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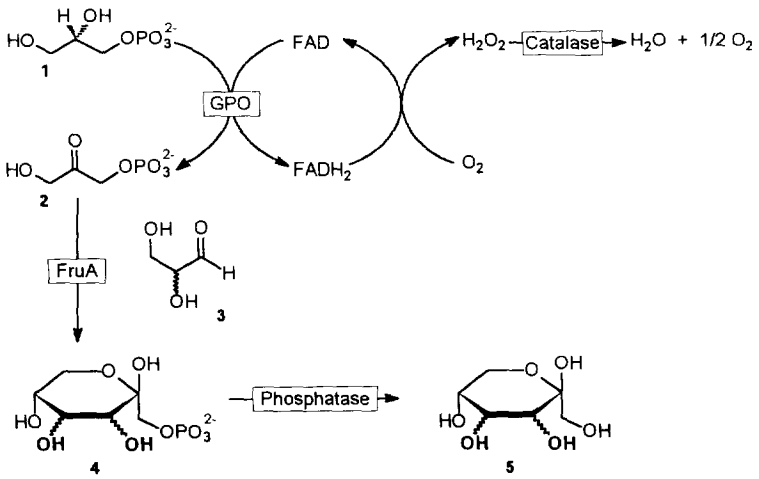
Coimmobilization of L-α-Glycerophosphate Oxidase with Catalase and its Application for the Synthesis of Dihydroxyacetone Phosphate

Leonie Krämer and Eberhard Steckhan*

Kekulé-Institut für Organische Chemie und Biochemie der Universität, Gerhard-Domagk-Str. 1, D-53121 Bonn, Germany

Abstract: The oxidation of L-α-glycerophosphate (**1**) to dihydroxyacetone phosphate (**2**) is carried out enzymatically under aerobic conditions by L-α-glycerophosphate oxidase coimmobilized with catalase, which serves for the decomposition of the generated hydrogen peroxide. **2** is trapped in an aldolase reaction with D/L-glyceraldehyde (**3**). The coimmobilized enzymes show an increased operational stability and can be reused in further transformations without loss of activity. © 1997 Elsevier Science Ltd.

Dihydroxyacetone phosphate (**2**) is the essential donor substrate for the enzymatic aldol reaction catalyzed by D-fructose 1,6-bisphosphate aldolase (FruA). The stereoselectivity of the enzymatic aldol reaction is high, not depending on the configuration of the substrates, since it is exclusively controlled by the enzyme. D-fructose 1,6-bisphosphate aldolase is specific for **2** as the donor but it accepts a wide range of differently substituted acceptor components. This makes aldolases ideal catalysts for the synthesis of carbohydrates and other highly functionalized compounds. Therefore, there is a need for an effective synthesis of dihydroxyacetone phosphate. The enzymatic oxidation of L-α-glycerophosphate(**1**) with the flavoenzyme L-α-glycerophosphate oxidase (GPO) generates dihydroxyacetone phosphate in one step[1].



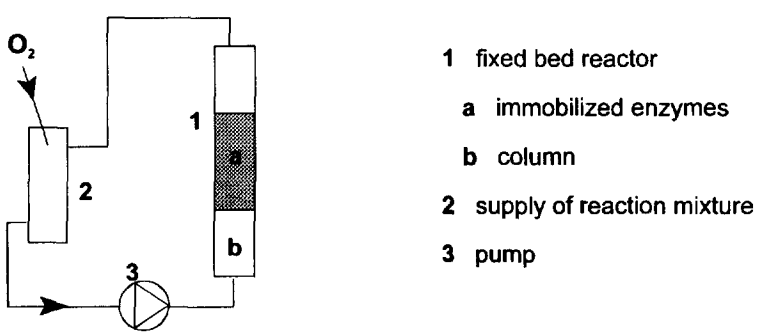
Scheme 1: Principle of the aerobic oxidation of L-α-glycerophosphate by L-α-glycerophosphate oxidase giving **2** which is trapped in an aldolase reaction to form sugar phosphates.

The prosthetic group flavin adenine dinucleotide is the electron carrier of the enzyme, which can be regenerated by oxygen. This leads to the formation of enzyme deactivating hydrogen peroxide which has to

be destroyed by catalase (scheme 1). The produced **2** was trapped in the aldolase reaction with racemic D/L-glyceraldehyde to build D-fructose 1-phosphate and L-sorbose 1-phosphate.

In preliminary studies the L-α-glycerophosphate oxidase showed a considerable loss of activity[2] during the oxidation of **1**. In order to obtain an increased operational stability of GPO and catalase during the reaction, to separate them easily from the reaction medium, and to be able to recover and to reuse the enzymes, they were immobilized to a non-soluble carrier. For this purpose, Eupergit C 250 L® was found to be the best immobilization matrix. Eupergit is an oxirane activated acrylic polymer in the form of beads. A covalent bond is provided between the carrier and the enzymes[3]. Coimmobilization of GPO and catalase could be easily achieved by suspending 100 mg Eupergit, 10 U L-α-glycerophosphate oxidase, and 20 kU catalase in 1.5 ml coupling buffer. The mixture was incubated for 16 h with gentle shaking. After the immobilization procedure the protein concentration in the filtrate was determined by the Bradford method[4]. The filtrate contained only 0.4 % of the protein starting concentration. Consequently, the enzymes are bound almost quantitatively to the matrix. However, the enzymes are partly losing activity in the course of the immobilization. The quantitative determination of the activity[5] of the immobilized enzymes, however, was not possible, since GPO and catalase influence each other during the assay procedure[6], which is based on the quantification of hydrogen peroxide. For that reason we examined the operational stability of the coimmobilized enzymes while studying the achieved turnover rate of **1** during the aerobic oxidation described in scheme 1 in consecutive runs. For this purpose, the enzymes were separated from the reaction medium after the reaction and were applied in the same reaction sequence, again. The turnover of L-α-glycerophosphate in the presence of the native enzymes and of the immobilized enzymes were compared with each other. The comparison of the turnover of L-α-glycerophosphate in the presence of native enzymes and immobilized enzymes permits the interpretation of the immobilization yield.

The enzymatic reactions were performed applying two different techniques: The *fed-batch* method in a stirred tank reactor and a modified fixed bed recirculation reactor (scheme 2). In the latter case, mass transfer is maintained by recirculating the reaction mixture continuously through a packed column containing the immobilized enzymes. Consecutive runs were carried out by simply replacing the substrate solution.



Scheme 2: Fixed bed recirculation reactor.

The aerobic oxidation was carried out in the presence of oxygen with the following starting concentrations: 1.14 mmol L-α-glycerophosphate, 1.5 mmol D/L-glyceraldehyde, coimmobilized GPO and catalase (coupling ratio: 100 U GPO / g carrier, 200 kU catalase / g carrier), 23 U FruA in 20 ml 0.1 M potassium phosphate buffer, pH 6.8. Because of the very low operational stability of rabbit muscle aldolase we had to add another 23 U during the reaction. After a period of 22 h or 48 h the turnover of L-α-glycerophosphate was determined by an enzymatic assay[7]. Then the formed sugar phosphates were dephosphorylated with acid phosphatase. After silylation[8] and gas chromatography the quantitative yield of the free sugars could be calculated.

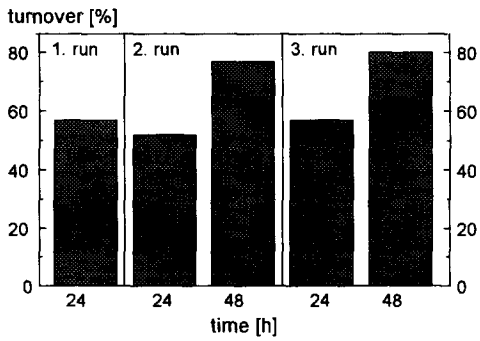
With the native enzymes we could achieve a turnover of L-α-glycerophosphate of 90 % after 20 h. The yield of isolated fructose and sorbose after dephosphorylation and purification by column chromatography was 34 % with respect to the turnover of L-α-glycerophosphate. Applying the native biocatalysts, separation and reuse of the oxidative enzyme is not possible. Therefore a second run was not practicable.

The application of the coimmobilized enzyme in the aerobic reaction sequence gave a turnover of L-α-glycerophosphate as high as with the native enzymes. The *fed-batch* method with the coimmobilized enzymes gave a turnover of L-α-glycerophosphate of 81 % after 24 h in the first run. After separation by filtration a turnover of L-α-glycerophosphate of 83 % could be achieved in a second run. This second reaction sequence yielded 24 % of fructose and 24 % sorbose determined by gas chromatography. Thus the turnover rate of the coimmobilized enzymes in a stirred tank reactor is as high as with the native enzymes since the turnover of L-α-glycerophosphate after 20 to 24 h is nearly identical. Consequently the activity yield of the immobilization must be very high. Moreover, the enzymes maintain their activity during oxidation of L-α-glycerophosphate since the turnover in the second run equals the turnover in the first run.

In the recirculation fixed bed reactor the turnover rate of L-α-glycerophosphate was lower in comparison with the other methods. This indicates that the mass transfer during recirculation is not as high as in a stirred tank reactor. In a first run 57 % of L-α-glycerophosphate were oxidized after 24 h. In a second run, again, after 24 h 52 % of L-α-glycerophosphate were consumed. However, after a prolonged reaction time of 48 h 80 % of L-α-glycerophosphate were oxidized. Then a third run with the same coimmobilized enzyme sample was performed resulting in a turnover of L-α-glycerophosphate of again 57 % after 24 h and a turnover of L-α-glycerophosphate of 80 % after 48 h (scheme 3). These results could be reproduced with another sample of the coimmobilized enzymes resulting in the conversion of 67 % of L-α-glycerophosphate after 24 h and of 91 % after 48 h. In a second run with the same enzyme sample the conversion reached 63 % and 88 % after 24 h respectively 48 h.

Consequently, also for this method the stability of the immobilized enzymes is again very high so that they may be reused without loss of activity. This indicates that the coimmobilization of GPO and catalase offers advantages in comparison with the native enzymes. Applying the recirculation fixed bed reactor the yield of fructose and sorbose after dephosphorylation is much lower than in a stirred tank reactor with coimmobilized or native enzymes. For this reason the operational stability of the aldolase seems to be lower

while circulating than in stirred solution. This conclusion was proved by utilizing both rabbit muscle aldolase and aldolase from bacterial sources.



Scheme 3: Turnover of L-α-glycerophosphate (**1**) in three consecutive oxidations in the recirculation fixed bed reactor after 24 h and 48 h of the respective run.

In conclusion, we have demonstrated the possibility of immobilizing L-α-glycerophosphate oxidase and catalase together to Eupergit® C 250 L. The coimmobilized enzymes were successfully applied for the oxidation of L-α-glycerophosphate with a turnover up to 83 %. This means that the immobilization yield was very high. After the reaction, the enzymes could easily be recovered without loss of activity. They maintained their activity also during consecutive runs. All these results indicate the advantages of the coimmobilization as a convincing method to increase the operational stability of L-α-glycerophosphate oxidase and catalase.

EXPERIMENTAL

General procedure for the comimmobilization of L-α-glycerophosphate oxidase and catalase:
Typically 100 mg of Eupergit® C 250 L (Fluka) are suspended in 1.5 ml of coupling buffer (1.0 M potassium phosphate of pH 8.0) in an Erlenmeyer flask. The height of the solution should not exceed 10 mm. Then, 0.01 mmol (50 µl of a 0.2 M solution) of glycerol-3-phosphate, 10 U of L-α-glycerophosphate oxidase (EC 1.1.3.21 aus *Pediococcus spec.*, Merck) dissolved in doubly distilled water (up to 5 mg/ml) and 20 kU catalase (40 µl suspension in doubly distilled water; EC 1.11.1.6 from bovine liver, Merck or Boehringer Mannheim) are slowly added. The mixture is incubated for 16 h with gentle shaking. After the immobilization procedure the beads are separated by a glas frit and washed for several times using a 0.1 M potassium phosphate solution of pH 7.0. The coimmobilized enzyme material is stored in a defined volume of 0.1 M potassium phosphate buffer of pH 7.0 at 4° C. The protein concentration in the filtrate is determined by the Bradford method[5]. For this purpose, the support material is separated by 20 min centrifugation. The sediment is washed three times and then again centrifugated. The separated solution is transferred into a Centricon ultrafiltration cartridge (Centricon-30, molecular weight cutoff 30000D) and centrifugated at 3000 Upm. Thus, the protein is retained by the ultrafiltration membrane within in the reservoir. The protein content of the thus obtained probes is quantitatively determined by using a calibration curve obtained with BSA standard solutions.

General procedure for the enzymatic oxidation with coimmobilized GPO/catalase in a stirred tank reactor (ultrafiltration cell) using the *fed-batch* method

The aerobic oxidation is carried out in an oxygen saturated buffer solution in a 50 ml ultrafiltration cell (Amicon, Series 8000, diameter 43 mm) equipped with an ultrafiltration membrane (Amicon, Diaflo YM 5, diameter 43 mm, molecular weight cutoff 5000 D). L-α-Glycerophosphate (1.14 mmol, 870 mg) and glyceraldehyde (1.50 mmol, 100 mg) are dissolved in 10 ml of 0.1 M phosphate buffer of pH 6.8. After adjusting the pH, the solution is saturated with oxygen. Then, the beads with the coimmobilized enzymes (30 - 40 U of GPO; coupling ratio: 100 U GPO / g carrier, 200 kU catalase / g carrier) and 23 U of D-fructose-1,6-bisphosphate aldolase (EC 4.1.2.13 from rabbit muscle, Boehringer Mannheim) are added together with 10 ml of the buffer solution. The conversion is followed by the enzyme assay described below. After 20 h, the reaction solution is pumped off under pressure. The retained enzymes are washed with buffer solution and then the cell is filled again with reaction mixture as before. The conversion of L-α-glycerophosphate is followed by an enzyme assay[7] based on the formation of NADH in the oxidation of L-α-glycerophosphate to dihydroxyacetone phosphate catalyzed by L-α-glycerophosphate dehydrogenase (EC 1.1.1.8 from rabbit muscle, Boehringer Mannheim). For this purpose, 10 µl probes of the reaction mixtures are quenched with 20 µl perchloric acid. The quantification of the sugar phosphate products fructose-1-phosphate and sorbose-1-phosphate is performed after dephosphorylation. Thus, the reaction mixture is heated to 75° C for 5 min to destroy the enzymes and subsequently filtrated. The pH of the solution is adjusted to pH 7.0, 4 equivalents of barium chloride with respect to the starting concentration of L-α-glycerophosphate are added, and the mixture is treated with 200 ml of acetone. At 4° C, the barium salts of the sugar phosphates are precipitating. The precipitate is suspended in water and then dissolved by using an acidic ion exchanger (DOWEX 50W-X8). The ion exchange resin is separated by filtration washed with water and the filtrate adjusted to pH 4-5 by using NaOH solution. This mixture is treated with 30 U of acidic phosphatase (EC 3.1.3.2 from potatoes, Boehringer Mannheim) and incubated over night at room temperature. Then, the mixture is filtrated over celite followed by lyophylization. After dissolving the residue in methanol, it is again filtrated over celite, and the solvent evaporated at reduced pressure. Subsequently, the quantification of the sugars is performed after silylation according to a literature procedure[8] by GC using a HP-1 column (Hewlett-Packard, 12 m, 0.25 mm inner diameter, 0.33 µm film thickness; carrier gas: nitrogen; starting temperature 120° C increased to 173° C at a rate of 10° C/min, after 4 min the temperature is further increased to 280° C at a rate of 60° C/min) and a Hewlett-Packard gas chromatograph (HP 5890, Series II). For the quantification, n-tetradecane is used as external standard.

General procedure for the enzymatic oxidation with coimmobilized GPO/catalase in a fixed bed recirculation reactor:

The reactor is designed as shown in Scheme 2. As fixed bed reactor a chromatography column (Pharmacia, diameter 3 cm) is applied filled with the Eupergit® beads at which the enzymes are coimmobilized. The inlet of the solution is at the bottom of the column. The top of the column is connected via Teflon® tubing to a reservoir formed by an acrylic glas block of 14 cm height into which a whole of 1 cm diameter is drilled. The oxygen inlet is connected to this reservoir. The reaction solution is circulated through the system by using a HPLC pump (Pharmacia, type 2150). The Eupergit® beads are slightly fluidized during the circulation process. All connections are made by Teflon® tubing and Latek connectors.

The volume of the whole reactor system is 20 ml. To fill the column, with the enzyme modified particles are suspended in the buffer solution and introduced into the column. Then, the pistons of the column are inserted in such a way that above the bed of the particles 2 cm of the solution is remaining. Then, the buffer solution is pumped through the system at a high rate to remove air bubbles. The substrates dissolved in 10 ml of buffer solution are introduced at the top of the reservoir and the aldolase is added. The reaction mixture is pumped at a flow rate of 0.5 ml/min. For the control of the turnover, samples can be taken from the top of the reservoir. The conversion is followed by the enzyme assay described above. At the end of the reaction, the solution is pumped off, the beads in the column are washed with buffer solution and the reactor is refilled with the reaction mixture. The analysis is performed as described before.

General procedure for the enzymatic oxidation using native GPO and catalase:

For comparison with the immobilized enzymes, enzymatic oxidations under aerobic conditions are performed in an Erlenmeyer flask of 20 ml volume using the native enzymes. Thus, L-α-glycerophosphate (1.14 mmol, 870 mg) and glyceraldehyde (1.50 mmol, 100 mg) are dissolved in 20 ml of 0.1 M phosphate buffer of pH 6.8. After adjusting the pH, the solution is saturated with oxygen. Then, 30 - 40 U of native GPO, 8 kU of native catalase and 23 U of D-fructose-1,6-bisphosphate aldolase are added. The flask is slowly shaken during the reaction. The conversion is followed by the enzyme assay described before. The work-up and analysis is performed as described for the immobilized enzymes.

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