Enzymatic cascade reactions involving phosphorylated intermediates: immobilization and process optimization
Babich, L.
A continuous flow reactor for the enzymatic synthesis of phosphorylated compounds

Part of this work has been published in:
L. Babich, A. F. Hartog, M. A. van der Horst, R. Wever
“Continuous-Flow Reactor-Based Enzymatic Synthesis of Phosphorylated Compounds on a Large Scale”
*Chemistry – A European Journal, 2012, 18, 6604-6609*
Chapter 3

Abstract

Acid phosphatase, an enzyme that is able to catalyze the regiospecific transfer of a phosphate group from cheap pyrophosphate to alcoholic substrates, was covalently immobilized on polymethacrylate beads with an epoxylinker (Immobeads-150 or Sepabeads EC-EP). After immobilization 70 % of the activity was retained and the immobilized enzyme was stable for many months when stored at 4 °C. With the immobilized enzyme we were able to produce and prepare D-glucose-6-phosphate, N-acetyl-D-glucosamine-6-phosphate, allylphosphate, glycerol-1-phosphate, inosine-5’-monophosphate, and N-acetyl-D-galactosamine-6-phosphate from the corresponding primary alcohol on gram scale using either a fed batch reactor or a continuous-flow packed-bed reactor. Other compounds, like dihydroxyacetone and glyceraldehyde, were phosphorylated to a good extent but were not isolated given their instability. Tripolyphosphate (PPP) was also successfully used as a phosphate donor with D-glucose and N-acetyl-D-glucosamine as substrates, reducing the amount of free phosphate produced. With PPP and N-acetyl-D-galactosamine as a substrate a higher conversion was also reached, because of the higher phosphate transferring capacity of the donor.

Introduction

The preparation of phosphate esters has always been a difficult task for chemists.[1] Phosphorylation is a key reaction not only in the chemistry of natural processes but also in organic synthesis. Nature assigns such remarkable role to phosphate esters based on their outstanding chemical stability combined with their facile manipulation by enzymes.[2] Due to their importance in living systems, phosphorylated intermediates are often used as prodrugs to improve drug delivery, are used as nutritional supplements or taste enhancer in the food industry, and as cosmetic ingredients in moisturizers, detergents, and new polymers.[3] Given the importance of phosphate esters, many phosphorylation methods – both chemical and biochemical – have been developed. In chemical methods usually harsh chemicals are used that often modify more functional groups on the molecule, thus requiring the use of protecting group chemistry.[4] In contrast, phosphorylating enzymes may be stereo- and regio-selective making the phosphorylation of polyhydroxy compounds in principle much more viable. In addition enzymes operate under mild conditions and in general produce less waste. In biological systems, kinases are involved in phosphorylation of a variety of compounds using ATP as a phosphate donor.
The disadvantage of using kinases in organic synthesis lies in their strict substrate acceptance and the need to regenerate the expensive ATP.\[5\] Hydrolytic enzymes, like phosphatases, can also perform phosphorylations. As demonstrated by Pradines, alkaline phosphatase is able to phosphorylate glycerol by both inorganic phosphate (P_i) and pyrophosphate (PP_i), with PP_i being the best donor.\[6,7\] Furthermore bacterial non specific acid phosphatases (NSAPs, EC 3.1.3.2) such as acid phosphatase from Shigella flexneri (PhoN-Sf) and from Shigella enterica (PhoN-Se) catalyze the regioselective monophosphorylation with PP_i of a wide range of compounds (Fig. 1) such as inosine, hexoses and pentoses, but also simple alcohols such as dihydroxyacetone, polyols and aromatic alcohols.\[8,9\]

![Phosphorylation reaction](image)

Fig. 1: Phosphorylation reaction carried out by acid phosphatase.

This phosphorylation reaction is in fact a transphosphorylation in which a phosphate group from a phosphate ester, such as PP_i, is transferred to the alcohol group. This transphosphorylation reaction has been studied in some detail for the acid phosphatase from Shigella flexneri (PhoN-Sf). It consists of a reversible two-step mechanism in which the affinity for PP_i, alcohol, or water determines whether hydrolysis, transphosphorylation of dephosphorylation occur.\[9,10\] A similar mechanism was reported by Asano and co-workers for acid phosphatase from Morganella morganii, for which a regioselective PP_i-nucleoside phosphotransferase mechanism has been proposed.\[11\] PhoN-Sf has a very broad substrate acceptance range, is regiospecific (phosphorylates only primary alcohols), is very stable under turnover, and has a broad pH optimum, which allows its use also at more alkaline pH values.\[8\] For these reasons PhoN-Sf has been also employed in multi-enzyme cascade reactions where phosphorylated intermediated are required.\[12-15\]

Despite the advantages of using enzymes in organic synthesis, costs, stability, and recycling of biocatalyst has always been an issue. Immobilization on solid supports is often performed to enable the recycling of a catalyst and improve its stability.\[16\] Moreover it allows for continuous processes and easy separation from the products, thus opening the way to industrial large-scale applications.\[17\] In fact in the last decade continuous reactors have progressively substituted batch syntheses, since they help to save time and money in the most challenging “post reaction” operations and allow automation, multi-steps syntheses, and scaling-up.\[18\]
We investigated the covalent immobilization of PhoN-Sf on two commercially available polymethacrylate porous beads, namely Immobeads-150 (Sigma) and Sepabeads EC-EP (Resindion). The beads are functionalized with epoxy-linkers, which react mainly with the amino groups of lysines present on the surface of the enzyme (Fig. 2). The covalent linkage of the enzyme to the beads has the additional advantage that it is possible to physically separate the phosphorylated products from the enzyme and to prevent the hydrolysis reaction. The immobilized acid phosphatase was then used either in a fed-batch reactor or in a packed-bed continuous reactor to phosphorylate a variety of primary alcohols in presence of PP. We investigated the conversion of D-glucose to D-glucose-6-phosphate, N-acetyl-D-glucosamine to N-acetyl-D-glucosamine-6-phosphate, dihydroxyacetone to dihydroxyacetone phosphate, glycerol to rac-glycerol-1-phosphate, allyl alcohol to allylphosphate, inosine to inosine-5'-monophosphate, glyceraldehyde to glyceraldehyde-3-phosphate, and N-acetyl-D-galactosamine to N-acetyl-D-galactosamine-6-phosphate using a continuous flow reactor with the immobilized enzyme. At last, the reaction conditions in flow-mode were optimized by using tripolyphosphate (PPP). This compound has a higher phosphate capacity than PP, but requires longer incubation times.

Materials & Methods

Immobilization of PhoN-Sf

Acid phosphatase from Shigella flexneri (PhoN-Sf) was expressed in E. coli as described elsewhere. After protein expression cells were disrupted by osmotic shock and centrifuged. The acid phosphatase was partially purified from the cell lysate by binding onto an ion exchange column (DEAE) and eluted with NaCl (1 M). The partly purified acid phosphatase was dialysed and concentrated for further use. All reagent used were of laboratory grade and purchased from Sigma-Aldrich. Free and immobilized enzyme activities were assayed spectrophotometrically by measuring the dephosphorylation of p-nitrophenyl phosphate (pNPP). 10 µL of soluble phosphatase (or 1 µL of immobilized phosphatase) was added to 490 µL of 10 mM pNPP, 100 mM acetate, pH 6 and
mixed for 1 minute. After incubation, the reaction was quenched with 500 µL of 1 M NaOH and the absorbance of p-nitrophenol was measured at 410 nm (ε = 16600 M⁻¹ cm⁻¹) using a Cary 50 UV spectrophotometer. PhoN-Sf was immobilized on Immobeads-150 (Sigma) or Sepabeads EC-EP (Resindion). The particle size for Immobeads and Sepabeads is 100-300 µm and 150-300 µm, respectively. Both carriers are methacrylate polymers with an average pore size of 10-20 nm and have epoxy functions to link the amino group of lysines present on the surface of the protein. 9 of the 10 lysines present in PhoN-Sf are exposed to the surface, thus available for binding. 10 mg of PhoN-Sf (300 U) were immobilized on 300 mg Immobeads-150 or 500 mg of Sepabeads EC-EP (containing 50 % water) in 15 mL of 1.25 M potassium phosphate, pH 8. The solution was shaken for 24 hours at 20 ºC. After 24 hours the beads were washed twice with 15 mL water and incubated with 2 volumes of 2 M glycine, pH 8.5 under stirring for 24 hours at 20 ºC to end-cap the unreacted epoxy groups of the beads. The beads were then washed twice with 4 mL water and twice with 0.1 M potassium phosphate pH 7 to remove glycine and stored at 4 ºC. The activity on the beads was tested by pNPP assay using 1 µL of settled beads. The activity and stability of the beads during turnover were evaluated in fed-batch incubations of 1 mL containing 400 mM glucose, 100 mM PPᵢ, 25 µL of settled Immobeads or Sepabeads at pH 4 and 5. After 24 hours the supernatant was removed and fresh reaction medium was added to the beads. The concentration of the remaining glucose and PPᵢ and Pᵢ formed was analyzed by HPLC using an Alltech OA 1000 organic acid column (0.65 x 30 cm) after 1 : 9 dilution in water. The column was eluted with 25 mM H₂SO₄ at a flow rate of 0.4 mL/min. The HPLC effluent was monitored at 210, 215, 275 and 320 nm and by a refractive index detector. The formation of D-glucose-6-phosphate was determined spectrophotometrically with a coupled enzymatic assay as described elsewhere.[⁹]

**Continuous-flow phosphorylation reaction**

In the packed-bed reactor experiment, an HPLC column 3 cm in length, 0.46 cm in diameter, and packed with 0.5 mL of the immobilized enzyme beads, was connected to a pump and washed with 0.1 M potassium phosphate at pH 7 (Fig. 4). The feed solution containing variable concentrations of the alcohol and 250 mM PPᵢ at different pH values was pumped at a flow rate of 0.03 mL/min to 0.05 mL/min. The outlet solution was collected into a flask or in tubes in a fraction collector. This continuous system was used to phosphorylate glucose, N-acetyl-D-glucosamine, allyl alcohol, glycerol, dihydroxyacetone, inosine, glyceraldehyde, and N-acetyl-D-galactosamine with PPᵢ as a phosphate donor. Optimal conversions were reached with a flow of 0.03 mL/min. Samples were
analyzed by HPLC as described above. Inosine and 5’-IMP were determined after 1 : 9 dilution with a Agilent 1100 HPLC using a Nucleosil 100-5 C18 column (0.45 × 15 cm; Macherey-Nagel). The column was eluted with 10 mM CH₃COOH-NH₂OH pH 5 at a flow rate of 1 mL/min. The HPLC effluent was monitored at 254 nm. Dihydroxyacetone phosphate was assayed by the enzyme system as described by Bergmeyer.[19] Glycerol-1-phosphate was determined by a coupled-enzymatic assay in which it is oxidized to dihydroxyacetone phosphate by glycerol-1-phosphate dehydrogenase with the concomitant reduction of NAD⁺ to NADH. An appropriate dilution of glycerol-1-phosphate was added to a solution containing 450 mM glycine, 274 mM hydrazine sulfate, 2.4 mM ethylenediaminetetraacetic acid, 2.5 mM NAD⁺ and 20 U/mL of α-glycerol-1-phosphate dehydrogenase. The reaction was incubated for 5 minutes at 20 °C and the absorbance recorded at 340 nm (ε = 6220 M⁻¹ cm⁻¹). Glucose-6-phosphate, N-acetyl-D-glucosamine, allylphosphate, and glycerol-1-phosphate were isolated as Ba²⁺-salt according to method previously published.[8] The mixtures containing PP₆, P₅, alcohol, and phosphorylated alcohol were stirred at room temperature with 5 % w/v barium acetate at pH 9 to remove free phosphate. After 2 hours the solution was filtered, the solid salt of barium phosphate was discarded, whereas the filtrate containing alcohol, phosphorylated alcohol, and acetate was concentrated, precipitated with 4 volumes of cold ethanol at pH 9, and kept at 4 °C. After one day, the solution was filtered and the barium salt of phosphorylated alcohol was dried and characterized. 5’-IMP and N-acetyl-D-galactosamine-6-phosphate were isolated by ion-exchange chromatography on DEAE-Sephadex A25. After the phosphorylation reaction, the pH of the solution containing inosine, 5’-IMP, and phosphate was set to 9. At this pH part of the phosphate crystallized and was easily removed from the solution. The phosphate contained in the solution of N-acetyl-D-galactosamine and N-acetyl-D-galactosamine-6-phosphate was removed by precipitation with an equimolar amount of Ba(OH)₂ at pH 8. Subsequently, 50 g of DEAE-Sephadex A25 were added to the solutions and stirred. The resin was loaded onto a column and the phosphorylated alcohols eluted with a gradient of NH₄HCO₃ pH 8.1 (75 mM, 150 mM and 250 mM, respectively). The fractions containing the phosphorylated alcohols were collected and water and buffer were removed by freeze-drying. 0.5 g 5’-IMP was obtained (50 to 60 % isolated yield) which was pure according to HPLC and UV-Vis. From 140 mL of solution containing 10 mM N-acetyl-D-galactosamine-6-phosphate, 95 mg of pure N-acetyl-D-galactosamine-6-phosphate were obtained corresponding to 20 % isolated yield, but a large amount of NH₄HCO₃ was still present.
Tripolyphosphate as phosphate donor

The use of tripolyphosphate (PPP₃) was first checked in batch reactions with immobilized PhoN-Sf. Reaction mixtures were prepared with 400 mM D-glucose, 5 % v/v PhoN-Sf Immobeads, and different concentrations of PPP₁ (250 and 400 mM) at pH 4.2 and incubated at room temperature. The amount of D-glucose consumed and free phosphate formed was determined by HPLC analysis, whereas D-glucose-6-phosphate was determined by the spectrophotometric assay already described. PPP₁ was also used as a phosphate donor with glucose and N-acetyl-D-glucosamine as acceptor alcohols at pH 4.2. With 300 mM glucose, 100 and 200 mM PPP₁ were used at different flow rates. With N-acetyl-D-glucosamine, PPP₁ concentration was varied from 65 to 130 mM and the flow from 0.01 to 0.03 mL/min in order to maximize the conversion. Also 100 mM N-acetyl-D-galactosamine was used with 200 mM PPP₁ at pH 4.2 in the flow reactor.

Results & Discussion

Immobilization of PhoN-Sf

For the covalent immobilization of PhoN-Sf two types of beads were used: Immobeads-150 and Sepabeads EC-EP. By measuring the activity of the non-bound enzyme present in the supernatant it was possible to monitor the immobilization process on each support. A decrease in activity in the supernatant indicates binding of the enzyme to the epoxy-groups on the beads or inactivation of the enzyme in solution. Therefore as a control acid phosphatase was incubated in buffer used in the immobilization procedure without beads. As shown in Fig. 3 no activity was present in the supernatants of both incubations with Immobeads and Sepabeads after 24 h, while the control shows that PhoN-Sf was stable under the conditions of the immobilization. The immobilization on Immobeads was complete after 5 hours whereas that on Sepabeads required longer time. After the end-capping of the unreacted epoxy groups by glycine, both types of beads showed phosphatase activity and in both cases the bound activity was 70 % of the initial total activity. Immobeads and Sepabeads showed specific activities of 333 U/g and 400 U/g, respectively. Both carriers performed very well but the immobilization was faster with Immobeads and therefore they were used in further experiments. Since upon incubation of the phosphatase in the immobilization buffer only 5 % of the initial activity was lost, the further loss of activity during the immobilization may be due to reaction of the essential lysine residue in the active site,[20] loss of flexibility, or reduced diffusion of substrates. For storage the buffer strength was decreased to
0.1 M and the pH to 7. In this buffer and storage at 4°C the beads retained 90 % of their initial activity for at least a period of one year.

![Graph](image)

**Fig. 3:** Time-course of binding of PhoN-Sf measured as decrease in phosphatase activity in the supernatant. PhoN-Sf was incubated with Immobeads-150 or Sepabeads EC-EP in 1.25 M potassium phosphate buffer, pH 8. The control consists of PhoN-Sf incubated in 1.25 M potassium phosphate, pH 8 without beads.

A fed-batch reaction was carried out in order to evaluate the phosphorylating activity of the beads and the possibility to reuse them. Reusability is one of the key parameters in enzyme immobilization techniques related to biocatalyst applications. The activity and reusability of the immobilized PhoN-Sf was determined by measuring the phosphorylation of D-glucose to D-glucose-6-phosphate (D-G6P) using PPi as a phosphate donor.\(^8\) Using the soluble enzyme and 400 mM of glucose a maximal concentration of 80 mM of D-G6P was reached after 300 min at pH 4 and 100 mM PPi.\(^9\) Once formed, D-G6P is hardly dephosphorylated even when all PPi is consumed. Therefore the incubation time to reach the maximal conversion is not critical. This is in contrast to other primary alcohols, which are rapidly dephosphorylated by PhoN-Sf when PPi is exhausted.\(^21\)

The fed-batch reaction was carried out with PhoN-Sf beads incubated with a 400 mM glucose and 100 mM PPi at pH 4 and 5. After 24 hours, the beads were separated from the supernatant and fresh reaction medium added. This was repeated 3 times. Table 1 shows the concentrations of D-G6P obtained after 4 cycles at two different pH values. The maximum concentration of phosphorylated sugar was obtained at pH 4 (80 mM), whereas at pH 5 the maximum concentration was only 29 mM. In both cases, the conversions are comparable to the data reported for the soluble PhoN-Sf.\(^8\) The table also shows that after 4 cycles only a slight reduction of the conversion in time occurs and the beads can be reused for at least 4 days.
Table 1: Concentration of D-glucose-6-phosphate at pH 4 and 5 in a fed-batch reaction over 4 cycles. Reaction mixtures contain 400 mM glucose, 100 mM PP_i, and 25 μL of PhoN-Sf Immobeads in 1 mL of solution (1 U/mL).

Continuous-flow phosphorylation reaction

One of the advantages of immobilized enzymes is their use in continuous-flow packed-bed reactors and even with small reactor volumes large amounts of product can be obtained.\(^{[18]}\) Furthermore, in our reaction the physical separation of the phosphatase and the phosphorylated product prevents the hydrolysis of the product and this increases the overall yield. We decided to use a packed-bed reactor in which the beads were packed in a small empty HPLC column of 0.5 mL connected to a pump (Fig. 4).

![Diagram of continuous-flow system](image)

**Fig. 4:** Scheme of the continuous-flow system with immobilized PhoN-Sf for the phosphorylation of primary alcohols using PP_i as a cheap phosphate donor.

Since the PhoN-Sf will also dephosphorylate (hydrolyse) the phosphorylated product, the residence time of the substrate molecules inside the reactor is important and this depends on the flow. The optimal residence time in the phosphorylation reaction mediated by PhoN-Sf and driven by pyrophosphate is one in which the phosphorylation dominates hydrolysis and this is determined by the concentration of PP_i still present and thus the flow rate. Thus one way to optimize the system is to modify the flow and to have a high concentration of PP_i present. To compare the productivity of the continuous-flow reactor to the fed-batch reactor, an experiment was performed with 400 mM glucose and 100 mM PP_i at pH 4, the optimal pH. At this lower concentration of PP_i the optimal flow was 0.05 mL/min and the maximum concentration of D-G6P reached was 70 mM. Since in fed-batch mode the same amount of product was obtained, the reactor is equally efficient with the additional advantage of a continuous production. The conversion as shown in Fig. 5 is stable for 2 days with 100 mM PP_i. After two days the concentration of PP_i was increased to 250 mM and the
flow lowered to 0.03 mL/min. The concentration of D-G6P increases to 130-150 mM and stays stable for 12 days. After 12 days a solution of 800 mL was collected containing 130 mM of D-G6P from which 32 grams of D-G6P as barium salt was isolated, corresponding to an isolated yield of 78%.

![Graph](image)

Fig. 5: Time course of D-glucose (400 mM) phosphorylation with PhoN-Sf Immobeads in a packed-bed continuous flow reactor at pH 4 with 100 mM PP, at flow rate 0.05 mL/min (day 1-2) and 250 mM PP, at 0.03 mL/min (day 3-12).

Given the structural similarity to D-glucose and the commercial value of N-acetyl-D-glucosamine-6-phosphate (NAG-P), N-acetyl-D-glucosamine (NAG) was chosen as a substrate for phosphorylation.\[^{22}\] NAG-P, is a high valuable compound which is used in cosmetic industry as additive for face and skin lotions. The enzymatic phosphorylation of NAG by polyphosphate-glucose phosphotransferase has been previously reported and patented.\[^{3}\] Since the phosphorylation of NAG by PhoN-Sf and PP, has not been previously studied, the time course of the phosphorylation by soluble PhoN-Sf at 3 different pH values was measured (Fig. 6). Because of its price 100 mM of NAG was used instead of 400 mM. As shown in Fig. 6, at pH 5 the reaction was quite slow and gave only 40% conversion. At pH 4.5 the reaction was faster and a good conversion (70%) was obtained. At pH 4 the reaction was very fast but after 5 hours dephosphorylation was observed. In this respect this substrate shows similarities with glucose in phosphorylation and dephosphorylation. The phosphorylation of glucose is fast at pH 4 but at this pH dephosphorylation occurs. At pH 4.5 the reaction slows down and dephosphorylation is not seen. Apparently phosphorylation of hexoses on position 6 is a very efficient reaction with almost quantitative conversions. This is probably due to the high affinity of PhoN-Sf for hexoses.\[^{21}\] Since at pH 4 the conversion is maximal but dephosphorylation occurs, further experiments were carried out at pH 4.2.
A flow reactor for the synthesis of phosphorylated compounds

![Graph](image)

**Fig. 6:** Time course of NAG phosphorylation by PhoN-Sf at three different pH values. Reaction mixtures contain 100 mM NAG, 250 mM PP<sub>i</sub>, 0.8 μM (ca. 1 U/mL) soluble PhoN-Sf, free enzyme, at pH 4, 4.5, and 5. The concentration of PP<sub>i</sub>, P<sub>i</sub>, NAG, and NAG-P<sub>i</sub> were determined by HPLC.

It was also possible to phosphorylate NAG using the continuous-flow reactor. At pH 6 only 25 % of the initial 100 mM NAG was converted into NAG-P<sub>i</sub> using two directly coupled 0.5 mL columns filled with PhoN-Sf beads and a flow of 0.03 mL/min. Much higher conversions were obtained at pH 5 and 4.8 (55 and 60 % conversion, respectively). At pH 4.2 the phosphorylation was so efficient that only one 0.5 mL column was sufficient to reach a conversion of 80 %. The beads were active and stable for over 16 days (data not shown). From 375 mL of a mixture of 60-65 mM NAG, 10.3 g of NAG-P<sub>i</sub> were isolated as a barium salt with an isolated yield of 82 %.

N-acetyl-D-galactosamine, another acetylated aminosugar, was studied. With 100 mM sugar, 250 mM PP<sub>i</sub>, pH 4.2, and a flow rate of 0.03 mL/min only 10 mM of phosphorylated sugar was detected. This is in accordance with the different affinity of PhoN-Sf between the non-acetylated counterparts, D-glucose and D-galactose. Previous studies on the substrate specificity of PhoN-Sf, reported a maximum phosphorylation of 80.4 mM of D-glucose, which dropped to only 30.4 mM for its isomer D-galactose,<sup>[8]</sup> suggesting that the lower affinity for the D-galactose is due to the sterical hindrance given by the C4 hydroxyl group in axial configuration. However, from the 140 mL of effluent, still 95 mg of N-acetyl-D-galactosamine-6-phosphate were isolated by column chromatography.

Allyl alcohol, a simple small primary alcohol, was also tested as substrate for PhoN-Sf immobilized on Immobeads packed in a column in a continuous flow system. Allylphosphate can be an interesting building block for further synthetic use in homogeneous catalysis. Phosphate may act as a good leaving group in palladium catalyzed C-allylation reactions similarly to allyl acetate.<sup>[23]</sup> Table 2 shows the concentration of allylphosphate reached with different concentrations
of allyl alcohol, with 250 mM PP, flow rate of 0.03 mL/min, at pH 4. At 400 mM allyl alcohol the effluent contained a concentration of 105 mM of allylphosphate. Interestingly, the amount of product formed increased considerably with higher substrate concentrations, but the conversion based on the alcohol was essentially constant. Thus, for synthetic purposes high substrate concentrations are required in order to obtain the maximum amount of phosphorylated alcohol possible. From a solution of 210 mL containing 89 mM allylphosphate, 4.6 g of allylphosphate were isolated as barium salt, corresponding to 90 % isolated yield.

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<th>Substrate</th>
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<th>Product (mM)</th>
<th>Conversion (%)</th>
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Table 2: Maximal concentration of allylphosphate and glycerol-1-phosphate obtained with different substrate concentrations in the flow reactor. Inlet solutions contain various concentrations of alcohol, 250 mM PP, pH 4 and a flow rate of 0.03 mL/min.

Glycerol is also phosphorylated by PhoN-Sf forming a racemic mixture of D- and L-glycerol-1-phosphate. A mixture of 100 mM glycerol and 100 mM PP, at pH 5.3 yielded 9 mM of glycerol-1-phosphate. With our continuous-flow system at a flow of 0.03 mL/min containing 500 mM glycerol, 250 mM PP, at pH 4 a concentration of 110 mM glycerol-1-phosphate was reached. As shown in Table 2 and similarly to allyl alcohol, the concentration of product formed and concentration of substrate used are closely related but the yield is more or less constant. Only after 14 day a slight decrease in activity (10 %) occurred. Continuous glycerol phosphorylation using a batch membrane reactor to phosphorylate glycerol by glycerol kinase coupled to an ATP regenerating system with acetate was previously reported. However, in this set-up only a few mM of glycerol phosphate was produced. Considerably higher yields were obtained by Pradine with a embedded batch reactor containing alkaline phosphatase immobilized on corn grits. Alkaline phosphatase is even able to phosphorylate glycerol using phosphate as donor at high pH. 168 mM glycerol-1-phosphate was synthesized using very high concentrations of glycerol (80 %
v/v) and P$_1$ (0.4 M) at pH 9. Such high concentrations of glycerol and phosphate do not allow a straightforward recovery of the product. We obtained 110 mM glycerol-1-phosphate from much lower concentrations of glycerol and this simplifies the isolation. From 530 mL of 83 mM glycerol-1-phosphate, 10 g of compound were isolated as barium salt, corresponding to 74 % isolated yield. The nucleotide inosine-5′-monophosphate (5′-IMP) is used as flavor enhancer (umami E630) in various foods.[28] This and related nucleotides are produced at a very large scale by phosphorylation of nucleosides by an acid phosphatase using PP$_i$ as a phosphate donor.[10,26] Although other methods exist using kinases, [27] they cannot compete with the above process. The regioselective phosphorylation of inosine by PhoN-Sf and PhoN-Se, a phosphatase from *Salmonella enterica* with high similarity to PhoN-Sf, was also studied.[9] PhoN-Sf phosphorylates inosine to 5′-IMP, but PhoN-Se phosphorylates also the 3′ position to yield inosine-3′-monophosphate (3′-IMP). In presence of 40 mM inosine, a maximal concentration of 7 mM of 5′-IMP was reached at pH 5 by PhoN-Sf.[6] With our packed-bed reactor 14 mM 5′-IMP was synthesized starting from a saturated inosine solution (100 mM), 250 mM PP$_i$, pH 5, and a flow rate of 0.03 mL/min. When the pH was lowered to 4, the conversion dropped to 9 mM and was re-established to 15 when the flow was lowered to 0.02 mL/min. This confirms the literature data claiming that the inosine phosphorylation by PhoN-Sf is slower at pH 4 compared to pH 5.[9] After 5 days, a solution of 200 mL containing 10 mM 5′-IMP, 90 mM inosine, and approximately 500 mM phosphate was collected and 5′-IMP was isolated by ion-exchange chromatography resulting in a fraction containing 0.5 g of pure 5′-IMP. Dihydroxyacetone phosphate (DHAP) is a very valuable compound that is the donor in aldol additions catalyzed by DHAP-dependent aldolases. DHAP chemical synthesis is rather complicated, but there are also enzymatic methods. [24,28] As shown by our group one of the simplest methods is the direct phosphorylation of dihydroxyacetone (DHA) using PhoN-Sf and PP$_i$.[12,13] DHAP has been used in cascade reactions in which DHA is phosphorylated by PhoN-Sf and in situ coupled to different aldehydes by an aldolase to yield a phosphorylated sugar which is readily dephosphorylated by PhoN-Sf. The soluble enzyme can phosphorylate up to 5.5 mM DHA starting from 100 mM DHA and 100 mM PP$_i$ at pH 4. In contrast to D-G6P under optimal conditions, DHAP is quickly dephosphorylated, thus the incubation time in a batch reaction and the residence time/flow in a continuous system need to be carefully controlled in order to achieve maximal yield. In the continuous flow system, 12 mM of DHAP was obtained from 100 DHA and 250 mM PP$_i$. When the concentration of DHA was increased to 500 mM, the conversion in the outlet jumped to 50 mM and remained constant for 14 days. However, unfortunately
DHAP could not be isolated as barium salt from the mixture because of its instability at high pH, which causes rapid chemical degradation to methylglyoxal. An option would be to use ion exchange chromatography to isolate DHAP. It was also tested whether glyceraldehyde could be phosphorylated using the flow reactor. However, at a concentration of 100 mM and 250 mM PP, at pH 4, only 5 mM of glyceraldehyde-3-phosphate was obtained. Due to the low concentration of glyceraldehyde-3-phosphate in respect to the unphosphorylated aldehyde and its instability at high pH values, this compound could not be isolated. Optimization of the reaction conditions, the use of long-chain polyphosphate as donor, and use of cosolvents to improve the solubility of the glyceraldehyde might increase the conversion in the flow reactor and allow the recovery of glyceraldehyde-3-phosphate. Once isolated in a pure form, glyceraldehyde-3-phosphate might be used as a substrate for the fructose-1,6-bisphosphate aldolase RAMA or directly converted into the highly valuable DHAP by triose phosphate isomerase. In this isomerisation reaction the thermodynamic equilibrium is shifted towards DHAP. Such a system could be an alternative synthetic pathway towards the large-scale synthesis of DHAP.

**Tripolyphosphate as phosphate donor**

PPP₃ was already used as a phosphate donor with alkaline phosphatase and with human deoxyribonucleoside kinases. It contains two energy-rich phosphodiester bonds instead of the one present in PP₃. Therefore from every molecule of PPP₃ two phosphates can be transferred by PhoN-Sf to a primary alcohol, increasing the phosphorylating capacity of the donor. Also the amount of P₃ liberated per phosphate molecule transferred is less than with PP₃. Moreover, PPP₃ is more soluble than PP₃ and solutions up to 500 mM can be prepared, in contrast to maximally 250-300 mM of PP₃. In order to investigate the suitability of PPP₃ in phosphorylation reaction, batch reactions were carried out with 400 mM D-glucose, immobilized PhoN-Sf and both PP₃ and PPP₃ at pH 4.2 and room temperature. D-glucose was chosen because of the high affinity for PhoN-Sf and good conversions previously obtained. Table 3 shows that whereas with 250 mM PP₃, 150 mM D-G6P is formed, 250 mM PPP₃ increases the yield to 200 mM D-G6P. Nevertheless, more phosphate is formed, from 350 to 400 mM. Increasing the concentration of PPP₃ to 400 mM does not increase the product concentration but less phosphate is formed, indicating that transfer of one phosphate from the PPP₃ moiety is preferred over the transfer of phosphate from the PP₃ moiety when both species are present in solution.
A flow reactor for the synthesis of phosphorylated compounds

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<thead>
<tr>
<th>Donor</th>
<th>Donor (mM)</th>
<th>D-G6P (mM)</th>
<th>Phosphate released (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP$_1$</td>
<td>250</td>
<td>150</td>
<td>350</td>
</tr>
<tr>
<td>PPP$_1$</td>
<td>250</td>
<td>200</td>
<td>400</td>
</tr>
<tr>
<td>PPP$_1$</td>
<td>400</td>
<td>200</td>
<td>350</td>
</tr>
</tbody>
</table>

**Table 3**: The effect of PP$_1$ or PPP$_1$, on the formation of D-glucose-6-phosphate and inorganic phosphate. The batch reactions were carried out with 400 mM D-glucose, 5 % v/v immobilized PhoN-Sf, PP$_1$ or PPP$_1$ with two concentrations at pH 4.2, 20 °C.

PPP$_1$ was then used with D-glucose in our flow-reactor and the conditions optimized in order to increase the yields and minimize the release of free phosphate. The feeding solution containing 300 mM glucose and 200 mM PPP$_1$ at pH 4.2 was pumped through the reactor composed of one packed column of 0.5 mL at three different flow rates (Fig. 7).

![Graph showing the concentration of G6P](image)

**Fig. 7**: Concentration of G6P given by the flow reactor with one or two columns at three different flow rate, starting from 300 mM glucose and 200 mM PP$_1$ at pH 4.2.

With 0.01 mL/min, 100 mM of G6P were formed together with only 64 mM of free phosphate formed. Increasing the flow rate to 0.02 mL/min and thus decreasing the residence time of each substrate molecule in the reactor, 120 mM of product was obtained with only 48 mM of free phosphate produced. An additional increase of flow rate to 0.03 mL/min caused a drop in conversion to 50 mM of product with 39 mM phosphate formed. From this experiment, it is obvious that in a continuous system the use of PPP$_1$ helps to drastically reduce the amount of free phosphate produces without affecting the yield of the reaction. This is certainly an advantage when phosphate interferes with next transformation, either chemical or enzymatic.

Fig. 7 also shows the output of the reactor after connecting a second column of
0.5 mL filled with PhoN-Sf Immobeads, which doubles the amount of catalyst. With this new setup, 120 mM of product and 41 mM phosphate were obtained with 0.03 mL/min. A lower flow rate causes a decrease in the yield and an increase in amount of phosphate. This demonstrates the importance of the residence time in this reaction where phosphorylation is followed by dephosphorylation.

Also the phosphorylation of N-acetyl-D-glucosamine (100 mM) by PPP$_1$ in the flow reactor was studied (Table 4). The concentrations of PPP$_1$ and the flow rate were varied. 250 mM PPP$_1$ gave 80 mM NAG-P$_1$ and 400 mM P$_i$ with a flow rate of 0.03 mL/min. Using PPP$_1$ and reducing the concentration to only 65 mM, 65 mM NAG-P$_1$ was obtained with only 110 mM P$_i$ formed at a flow rate of 0.02 mL/min. With a slightly higher concentration of PPP$_1$, 130 mM, and low flow rate, 0.01 mL/min, 75 mM of NAG-P$_1$ was synthesized with 190 mM of free phosphate formed. This conversion is comparable to one given by using PP$_i$, with the difference that the time/space yield is lower, but much less phosphate is produced. At the same concentrations of reactants, the effect of the flow rate on the phosphorylation was studied. At 0.01 mL/min 75 mM NAG-P$_1$ and 190 mM P$_i$ were formed. These concentrations decreased at 0.03 mL/min to 50 and 65 mM NAG-P$_1$ and P$_i$, respectively. It is clear that at slow flow a higher concentration of product is formed though more PPP$_1$ is hydrolyzed to P$_i$ than at faster flow.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Donor (mM)</th>
<th>Flow rate (mL/min)</th>
<th>NAG-P$_1$ (mM)</th>
<th>Phosphate released (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP$_i$</td>
<td>250</td>
<td>0.03</td>
<td>80</td>
<td>400</td>
</tr>
<tr>
<td>PPP$_i$</td>
<td>65</td>
<td>0.02</td>
<td>65</td>
<td>110</td>
</tr>
<tr>
<td>PPP$_1$</td>
<td>130</td>
<td>0.01</td>
<td>75</td>
<td>190</td>
</tr>
<tr>
<td>PPP$_i$</td>
<td>130</td>
<td>0.02</td>
<td>60</td>
<td>85</td>
</tr>
<tr>
<td>PPP$_1$</td>
<td>130</td>
<td>0.03</td>
<td>50</td>
<td>65</td>
</tr>
</tbody>
</table>

*Table 4:* Concentration of NAG-P$_1$ and P$_i$ formed in the flow reactor with 100 mM NAG and different concentrations of phosphate donors at pH 4.2

PPP$_1$ was also used to phosphorylate continuously N-acetyl-D-galactosamine. This sugar (100 mM) was phosphorylated only to a small extent by 250 mM PP$_i$, giving only 10 mM of product. In this case, the use PPP$_1$ was found to be very advantageous, because with 200 mM of PPP$_1$, 27 mM of N-acetyl-D-galactosamine-6-phosphate was produced at flow rate of 0.02 mL/min.

Table 5 gives an overview of the yields of the various phosphorylated compounds that were obtained using the continuous flow system.
A flow reactor for the synthesis of phosphorylated compounds

<table>
<thead>
<tr>
<th>R-OH to R-OP&lt;sub&gt;i&lt;/sub&gt;</th>
<th>R-OH concentration</th>
<th>R-OP&lt;sub&gt;i&lt;/sub&gt; concentration</th>
<th>Isolated yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose to D-Glucose-6-phosphate</td>
<td>400 mM</td>
<td>150 mM (12 days)</td>
<td>32 g (78 %)</td>
</tr>
<tr>
<td>N-acetyl-D-glucosamine (NAG) to NAG-P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>100 mM</td>
<td>80 mM (16 days)</td>
<td>10.3 g (82 %)</td>
</tr>
<tr>
<td>Allyl alcohol to allylphosphate</td>
<td>400 mM</td>
<td>105 mM (5 days)</td>
<td>4.6 g (90 %)</td>
</tr>
<tr>
<td>Glycerol to glycerol-1-phosphate</td>
<td>500 mM</td>
<td>110 mM (14 days)</td>
<td>10 g (74 %)</td>
</tr>
<tr>
<td>Inosine to inosine-5'-monophosphate</td>
<td>100 mM</td>
<td>14 mM (5 days)</td>
<td>0.5 g (50 %)</td>
</tr>
<tr>
<td>N-acetyl-D-galactosamine to N-acetyl-D-galactosamine 6-phosphate</td>
<td>100 mM</td>
<td>10 mM (5 days) 25 mM with PPP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>95 mg (20 %) n.a.</td>
</tr>
<tr>
<td>Dihydroxyacetone to dihydroxyacetone phosphate</td>
<td>500 mM</td>
<td>50 mM (14 days)</td>
<td>-</td>
</tr>
<tr>
<td>DL-glyceraldehyde to DL-glyceraldehyde-3-phosphate</td>
<td>100 mM</td>
<td>6 mM (7 days)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5: List of all the alcohols and carbohydrates phosphorylated with the packed-bed flow reactor and 250 mM PP<sub>i</sub> as phosphate at pH 4.2 and room temperature.

Conclusions

In this chapter, a method for the covalent immobilization of the acid phosphatase PhoN-Sf on Immobeads is reported. The binding efficiency was 100 % with a remaining catalytic efficiency of 70 %. Immobilization did not affect the overall properties of PhoN-Sf such as stability and pH optimum and the beads were fully active under turnover for at least two weeks. The excellent stability of the immobilized enzyme in combination with a continuous-flow reactor offered the possibility to produce a wide range of phosphorylated alcohols at a scale of grams or more using cheap PP<sub>i</sub> as the phosphate donor. A small column of 0.5 mL was sufficient and scale-up of the process to synthesize larger amounts seems easily feasible. Conditions, conversions, and isolated yields are summarized in Table 5. We describe also how the use of a donor with higher phosphate transferring capacity like PPP<sub>i</sub> helps to increase the yields and reduces the amount of free phosphate produced by the reactor. This may facilitate the follow-up chemistry.

Furthermore we envision that there are interesting possibilities in the field of
new multi-enzyme continuous processes where phosphorylated intermediates are involved.\textsuperscript{[12-14,30]} This work also shows that continuous-flow reactors with immobilized enzymes have large advantages compared to classical synthetic chemistry methods, that are not only labour intensive and require protection of reacting groups, but are also difficult to scale up.

Bibliography