.pne}$ enzymatic activity.

To reduce the synthetic cost of rare sugars, $GPO_{S.pne}$ pure enzyme was employed in the one-pot system instead of the commercial $GPO_{S.the}$ we previously used. To optimize the one-pot system, firstly the optimal temperature and pH of $GPO_{S.pne}$ were determined. The results showed that $GPO_{S.pne}$ exhibited the highest activity at 30 °C and the relative activity at 50 °C remained more than 60% of the maximum activity. However, $GPO_{S.pne}$ almost lost its activity at 60 °C (Fig. 3). Furthermore, $GPO_{S.pne}$ could tolerate a broad pH range and the optimal pH value was 8.0 (Fig. 4). Considering the optimal pH values of aldolases are around 7.0 and $GPO_{S.pne}$ exhibits 85% of the highest activity at pH 7.0, we chose pH 7.0 to carry out the one-pot reactions.

As a proof-of-concept experiment, $GPO_{S.pne}$ and $RhaD_{E.coli}$ were firstly employed in the one-pot system with D-glyceraldehyde as the acceptor. Consistent with our previous result, the product D-psicose and D-sorbose were simultaneously generated and the ratio (D-psicose/D-sorbose = ~2:1) was determined by ion-exchange HPLC after calibration with standard curves. After purification by silica gel chromatography and gel filtration, a mixture of D-psicose and D-sorbose was obtained (52% total yield, see Table 1). The mixture containing only two rare sugars could be well separated by Ca^{2+} exchange resin chromatography at 70 °C to obtain pure D-sorbose and D-psicose. Alternatively, rare sugar L-fructose was synthesized with L-glyceraldehyde as the acceptor instead of the D-isomer (49% yield, see Table 1).

To demonstrate that $GPO_{S.pne}$ is compatible with other DHAP-dependent aldolases, $FucA_{T.HB8}$ and $FruA_{S.car}$ were also employed in this one-pot system together with $GPO_{S.pne}$ under the same reaction conditions. Our results showed that when $FucA_{T.HB8}$ accepting D-glyceraldehyde as the acceptor, rare sugar D-psicose was synthesized as the dominated product with rare sugar D-sorbose as the minor product and the ratio (D-psicose/D-sorbose = ~9:1) was also determined by ion-exchange HPLC (70% total yield, see Table 1).

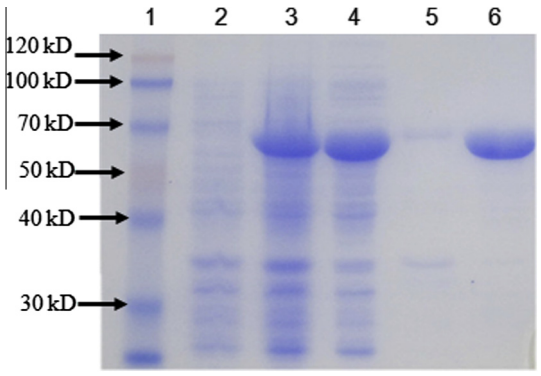


Figure 1. SDS–PAGE analysis of $GPO_{S.pne}$ expression and purification. Lanes: 1, protein marker; 2, whole cells not induced; 3, whole cells induced for 16 h; 4, supernatant of cell lysate; 5, precipitate of cell lysate; 6, purified GPO.

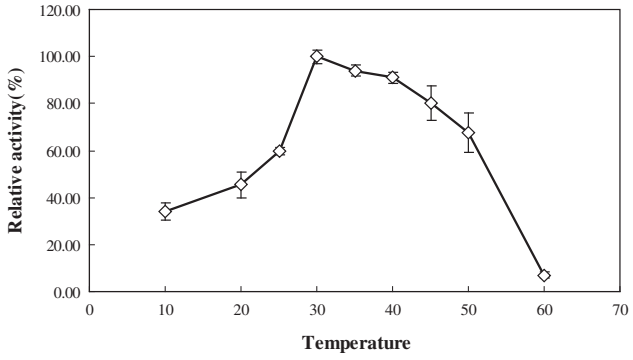


Figure 3. Effect of temperature on the activity of $GPO_{S.pne}$. Error bars represent the standard deviation from three parallel experiments.

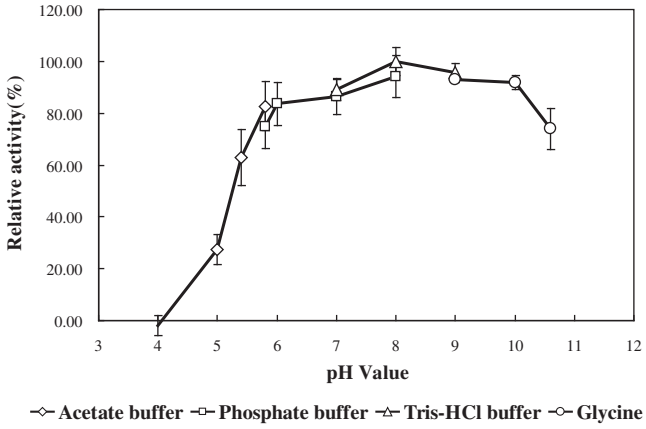
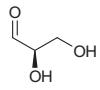
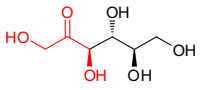
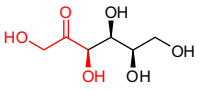
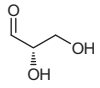
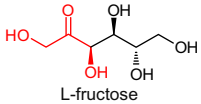
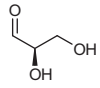
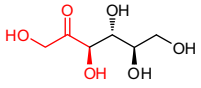
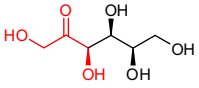
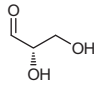
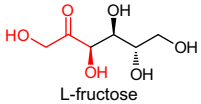
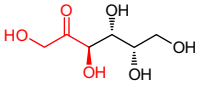
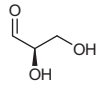
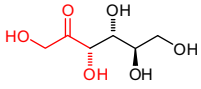
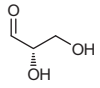
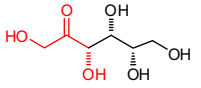


Figure 4. Effect of pH on the activity of $GPO_{S.pne}$. Error bars represent the standard deviation from three parallel experiments.

Table 1
One-pot four-enzyme synthesis of ketoses with $GPO_{S.pne}$ and aldolases

Enzyme	Acceptor	Product(s)		Yield (%)	Ratio
$GPO_{S.pne}$, $RhaD_{E.coli}$	 D-glyceraldehyde	 D-psicose	 D-sorbose	52	2:1
$GPO_{S.pne}$, $RhaD_{E.coli}$	 L-glyceraldehyde	 L-fructose		49	1:0
$GPO_{S.pne}$, $FucA_{T.HB8}$	 D-glyceraldehyde	 D-psicose	 D-sorbose	70	9:1
$GPO_{S.pne}$, $FucA_{T.HB8}$	 L-glyceraldehyde	 L-fructose	 L-tagatose	52	1.1:1
$GPO_{S.pne}$, $FruA_{S.car}$	 D-glyceraldehyde	 D-fructose		56	1:0
$GPO_{S.pne}$, $FruA_{S.car}$	 L-glyceraldehyde	 L-sorbose		54	1:0

Alternatively, when $FucA_{T.HB8}$ accepting L-glyceraldehyde as the acceptor, both rare sugars L-fructose and L-tagatose were simultaneously generated (52% total yield) and the ratio was L-fructose/L-tagatose = ~1.1:1 (Table 1), which was consistent with our previous results.[11] Furthermore, D-fructose and L-sorbose were synthesized catalyzed by $GPO_{S.pne}$ and $FruA_{S.car}$ with D- and L-glyceraldehyde as the acceptor, respectively. After purification by silica gel chromatography and P-2 gel filtration, the yields for D-fructose and L-sorbose were 56% and 54%, respectively (Table 1). With these results, we are delighted to see that $GPO_{S.pne}$ is very compatible with aldolase $RhaD_{E.coli}$, $FucA_{T.HB8}$ or $FruA_{S.car}$ in the one-pot system. Moreover, $GPO_{S.pne}$ exhibits comparable activity to the previously used commercial $GPO_{S.the}$, because similar synthetic yields were observed.[11–13] However, it is worth noting that $GPO_{S.pne}$ contains an easily dissociable FAD and displayed optimum activity at 30 °C while the commercial one contains FAD as a tightly bound cofactor and is optimally active at 37 °C.

In summary, we successfully expressed $GPO_{S.pne}$ in *E. coli* heterologously and characterized the enzymatic properties of this enzyme. Using the one-pot four-enzyme system we developed before (Scheme 1), a series of ketoses including four rare sugars were synthesized by replacing the commercial $GPO_{S.the}$ with $GPO_{S.pne}$. The scale of the preparative synthesis is about 100 mg in this study and the reaction system could be easily magnified if necessary. It demonstrated that $GPO_{S.pne}$ could serve as a promising alternative to the relatively expensive commercial $GPO_{S.the}$ in large scale synthesis or fermentation. Considering the useful application of GPO in generating the expensive substrate DHAP, we believe this method could contribute to the preparation of more other useful carbohydrates and derivatives.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.12.032.

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