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Resilient Enzymes through Immobilisation: Stable NDP Polyphosphate Phosphotransferase from *Ruegeria pomeroyi* for Nucleotide Regeneration

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Abstract: Immobilisation plays an important role in the industrial application of enzymes. The stabilisation and reusability of immobilised enzymes reduce the cost of the catalyst and facilitate their use in continuously operated reactors. For this purpose, an applicable type of immobilisation needs to be identified. In this study, we investigate the conversion of CDP and PolyP to CTP by NDP polyphosphate phosphotransferase 3 from *Ruegeria pomeroyi* (*Rp*PPK2-3) and describe the covalent immobilisation of *Rp*PPK2-3. In order to select a suitable carrier for the immobilisation of *Rp*PPK2-3, a screening with different amino methacrylate (glutaraldehyde-pre-activated) and epoxy methacrylate carriers was carried out. The epoxy methacrylate carrier ECR8209M (Purolite®) was found to be the most suitable. With a half-life of 462 d when stored at 6 °C and a 50-fold reusability with a 93% residual activity, the immobilised enzyme showed a higher stability compared to the soluble enzyme with a half-life of 0.04 d. Although the half-life of the soluble enzyme could be increased to 32 d by adding PP_i, it could not reach the stability of the immobilisate. Due to the resilience of the immobilisate, it is suitable for application in continuous reactor set-ups, e.g., packed-bed reactors.

Keywords: immobilisation; polyphosphate kinase 2; *Ruegeria pomeroyi*; polyphosphate; cytidine 5′-triphosphate; nucleotide regeneration

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1. Introduction

Polyphosphate kinases (PPKs) use inorganic polyphosphate (PolyP) as a phosphate donor. PolyP is a high-energy phosphoanhydride-bound linear polymer consisting of tens or hundreds of orthophosphates (P_i), representing a rich source of energy [1,2]. While the linear form is the most common form in living organisms, a cyclic (metaphosphate) or branched (ultraphosphate) form can also occur [3]. PolyP is found in all living organisms and has many biological functions, such as the generation of adenosine triphosphate (ATP) in kinase reactions, the storage of phosphate (P_i), the chelation of metal ions like Mg^{2+}, molecular chaperone activity on bacterial virulence, etc. [2,4,5]. However, it also has industrial applications, for example as an antibacterial agent in processed meat, poultry or fish products or for the regeneration of the expensive substrate ATP using PPK in enzymatically catalysed reactions [2,6].

Due to the moderate price of PolyP (63 EUR·kg^{-1}, sodium polyphosphate, Merck KGaA, retrieved: January 2024) as a substrate, the use of PPKs is particularly promising for the regeneration of expensive nucleotide triphosphates (NTPs) such as cytidine triphosphate (CTP) (12,130 EUR·kg^{-1}, cytidine 5′-triphosphate disodium salt, Biosynth, retrieved: January 2024). Other enzymes for CTP regeneration, such as pyruvate kinase with phosphoenolpyruvate [7], acetate kinase with acetyl phosphate [8] or creatine kinase

with creatine phosphate [9], have also been described [10]. However, all these donors are much more expensive than PolyP.

According to their functionality, PPKs can be divided into two families: PPK1 and PPK2 (Figure 1) [5,11]. PPK1s preferentially synthesise long PolyP chains by the reversible transfer of the terminal P_i from ATP, but they also accept all other NTPs [1,4,12]. PPK2s preferentially catalyse the degradation of PolyP to phosphorylate nucleotide monophosphate (NMP) or nucleotide diphosphate (NDP) and are classified into three subfamilies [12–14]. Many genomes encode two or three paralogs of PPK2, most of which are one-domain PPK2s of approximately 230 residues or two-domain PPK2s of 496–544 residues [15]. The one-domain PPK2s generate NTP from NDP and PolyP (class I subfamily), whereas the two-domain PPK2s generate NDP from NMP and PolyP (class II subfamily) [15,16]. In addition, a PPK2 class III subfamily with one-domain PPK2s catalyses both NMP and NDP phosphorylation with PolyP [15]. In this study, an NDP polyphosphate phosphotransferase from the bacterium *Ruegeria pomeroyi* (EC 2.7.4.1, gene: SPO1727, UniProt KB: Q5LSN8) is examined. This enzyme was first characterised by Achbergová and Nahálka in 2014 and named *Rp*PPK2-3 [17]. The abbreviation *Rp*PPK2-3 erroneously suggests that it is a PPK2 subfamily III. However, no NMP phosphorylation was detected. *Rp*PPK2-3 belongs to the PPK2 family, a class I subfamily based on the classification that was proposed by Motomura and co-workers as well in 2014 [15].

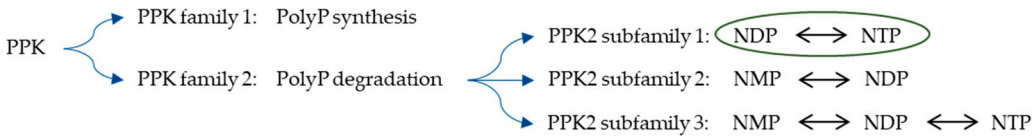
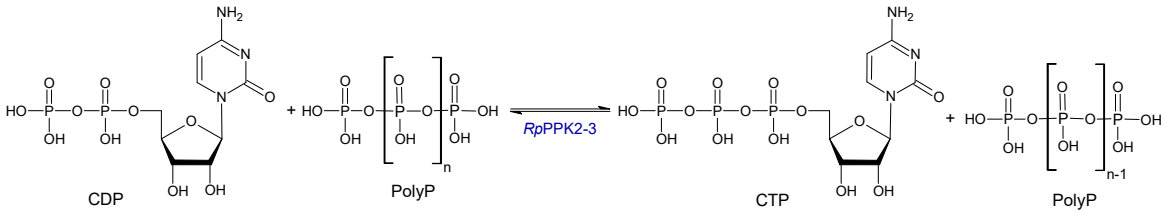


Figure 1. Polyphosphate kinase (PPK) families and PPK2 subfamilies (RpPPK2-3 belongs to PPK family 2 subfamily 1, circled in green).

*Rp*PPK2-3 converts NDP to NTP using PolyP as a donor and accepts adenosine diphosphate (ADP), cytidine diphosphate (CDP), uridine diphosphate (UDP), guanosine diphosphate (GDP) and deoxythymidine diphosphate (dTDP) with a preference for UDP, GDP and CDP [17–19]. In this study, the conversion of CDP and PolyP to CTP is investigated (Scheme 1).



Scheme 1. RpPPK2-3-catalysed conversion of CDP and PolyP to CTP.

The use of *Rp*PPK2-3 in biocatalytic synthesis has already been described in the literature, and examples are given below. Due to its acceptance of all NDPs, different methods for its utilisation are possible. Nahálka et al. [18] described the use of *Rp*PPK2-3 and cytidine 5′-monophosphate kinase (CMP kinase) as an active inclusion body system for CTP regeneration starting from CMP combined with whole cells, co-expressing two enzymes for the synthesis of CMP-sialic-acid. Gottschalk et al. [19] developed a one-pot synthesis for hyaluronic acid with a combined ATP and UTP regeneration using *Rp*PPK2-3. The use of *Rp*PPK2-3 provided a solution for the limitations of ATP, ADP and UDP inhibition [19]. His-tag-immobilised enzymes on Ni^{2+}/nitrilotriacetic acid magnetic beads were used, and almost the highest possible loading capacity according to the manufacturer's specifications for the immobilised *Rp*PPK2-3 with an increased maximum specific activity was achieved [20]. Mahour et al. [21] described the use of *Rp*PPK2-3 to regenerate ATP and UTP in a five-enzyme cell-free cascade to produce UDP-GlcNAc.

The industrial application of enzymes is often challenged by a lack of long-term stability and the difficulty of enzyme recovery and reuse [22]. Immobilisation can increase enzyme stability and positively influence their specificity and selectivity and can also potentially reduce inhibition [23]. The application of immobilised enzymes in different reactor configurations, such as continuous packed-bed reactors (PBR), is another advantage [23]. The PBR reactor configuration can prevent product contamination by enzymes, allowing for the use of high enzyme concentrations, as there is no risk of enzyme aggregation, and for reducing enzyme costs due to enzyme reusability. Furthermore, it allows for the conversion under high pressures, and the performance of multi-enzyme cascades with co-immobilised enzymes is such that even cofactor regeneration can be implemented [23,24]. Thus, immobilisation could offer many advantages for the development of low-cost production processes. Enzyme immobilisation can be achieved by carrier binding, encapsulation or cross-linking [22]. Covalent carrier binding offers the advantage of stable bonds that prevent leaching of the enzyme as well as its easy recovery and reuse [23,25]. For continuous-flow reactors, immobilisation on the reactor wall or on carrier materials such as particles or monoliths has been demonstrated [25]. In this study, epoxy methacrylate particles with glutaraldehyde-pre-activated amino methacrylate particles as supports were used to covalently immobilise *Rp*PPK2-3. The covalently immobilised *Rp*PPK2-3 was then used in a CTP regeneration system in a multi-enzyme cascade performed in a PBR to synthesise the human milk oligosaccharide (HMO) sialyllactose.

2. Results

2.1. Expression and Purification of RpPPK2-3

The fed-batch fermentation using the expression strain *Escherichia coli* (*E. coli*) BL21(DE3) pET-22b(+)_rpppk2-3 with a total culture volume of 1.4 L resulted in an activity yield of up to 100,000 $U \cdot L_{culture}^{-1}$ with a specific activity of 24 $U \cdot mg_{enzyme}^{-1}$. The purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S1). The His_6-tagged *Rp*PPK2-3 with a calculated mass of 36 kDa clearly showed bands at this level. The enzyme was lyophilised in 50 $mmol \cdot L^{-1}$ sodium phosphate buffer at pH 7.4 and was soluble and stable for at least six months when stored at −20 °C.

2.2. Stability of the Soluble RpPPK2-3

The stability of an enzyme is important for its use in catalysis and to ensure that its activity is maintained during the storage and the immobilisation process. Tris(hydroxymethyl) aminomethane (Tris) buffer was used for the reaction cascade in which the *Rp*PPK2-3 was integrated. Therefore, we investigated the stability when the *Rp*PPK2-3 was stored in Tris buffer and additionally studied the influence of the additives PolyP, $MgCl_2$ and PP_i on the enzyme's stability (Section 2.2.1). A sodium phosphate buffer was used for the immobilisation because this buffer ensures enzyme stability over the duration of the immobilisation process. The stability was analysed at different sodium phosphate concentrations (Section 2.2.2).

2.2.1. Influence of Additives on the Stability of the Soluble RpPPK2-3

For some enzymes, the absence of a substrate leads to a loss of stability [26]. A stability study was performed to investigate the effect of the substrate on RpPPK2-3's stability. For this purpose, the enzyme was stored in 50 $mmol \cdot L^{-1}$ Tris at pH 7.8, and the stability was also investigated with respect to the influence of the addition of PP_i, PolyP or PolyP with Mg^{2+} to the buffer (Table 1, Figures S2 and S3). The deactivation constant k_d was determined by exponential fitting to calculate the half-life according to Equation (4).

When stored without the addition of the substrate, the half-life was 0.04 d. The half-life could be increased to 14 d by the addition of PolyP. A stabilising effect of PolyP on PPKs has already been described [15,27,28]. Pyrophosphate (PP_i) also had a stabilising effect with an increased half-life of 32 d. However, PP_i has also been described as an inhibitor of PPKs [19], which had to be considered. Mg^{2+} seemed to have a negative effect on the

stability of the enzyme, as the half-life was reduced from 14 d to 6 d when $MgCl_2$ was added. To verify these results, further investigations with different combinations of PolyP and Mg^{2+} concentrations have to be performed. The use of a Tris buffer was carried out due to its possible stabilising effect on the substrate PolyP for catalysis, but the Tris buffer without any additive is not suitable for storage of the enzyme due to the short half-life of 0.04 d.

Table 1. Stability of *Rp*PPK2-3 upon addition of substrate.

Additive *	$t_{1/2}$/d
None	0.04
PolyP	14
PolyP + $MgCl_2$	6
PP_i	32

* Additive concentrations: 10 mmol·L^{-1} PP_i, 8.5 g·L^{-1} PolyP, 33 mmol·L^{-1} $MgCl_2$; storage conditions: 22 or 30 °C, 40 mg·L^{-1} *Rp*PPK2-3, 50 mmol·L^{-1} Tris buffer, pH 8.

2.2.2. Influence of Buffer Concentration on the Stability of the Soluble RpPPK2-3

To carry out the immobilisation, the enzyme was required to be stable over the immobilisation period of 3 days. A suitable buffer had to found for the immobilisation. As shown in Section 2.2.1, the Tris buffer was not suitable for storage. However, a Tris buffer cannot be used for covalent immobilisation anyway, as the amino group of the Tris buffer would bind to the functional aldehyde or epoxy groups of the support. Therefore, we analysed the stability of the enzyme in sodium phosphate buffer at different concentrations at 20 °C (Table 2).

Table 2. Stability study with different sodium phosphate concentrations at pH 7.4 (soluble enzyme).

C (Buffer)/mmol·L^{-1}	Activity * Retention after 3 Days/%
20	73
250	85
500	98
1000	100

* Reaction conditions activity assay: 4 mg·L^{-1} *Rp*PPK2-3; T = 20 °C, 500 rpm, 50 mmol·L^{-1} Tris buffer, pH 7.8, 30 mmol·L^{-1} $MgCl_2$, 7.3 g·L^{-1} PolyP, 5 mmol·L^{-1} CDP, V = 1 mL, reaction time: 2.5 min; storage conditions: 40 mg·L^{-1} *Rp*PPK2-3, T = 20 °C, 300 rpm, 20–1000 mmol·L^{-1} sodium phosphate buffer, pH 7.8.

When stored in the 20 mmol·L^{-1} sodium phosphate buffer, an activity retention of at least 73% could be achieved after three days of storage. At a sodium phosphate buffer concentration of 1000 mmol·L^{-1}, 100% of the activity was retained. At high ionic strengths, the strong kosmotropic properties of buffer salts can contribute to the solvation and maintenance of spatial structures [29]. The sodium phosphate buffer was therefore suitable for both storage and the immobilisation process.

2.3. Immobilisation

2.3.1. Screening of Different Carriers for Immobilisation of RpPPK2-3

In order to select a suitable carrier for the immobilisation of *Rp*PPK2-3, five different carriers were compared and evaluated based on the characteristic variables of the immobilisation process and the immobilised enzyme (Table 3).

All five carriers showed a high immobilisation yield, meaning that a large proportion of the enzyme used for immobilisation was bound to the carrier. Comparable values for the yield in the immobilisation process were also described by Petermeier et al. [30]. The low immobilisation efficiency and activity yield were probably due to conformational changes in the enzyme or an inaccessible active site due to the undirected covalent bonding. Low activity yields with these carriers have also been described by De Santis et al., with yields ranging from 0.4 up to 4% [31], which compares well with our calculated yield of from 0.6

to 2.5%. The immobilisation efficiencies of the two epoxy methacrylate carriers were 2.5% (ECR8209M) and 2.3% (ECR8204M), respectively, which were higher than the others. The same was true for the activity yields, which were 2.5% (ECR8209M) and 2.2% (ECR8204M). The higher activity yield of the immobilised enzymes on the epoxy methacrylate carriers compared to the amino methacrylate carriers could be explained by the fact that the binding to the aldehyde group of the pre-activated amino methacrylate carrier is via an amino group, whereas the binding to the epoxy group is also possible via thiol, carboxyl and phenol groups [32–34]. The epoxy bond may lead to a better orientation of the active site. The lower activity yield of the epoxy/butyl methacrylate carrier (ECR8285) could be due to the hydrophobic surface of the carrier and a resulting less favourable orientation of the enzyme. Thus, the two epoxy methacrylate carriers proved to be the most suitable carrier materials in terms of size for the immobilisation process. The carrier loading was significantly higher for the amino methacrylate carrier than for the epoxy methacrylate carrier, despite the comparable high immobilisation yields. This was due to the use of less enzyme mass per carrier mass for the epoxy and epoxy/butyl methacrylate carrier. Despite the lower carrier loading, the carrier specific activity of the epoxy methacrylate carrier with 19.6 $U \cdot g_{carrier}^{-1}$ (ECR8209M) and 17.6 $U \cdot g_{carrier}^{-1}$ (ECR8204M) was significantly higher than that of the other immobilisates with specific activities of around 10 $U \cdot g_{carrier}^{-1}$. Looking at the results of the amino methacrylate carrier, no relevant difference was observed between the use of a short spacer (C2-spacer, ECR8309M) and a long spacer (C6-spacer, ECR8409M). There was an insignificant difference between the results of the smaller pores (300–600 Å, ECR8204M) and the larger pores (600–1200 Å, ECR8209M) of the epoxy methacrylate carrier. Small pores can lead to blocking or material transport limitations, while pores that are too large can reduce carrier loading due to the smaller surface area [35]. Both factors (spacer/pore size) could have had an influence here, but they resulted in similar enzyme activities. Thus, the two epoxy methacrylate carriers also proved to be the most suitable carriers in terms of the characteristic variables of the immobilised enzymes. To prove the stability of the immobilisates, two different stability studies were performed: a storage stability study over 15 days (Figure 2A) and a reusability study (Figure 2B) over 20 cycles.

Table 3. Characteristic parameters for the immobilisation process and the immobilised *Rp*PPK2-3 with different carriers.

Characteristic Variable	ECR8309M	ECR8409M	ECR8209M	ECR8204M	ECR8285
Immobilisation yield/%	93.0	91.9	98.6	94.9	97.5
Immobilisation efficiency/%	0.7	0.8	2.5	2.3	1.4
Activity yield/%	0.6	0.7	2.5	2.2	1.4
Carrier loading/$mg_{enzyme} \cdot g_{carrier}^{-1}$	83.3	83.3	38.5	35.1	38.2
Carrier spec. activity */$U \cdot g_{carrier}^{-1}$	10.1	10.8	19.6	17.6	10.9
Spec. activity */$U \cdot mg_{enzyme}^{-1}$	0.1	0.1	0.5	0.5	0.3

* One unit (U) is defined as a product formation of 1 µmol per min; reaction conditions of activity assay: 7 $g \cdot L^{-1}$ immobilisate, T = 20 °C, 1000 rpm, 50 $mmol \cdot L^{-1}$ Tris buffer, pH 7.8, 30 $mmol \cdot L^{-1}$ $MgCl_2$, 7.3 $g \cdot L^{-1}$ PolyP, 5 $mmol \cdot L^{-1}$ CDP, V = 1.5 mL, reaction time: 2.5 min.

In the storage stability study, a relative activity of >85% was measured for all carriers after 14 or 15 days, indicating no relevant loss of activity. In the reusability study, a relative product activity of >86% was measured for all immobilisates after 20 cycles of 15 min of reaction time each, so that no relevant loss of activity could be detected. Partially, the loss of activity might be due to some slight abrasion of the carrier by mechanical stress. No enzyme leaching was detected in the reusability study and the storage stability study.

In comparison of all tested carriers, the epoxy methacrylate carriers ECR8209M and ECR8204M proved to be the most suitable carriers for the immobilisation of *Rp*PPK2-3. They showed the best results in terms of the characteristic variables of the immobilisation process and the immobilised enzyme. All carriers proved to be stable in terms of storage and

reuse. Therefore, based on the highest activity, for further experiments with immobilised *Rp*PPK2-3, the epoxy methacrylate carrier ECR8209M was selected.

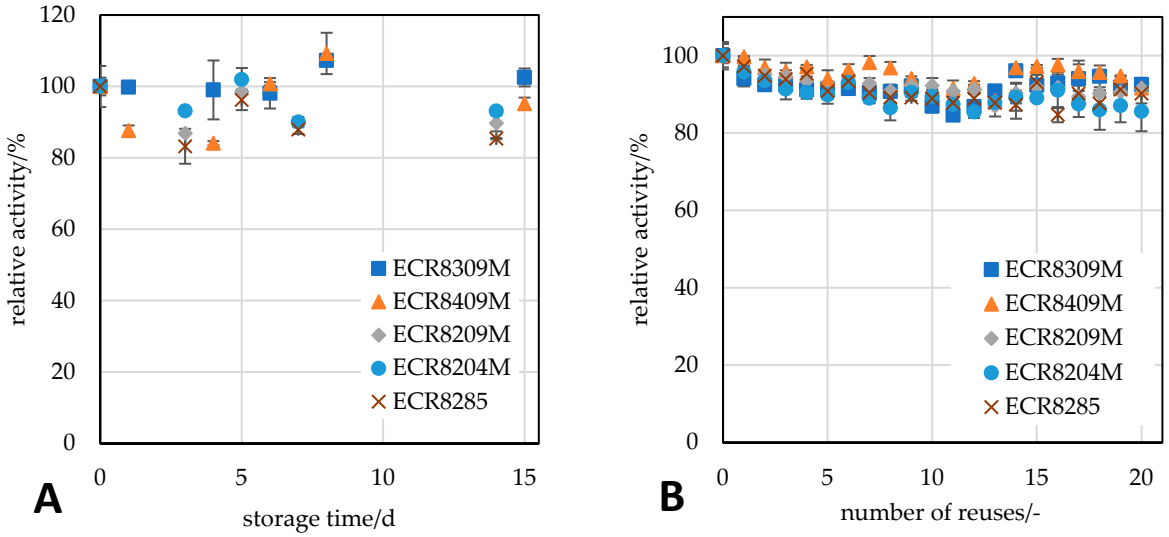


Figure 2. Stability analysis of *Rp*PPK2-3 immobilised on different carriers, (**A**): storage stability study, (**B**): reusability study (storage conditions for A: T = 6 °C, 20 mmol·L^{-1} sodium phosphate buffer pH 7.4; reaction conditions of activity assay for A: 7.3 g·L^{-1} immobilisate, T = 20 °C, 1000 rpm, 50 mmol·L^{-1} Tris buffer, pH 7.8, 30 mmol·L^{-1} $MgCl_2$, 7.3 g·L^{-1} PolyP, 5 mmol·L^{-1} CDP, V = 1.5 mL, reaction time: 2.5 min; reaction conditions of activity assay for B: 7 g·L^{-1} immobilisate, T= 20 °C, 1000 rpm, 50 mmol·L^{-1} Tris buffer, pH 7.8, 30 mmol·L^{-1} $MgCl_2$, 7.3 g·L^{-1} PolyP, 5 mmol·L^{-1} CDP, V = 1.5 mL, reaction time: 15 min; after each measurement, the immobilisate was washed twice with 1 mL of 20 mmol·L^{-1} sodium phosphate buffer at pH 7.4). Error bars show standard deviations of two independent experiments.

2.3.2. Optimisation of the Immobilisation Process

Immobilisation of *Rp*PPK2-3 on epoxy methacrylate ECR8209M was performed according to the manufacturer's instructions [36]. The manufacturer describes more efficient immobilisation on epoxy-functionalised carriers when using highly concentrated buffer solutions with a concentration of >1 mol·L^{-1}. In our previous immobilisations, precipitation occurred after dissolving lyophilised *Rp*PPK2-3 in 1 mol·L^{-1} sodium phosphate buffer, resulting in loss of the enzyme. To avoid this loss, we investigated the effect of using highly concentrated buffer solutions for immobilisation. Four immobilisations were performed and compared using sodium phosphate buffer concentrations of 20, 250, 500 and 1000 mmol·L^{-1} (Table 4). When the lyophilisate was dissolved in the 20, 250 and 500 mmol·L^{-1} buffers, up to 15% of the enzyme was lost by precipitation, and 35% was lost in the 1000 mmol·L^{-1} buffer. Higher salt concentrations may favour enzyme precipitation [29]. This makes it difficult to use a 1000 mmol·L^{-1} buffer for large-scale applications. The use of a different buffer salt (such as MOPS or HEPES) and the addition of stabilisers could reduce the precipitation. In addition, the precipitation was also caused by mechanical stress from mixing during the immobilisation.

The characteristics of the immobilisation with the 20, 250 and 500 mmol·L^{-1} sodium phosphate buffers gave similar results, whereas the use of the 1000 mmol·L^{-1} immobilisation buffer was a deviant. When the 1000 mmol·L^{-1} immobilisation buffer was used, the carrier with a carrier loading of 54.5 $mg_{enzyme}{\cdot}g_{carrier}{}^{-1}$ was loaded with only about half the enzyme compared to the other immobilisates. The carrier specific activity was approximately the same for all the immobilisates. The specific activity of 0.4 $U{\cdot}mg_{enzyme}{}^{-1}$ was, therefore, twice that of the other immobilisates, resulting in a higher immobilisation efficiency of 2.3% and an activity yield of 1.9%. The increased efficiency is promising for further immobilisations. However, this is only the case if a solution to the solubility

problem of *Rp*PPK2-3 in 1000 mmol·L^{-1} sodium phosphate buffer is found. The result with the 1000 mmol·L^{-1} buffer was different from the results of the screening experiment shown in Section 2.3.1, where a 1000 mmol·L^{-1} concentration of sodium phosphate buffer was also used. The carrier loading was lower with 38.5 $mg_{enzyme}·g_{carrier}^{-1}$, but the carrier specific activity was almost the same with 19.6 $U·g_{carrier}^{-1}$. It seems possible to achieve the same activities with a lower loading of the carrier, which would also reduce the amount of enzyme used. Further experiments with lower carrier loadings and blocking of the remaining free active groups on the support at the immobilisation endpoint could lead to an increase in activity yield and a further optimisation of the immobilisation process.

Table 4. Characteristic variables of immobilisation using different concentrations of the immobilisation buffer (sodium phosphate, pH 7.4).

Characteristic Variable	20 mmol·L^{-1}	250 mmol·L^{-1}	500 mmol·L^{-1}	1000 mmol·L^{-1}
Immobilisation yield/%	78.5	82.3	72.9	83.3
Immobilisation efficiency/%	1.2	1.3	1.4	2.3
Activity yield/%	1.0	1.1	1.0	1.9
Carrier loading/$mg_{enzyme}·g_{carrier}^{-1}$	100.0	99.4	102.3	54.5
Carrier spec. activity */$U·g_{carrier}^{-1}$	18.1	20.2	18.4	21.5
Spec. activity */$U·mg_{enzyme}^{-1}$	0.2	0.2	0.2	0.4

* One unit (U) is defined as a product formation of 1 µmol per min; reaction conditions of activity assay: 7 g·L^{-1} immobilisate, T = 20 °C, 1000 rpm, 50 mmol·L^{-1} Tris buffer, pH 7.8, 30 mmol·L^{-1} $MgCl_2$, 7.3 g·L^{-1} PolyP, 5 mmol·L^{-1} CDP, V = 1.5 mL, reaction time: 2.5 min.

2.4. Stability of the Immobilised RpPPK2-3

To investigate the stability of the immobilised *Rp*PPK2-3, a long-term storage stability study (Table 5, Figure S4) and a 50-time reusability study (Figure 3) were performed. The long-term storage stability study determined the stability of the enzyme at different temperatures (6, 20 or 40 °C), when stored in water or is 20 mmol·L^{-1} sodium phosphate buffer and when under mechanical stress by shaking. Stability was assessed based on the half-life ($t_{1/2}$) (Table 5).

Table 5. Storage, temperature and mechanic stability of *Rp*PPK2-3 immobilised on epoxy methacrylate ECR8209M.

Storage Temperature/°C	Shake Frequency/rpm	Storage Solution	$t_{1/2}$/d
6	0	water	462
6	0	buffer	433
20	0	buffer	231
20	300	buffer	248
40	0	buffer	12

For the immobilised *Rp*PPK2-3 stored in water or in buffer at 6 °C, the half-life time was almost the same at 462 d and 433 d, respectively. So, the immobilisate can be stored in both of them. At higher temperatures, the stability decreased. At 20 °C, only half of the stability could be determined with a half-life of 231 d, compared to 433 d at 6 °C. At 40 °C, the half-life was reduced to 12 d, so the temperature has a significant effect on the enzyme's stability. Mechanical stress by shaking the immobilisate at 300 rpm showed no loss of stability. In previous studies, we have already demonstrated high half-lives for covalently immobilised enzymes of up to >179 d when stored at 6 °C [37].

The reusability study demonstrated a 50-fold reuse of the immobilisate with a 15 min reaction time per cycle (Figure 3). After 50 cycles, a relative activity of 93% was measured compared to the first use, so no significant loss of activity could be detected. In our previous study, we have already demonstrated the reusability of covalently immobilised enzymes over 50 cycles, which exhibited robust characteristics and could also be used under high pressures of up to over 100 MPa [37].

The storage stability study and the reusability study showed that the immobilisate is stable and resilient. No leaching was detected in these studies. Therefore, it is suitable for use in a continuously operated packed-bed reactor.

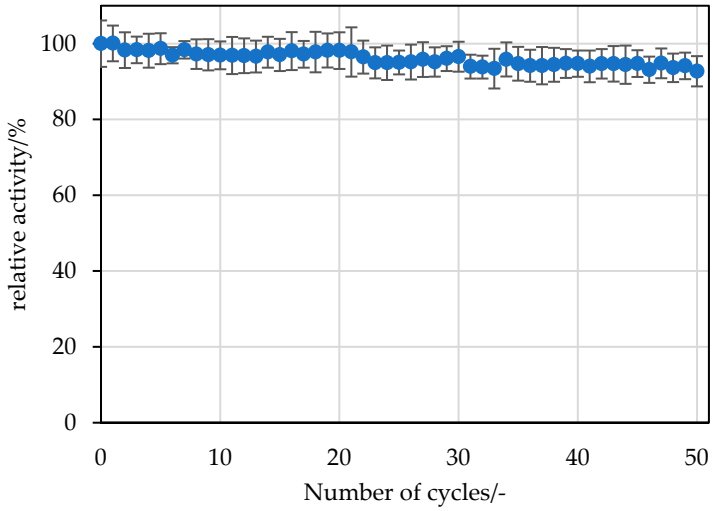


Figure 3. Reusability study of *Rp*PPK2-3 immobilised on epoxy methacrylate ECR8209M (reaction conditions of activity assay: 5 mmol·L^{-1} CDP, 7.3 g·L^{-1} PolyP, 30 mmol·L^{-1} $MgCl_2$, 50 mmol·L^{-1} Tris, pH 7.8, T = 20 °C, 1000 rpm, V = 1.5 mL, 7 g·L^{-1} immobilisate, reaction time = 15 min). Error bars show standard deviations of two independent experiments.

3. Materials and Methods

3.1. Chemicals

The following chemicals were used in this study with the following manufacturers and order number: acetonitrile (VWR International GmbH, Darmstadt, Germany, 83640.320), albumin from bovine serum (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, A7906), amino C2 methacrylate (Purolite Ltd., Wales, UK, ECR8309M), amino C6 methacrylate (Purolite Ltd., ECR8409M), Bradford reagent (Sigma-Aldrich, B6916), cytidine 5′-triphosphate disodium salt (Shanghai Tianqi Chemical Limited, Zhengzhou, China), cytidine 5′-diphosphate disodium salt (Biosynth Ltd., Compton, UK, NC09380), dipotassium hydrogen phosphate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany, 6875.1), disodium hydrogen phosphate dihydrate (VWR, 28029.260), epoxy/butyl methacrylate (Purolite Ltd., ECR8285), epoxymethacrylate (Purolite Ltd., ECR8209M/ECR8204M), glutaraldehyde (Carl Roth, 3778.1), 2-propanol (VWR, 20880.320), magnesium chloride hexahydrate (Sigma-Aldrich, M9272), potassium dihydrogen phosphate (Carl Roth, 3904.1), sodium chloride (VWR, 27810.295), sodium phosphate monobasic monohydrate (Sigma-Aldrich, 71507), sodium polyphosphate (Merck KGaA, Darmstadt, Germany, 106529), tetrabutylammonium bromide (Carl Roth, 6633.3) and Tris (AppliChem GmbH, Darmstadt, Germany, A1379). All chemicals were of analytical grade.

3.2. Expression and Purification

The gene for *Rp*PPK2-3 (SPO1727) cloned into the pET22-b(+) expression vector was kindly provided by the RWTH Aachen University, Laboratory for Biomaterials [19]. The *E. coli* BL21(DE3) was transformed with the plasmid using heat shock. The expression of the C-terminal hexahistidine (His6)-tagged *Rp*PPK2-3 was performed as a fed-batch cultivation in a bioreactor with a working volume of up to 2 L. Immobilised metal affinity chromatography (IMAC) was used for enzyme purification. The buffer was exchanged to 50 mmol·L^{-1} sodium phosphate buffer at pH 7.4, performed by dialysis or tangential flow filtration, and the enzyme was lyophilised and stored at −20 °C. The purity of the enzyme was qualified by SDS-PAGE. The enzyme concentration was determined by a Bradford assay using albumin from bovine serum (BSA) as a reference.

3.3. Immobilisation

Epoxy- and amino-functionalised carriers were used for the covalent immobilisation of the *Rp*PPK2-3 (Table 6). Epoxy-functionalised carriers form stable covalent linkages with the thiol, amino, carboxylic or phenolic groups of the enzyme. The amino-functionalised carriers were pre-activated with glutaraldehyde. The terminal amino groups of the enzyme formed multipoint covalent bonds with the aldehyde group of the resin, resulting in an imino bond [32]. Immobilisation was performed according to the manufacturer's instructions [36] and as described in previous studies [37]. A sodium phosphate buffer at pH 7.4 with a concentration ranging from 20 to 1000 mmol·L^{-1} was used as the immobilisation buffer for the epoxy-functionalised carriers and a concentration of 20 mmol·L^{-1} for the amino-functionalised carriers. For the mixing during the immobilisation process, a rotary mixer (Sample Mixer MXICI, Dynal) was used. For vacuum filtration, a vacuum pump (Chemistry diaphragm pump ME 2C NT, Vacuubrand GmbH + Co. KG, Wertheim, Germany), a filter attachment (Bottletop-filter, Thermo Fisher Scientific Inc., Waltham, MA, USA) and a membrane filter (Labsolute® CA membrane filter, Th. Geyer GmbH & Co. KG, Renningen, Germany) was used. The carrier was equilibrated by washing 3 times with immobilisation buffer with a carrier-to-buffer ratio of 1:2 (*w*/*v*). To pre-activate the amino methacrylate carriers, an additional step was performed with a 1% glutaraldehyde (*v*/*v*) solution in immobilisation buffer with a ratio of 1:4 (*w*/*v*). After the addition of the 1% glutaraldehyde solution, mixing was carried out for 1 h at room temperature and at 10 rpm. The pre-activated amino methacrylate carriers were then washed 3 times with immobilisation buffer (ratio of 1:4 (*w*/*v*)). A 5 mg·mL^{-1} enzyme solution in immobilisation buffer was added to the filtered equilibrated and pre-activated carriers with an enzyme loading of 100 $mg_{ezyme} \cdot g_{carier}{}^{-1}$ and was mixed at 20 °C and at 10 rpm for 18 h. The epoxy methacrylate carrier was additionally stored for 20 h at 20 °C without mixing. The immobilisates were washed with immobilisation buffer containing 500 mmol·L^{-1} sodium chloride for the desorption of non-covalently bound proteins and stored in immobilisation buffer at 6 °C. The specific enzyme activity and enzyme concentration (Bradford assay) was determined in the enzyme solution before and after immobilisation. The carrier specific activity was determined with the immobilisate.

Table 6. Carriers used for immobilisation (Purolite® Lifetech™ ECR enzyme immobilisation resins [32]).

Carrier/Order Number	Immobilisation Type	Pore Diameter/Å	Particle Diameter/µm	Hydrophobicity
Amino C2 methacrylate/ECR8309M	Covalent via pre-activation with glutaraldehyde	600–1200	300–710	Hydrophilic
Amino C6 methacrylate/ECR8409M	Covalent via pre-activation with glutaraldehyde	600–1200	300–710	Hydrophilic
Epoxy methacrylate/ECR8209M	Covalent	600–1200	300–710	Hydrophilic
Epoxy methacrylate/ECR8204M	Covalent	300–600	300–710	Hydrophilic
Epoxy/butyl methacrylate/ECR8285	Covalent	450–650	250–1000	Hydrophobic

3.3.1. Screening of Different Carriers

To select a suitable support, a screening with five different supports was performed (Table 6). To obtain a stable immobilisate, carrier materials with a covalent bond toward the enzyme were selected. The support materials differed regarding their functional group, pore and particle diameter, spacer length and hydrophobicity. The *Rp*PPK2-3 was immobilised on all the supports and compared in terms of the immobilisation yield, immobilisation efficiency, activity yield, carrier loading and carrier specific activity. In addition, the stability of the immobilised enzyme was determined in a reusability and storage study. The immobilisation buffer used for the epoxy-functionalised carriers was a 1000 mmol·L^{-1} sodium phosphate buffer at pH 7.4, and for the amino-functionalised carriers, a 20 mmol·L^{-1} sodium phosphate buffer at pH 7.4 was used.

3.3.2. Optimisation of the Immobilisation Process

To determine the effect of the buffer concentration on the immobilisation efficiency, the immobilisation was performed with different buffer concentrations. Four immobilisations with sodium phosphate buffer concentrations of 20, 250, 500 and 1000 mmol·L^{-1} were performed and compared in terms of the immobilisation yield, immobilisation efficiency, activity yield, carrier loading and carrier specific activity.

3.3.3. Calculation of Characteristic Parameters of the Immobilisation Process

The quality of enzyme immobilisation can be assessed by characteristic parameters describing both the immobilisation process and the successfully immobilised enzyme. To characterise the immobilisation process, the immobilisation yield, immobilisation efficiency and activity yield were calculated as described by Syldatk et al. [38]. The immobilisation yield (Y_{Immo}) describes the percentage of the theoretical maximum enzymatic activity (EA_{total}) (Equation (1)).

$$Y_{immo} = \frac{EA_{total} - EA_{free}}{EA_{total}} \cdot 100\% \quad (1)$$

EA_{free} is the activity remaining in the enzyme solution after immobilisation. The immobilisation efficiency (Eff_{Immo}) is the percentage of the measured immobilised activity (apparent activity, EA_{app}) of the theoretical immobilised activity (Equation (2)).

$$Eff_{immo} = \frac{EA_{app}}{EA_{total} - EA_{free}} \cdot 100\% \quad (2)$$

The activity yield (Y_{EA}) describes the percentage of the apparent activity (EA_{app}) from the theoretical maximum activity (EA_{total}) and is the product of the immobilisation yield and the immobilisation efficiency (Equation (3)).

$$Y_{EA} = \frac{EA_{app}}{EA_{total}} \cdot 100\% \quad (3)$$

The activity of the enzyme solution and the immobilisate were measured using the activity assay described in Section 3.4. The enzyme concentration was determined by a Bradford assay using bovine serum albumin (BSA) as a reference.

3.4. Activity Assay

The activity assay was performed in duplicate in a 2 mL reaction tube using a thermoshaker with a reaction volume of 1 mL for the soluble enzyme and 1.5 mL for the immobilised enzyme, respectively. The reaction conditions were 20 °C with a rotation speed of 500 rpm for the soluble enzyme and 1000 rpm for the immobilised enzyme, respectively. The reaction contained 5 mmol·L^{-1} CDP, 7.3 g·L^{-1} PolyP, 30 mmol·L^{-1} $MgCl_2$, 50 mmol·L^{-1} Tris at pH 7.8 and 4 mg·L^{-1} free enzyme or 7 g·L^{-1} immobilised enzyme. The reaction was stopped after 2.5 min with 2-propanol. CDP and CTP were quantified on an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, USA). A Phenomenex Luna 3u C18(2) 100 Å (150 × 4.6 mm, 3 µm particles) column was used at 40 °C and at a flow rate of 0.8 mL·min^{-1}. The isocratic eluent consisted of 46% acetonitrile and 54% 20 mmol·L^{-1} potassium phosphate buffer containing 20 mmol·L^{-1} tetrabutylammonium bromide (TBAB) as an ion-pair reagent at pH 5.9. Cytidine nucleotides were detected at 272 nm (variable wavelength detector (VWD, Agilent Technologies)) with a retention time of 2.1 (CMP), 2.5 (CDP) and 2.9 min (CTP) (Figure S5).

3.5. Stability Studies

3.5.1. Stabilisation of the Soluble RpPPK2-3 by Additives

To investigate the influence of PolyP, pyrophosphate (PP_i) and Mg^{2+} on the stability of the *Rp*PPK2-3, a storage stability study was performed. To analyse the stability without the addition of additives, the *Rp*PPK2-3 was stored in 50 mmol·L^{-1} Tris at pH 7.8 with an

enzyme concentration of 0.04 g·L^{-1} at 22 °C for 3 h in a 1.5 mL reaction tube. To investigate the influence of additives on enzyme stability, additional analyses were performed with added PP_i, PolyP and PolyP with Mg^{2+}. PP_i was added at a concentration of 10 mmol·L^{-1}, PolyP at 8.5 g·L^{-1} and $MgCl_2$ at 33 mmol·L^{-1}. The *Rp*PPK2-3 was stored in 50 mmol·L^{-1} Tris at pH 8.0 with an enzyme concentration of 0.04 g·L^{-1} at 30 °C for up to 45 d in a 1.5 mL reaction tube on a thermoshaker. The activities were analysed according to Section 3.4.

3.5.2. Stability of the Soluble RpPPK2-3 in Sodium Phosphate Buffer

To analyse the stability of the *Rp*PPK2-3 in sodium phosphate buffer, a storage stability study was performed. The enzyme was stored in sodium phosphate buffer with concentrations of 20, 250, 500 or 1000 mmol·L^{-1} at pH 7.8 with an enzyme concentration of 0.04 g·L^{-1} at 20 °C and 300 rpm in a 2 mL reaction tube on a thermoshaker. The activity was measured according to Section 3.4 at the beginning of the study and after 3 days of storage.

3.5.3. Reusability Studies of the Immobilisate

The reusability was analysed in duplicate with 10 mg of immobilised *Rp*PPK2-3 in a 2 mL reaction tube using a thermoshaker. The reaction was started by adding 1.5 mL of substrate solution (see Section 3.4). After 15 min, a sample of the reaction mixture was analysed for product formation at a conversion of 25% at the first use. The remaining substrate was then pipetted off, and the supports were washed twice with 20 mmol·L^{-1} sodium phosphate buffer at pH 7.4 and used for the next cycle or stored at 6 °C until the next experiment. For the next experiment, the wash buffer was removed by pipetting, and the experiment was started by adding the substrate as described above. For the screening experiment with different carriers, 20 replicate batches were performed. For the selected carrier (ECR8209M), 50 replicate batches were performed.

3.5.4. Storage Stability Studies of the Immobilisate

For the stability studies of the immobilisate, aliquots containing 10 mg of immobilised *Rp*PPK2-3 in 2 mL reaction tubes were stored under six different conditions: in 1 mL of 20 mmol·L^{-1} sodium phosphate buffer at pH 7.4 or water at 6 °C, in 1 mL of 20 mmol·L^{-1} sodium phosphate buffer at pH 7.4 at 20 °C with and without shaking at 300 rpm, and in 1 mL of 20 mmol·L^{-1} sodium phosphate buffer at pH 7.4 at 40 °C. The aliquots were stored for 200 days, and the activity was measured at regular time points over the storage period. At each time point, the initial activity was analysed using the standard activity assay (Section 3.4). The stability was assessed by the half-life, which is the time taken to halve the initial activity. The deactivation constant k_d was determined by exponential fitting, and the half-life time was calculated according to the following equation [39]:

$$t_{1/2} = \frac{\ln(2)}{k_d} \quad (4)$$

4. Conclusions

The industrial application of immobilised enzymes in a continuous reactor set-up requires a stable and leaching-free enzyme–carrier connection. For this reason, we have immobilised the enzyme *Rp*PPK2-3 by a covalent immobilisation technique. In an immobilisation screening with different amino methacrylate (pre-activated with glutaraldehyde) and epoxy methacrylate carriers, the epoxy methacrylate carrier ECR8209M (Purolite®) was selected. With this carrier, a high specific activity of 19.6 U·$g_{carrier}$$^{-1}$ was achieved. The analysed relatively low activity yield of 2.5% can be balanced by a high recyclability and strong stability of the immobilisate. After 50 cycles, the immobilised *Rp*PPK2-3 showed a 93% residual activity, and in a storage stability study, a long half-life of 462 days under storage conditions (6 °C) and 12 days at a higher temperature (40 °C) in sodium phosphate buffer was observed. Mechanical stress did not have an effect on the stability of the immobilised enzyme. In addition, the stability of the soluble enzyme was increased from 0.04 days to 14 days by adding the substrate PolyP to the enzyme solution. Therefore, it is

expected that the immobilisate will be additionally stabilised under reaction conditions. The high stability of the immobilisate demonstrates its qualification for long-term application in a continuous reactor set-up. In further studies, *Rp*PPK2-3 immobilised on epoxy methacrylate ECR8209M will be investigated in a CTP regeneration cascade in a continuous packed-bed reactor.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal14030165/s1, Figure S1: SDS-PAGE of *Rp*PPK2-3; Figure S2: Storage stability study of RpPPK2-3 in Tris buffer; Figure S3: Storage stability study of RpPPK2-3 in Tris buffer with added substrate; Figure S4: Stability study of RpPPK2-3 immobilised on epoxy methacrylate ECR8209M; Figure S5: HPLC chromatogram of samples taken at different times in the activity assay.

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