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Immobilization of Phenylalanine Ammonia-Lyase on Single-Walled Carbon Nanotubes for Stereoselective Biotransformations in Batch and Continuous-Flow Modes

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Dedicated to Professor János Rétey on the occasion of his 80th birthday.

Carboxylated single-walled carbon nanotubes ($SwCNT_{COOH}$) were used as a support for the covalent immobilization of phenylalanine ammonia-lyase (PAL) from parsley by two different methods. The nanostructured biocatalysts ($SwCNT_{COOH}$-PAL^{I} and $SwCNT_{COOH}$-PAL^{II}) with low diffusional limitation were tested in the batch-mode kinetic resolution of racemic 2-amino-3-(thiophen-2-yl)propanoic acid (**1**) to yield a mixture of (*R*)-**1** and (*E*)-3-(thiophen-2-yl)acrylic acid (**2**) and in ammonia addition to **2** to yield enantiopure (*S*)-**1**. $SwCNT_{COOH}$-PAL^{II} was a stable biocatalyst (>90% of the original activity remained after six cycles with **1** and after three cycles in 6 M NH_3 with **2**). The study of ammonia addition to **2** in a continuous-flow microreactor filled with $SwCNT_{COOH}$-PAL^{II} (2 M NH_3, pH 10.0, 15 bar) between 30–80 °C indicated no significant loss of activity over 72 h up to 60 °C. $SwCNT_{COOH}$-PAL^{II} in the continuous-flow system at 30 °C was more productive (specific reaction rate, $r_{flow} = 2.39$ μmol min^{-1} g^{-1}) than in the batch reaction ($r_{batch} = 1.34$ μmol min^{-1} g^{-1}).

Introduction

New biocatalytic methods are gaining ever more importance in organic synthesis on a laboratory and industrial scale as biocatalysts are selective, easy-to-handle, and environmentally friendly.[1] Catalytic activity, substrate, specificity, selectivity, and enzyme stability are key factors that affect the usefulness of biocatalysts.[1,2] Immobilization can often modify and improve the properties of enzymes and may also enable their recovery and reuse.[3,4] Immobilization may improve enzyme properties in several ways. It can provide a favorable environment for the enzyme, may increase its rigidity, or prevent subunit dissociation by multipoint, multi-subunit immobilization.[3,5] Immobilization may also modify thermal and operational stability, diffusion features, mechanical, and other properties of the native enzyme.[3,5] The covalent immobilization of enzymes is applied frequently to prepare carrier-bound biocatalysts.[6] The nature of the functionalities and linker length on the carrier surface can influence the properties of the resulting immobilized enzyme preparations.[7]

The nano-sized diameter of carbon nanotubes (CNTs) with micron-sized length offers a support with a large available surface, low diffusion limitation, and easy recovery. Thus, CNTs are used widely for the immobilization of bio-macromolecules, which exploits their mechanical, thermal, and electrical properties and general biocompatibility.[8] Covalent and noncovalent immobilization of enzymes on CNTs is often a key step in their biological applications.[9] However, claims of covalent attachment have often been unfounded and immobilization was only caused by adsorption.

Although noncovalent immobilization preserves the structure of both the nanomaterial and the enzyme well, a gradual leak of the enzyme from the CNT cannot be excluded.[7] Immobilization by physical adsorption on CNTs can be effected by hydrophobic interactions,[10] electrostatic interactions,[11] or hydrogen bonding[12] and can be modulated by surfactants (such as Triton-X100[13] or sodium cholate[14]) or adsorbed polymer

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layers[15,16] (such as chitosan,[17] sulfonated polyaniline,[18] and poly(sodium 4-styrenesulfonate)[19]).

The direct covalent linking of an enzyme onto CNTs results in more stable immobilization but may reduce enzyme activity. Covalent immobilization onto carboxylated CNTs has been performed by diimide-activated amidation with the surface-exposed amino groups of the proteins.[20,21] Lipases[22,23] and other enzymes have been immobilized in this way.[23,24]

The immobilization of enzymes onto CNTs has also been performed using linker molecules such as *N*-succinimidyl 1-pyrenebutanoate for horseradish peroxidase[25] and glucose oxidase,[26] 1-aminopyrene for laccase[27] or polyethylene glycol for perhydrolase,[28] and glutardialdehyde was applied as a crosslinking agent for lipases and esterases.[8]

In the past decades several so-called enabling technologies have been developed that have transformed the methodology of organic synthesis.[29] The development of heterogenized homogeneous catalysts and the use of new reactor designs such as continuous-flow micro- and minireactors are important trends in enabling technologies.[29] Although continuous-flow systems allow the rapid preparation of compounds with minimum workup and are often superior to batch reactions,[29,30] microreactor technology using immobilized biocatalysts embedded in a structured flow-through reactor is still a rather unexplored platform for biotransformations.[31] Various enzyme-catalyzed biotransformations have been performed in different continuous-flow mini- and microreactors,[31] but the usefulness of packed-bed continuous-flow bioreactors to study the effects of temperature, pressure, and flow rate have been mainly demonstrated for lipase-catalyzed kinetic resolutions.[32]

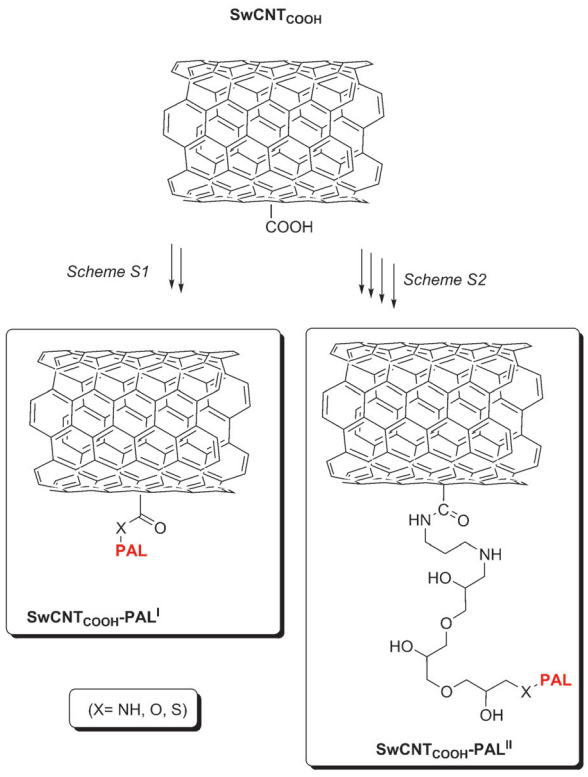
Phenylalanine ammonia-lyases (PAL; EC 4.3.1.24 and EC 4.3.1.25) are homotetrameric enzymes that perform the nonoxidative deamination of L-phenylalanine into (*E*)-cinnamic acid.[33,34] In plants PALs play a key role as the starting point of the phenylpropanoid pathway.[35] PAL is also present in fungi[36] and in some bacteria.[37] In medical applications PAL has shown potential in the enzyme-replacement therapy of phenylketonuria (PKU)[38] and may be a therapeutic enzyme in cancer treatment.[39] PALs of plant and yeast origin are also useful as biocatalysts in the preparation of various unnatural L- and D-α-amino acids.[34,40] The synthetic potential of PAL as a biocatalyst has been utilized in the production of L-phenylalanine (L-Phe) and (*S*)-2-chlorophenylanine by the addition of ammonia to (*E*)-cinnamic acid[41] and (*E*)-2-chlorocinnamic acid,[42] respectively. Although immobilized whole cells that contain PAL were applied in the continuous-flow mode for the production of L-Phe,[43] the behavior of an immobilized PAL in a continuous-flow microreactor has never been explored. This may be because native PALs were immobilized by entrapment in microcapsules,[44] as cross-linked enzyme aggregates (CLEAs),[45] as bovine serum albumin (BSA) co-CLEAs,[46,47] and cross-linked films.[48] None of these systems could be used in continuous-flow packed-bed columns because of their inappropriate shape, particle size, and mechanical properties. Mesoporous silica gel as a support material for cross-linked PAL (MSG-CLEA) improved this situation,[49] but this form still suffered from diffusion limitations. Nanofibrous single-walled carbon nanotubes (SwCNTs) with micron-sized length, however, are ideal supports in a packed-bed microreactor because of their large available surface with quite low diffusion limitation and low hydrostatic resistance.

In this study our aim was the covalent immobilization of PAL from *Petroselinum crispum* (*Pc*) on carboxylated single-walled carbon nanotubes ($SwCNT_{COOH}$) to prepare PAL biocatalysts with improved properties for continuous-flow applications. This study demonstrates for the first time the usefulness of SwCNT-PALs as a convenient biocatalyst for stereoselective biotransformations both in batch mode and in continuous-flow reactors. Moreover, the temperature-dependent properties and durability of SwCNT-PALs were also characterized.

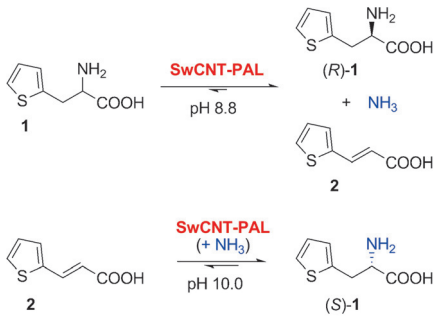
Results and Discussion

The aim of this study was to immobilize PAL covalently on functionalized SwCNTs and to test the activity and reusability of the resulting immobilized PAL in reactions performed in both batch and continuous-flow modes.

For this purpose SwCNTs functionalized with carboxy groups were tested ($SwCNT_{COOH}$; Scheme 1 and Schemes S1 and S2). The immobilized SwCNT-PALs obtained in this way were tested in ammonia elimination and addition reactions using racemic 2-amino-3-(thiophen-2-yl) propanoic acid (**1**) and 3-(thiophen-2-yl)acrylic acid (**2**) as substrates (Scheme 2 and Table 1).



Scheme 1. Covalent immobilization of *Pc*PAL on $SwCNT_{COOH}$. See Schemes S1 and S2 in the Supporting Information for details.



Scheme 2. SwCNT-PALs for ammonia elimination from **1** and ammonia addition to **2**.

Table 1. Conversion of the reactions catalyzed by the two forms of *Pc*PAL immobilized on $SwCNT_{COOH}$ (25 °C, 17 h).

Run	Elimination from **1** (pH 8.8, Tris buffer) Conversion [%]		Addition to **2** (4.5 mM in 6 M NH_3, pH 10.0) Conversion [%]	
	$SwCNT_{COOH}$-PAL^{I}	$SwCNT_{COOH}$-PAL^{II}	$SwCNT_{COOH}$-PAL^{I}	$SwCNT_{COOH}$-PAL^{II}
1	48.4	49.2	36.9	36.7
2	49.2	48.5	24.2	37.2
3	49.0	48.8	3.8	36.2
4	49.3	47.4	–	34.2
5	45.6	46.3	–	17.5
6	43.7	42.4	–	5.7
7	42.5	42.3	–	2.2

Covalent immobilization of PAL on $SwCNT_{COOH}$

In our first attempt, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC·HCl) was used for the activation of carboxylic groups on the surface of $SwCNT_{COOH}$ (Scheme 1 and Scheme S1). Although the immobilization yield was quite high (89.5% of the *Pc*PAL was bound to $SwCNT_{COOH}$), the product obtained by the diimide-activation approach ($SwCNT_{COOH}$-PAL^{I}) was not stable under the harsh conditions (6 M NH_3) of the ammonia addition reaction to **2**. A TEM investigation revealed the almost complete removal of PAL from the surface after only two cycles (Figure S1). These results may be rationalized by assuming that in the "direct" immobilization that leads to $SwCNT_{COOH}$-PAL^{I} the durable amide bonds were formed only as a minor fraction besides other weaker bonds.

Recently, glycerol diglycidyl ether (GDE) has been applied as a cross-linker to prepare PAL CLEAs superior to those cross-linked with glutardialdehyde.[49] Thus, with the aim to create more stable SwCNT-PAL immobilized covalently with only amide bonds from the carboxylic acids on $SwCNT_{COOH}$, GDE was used as the linker to attach *Pc*PAL onto the surface of propane-1,3-diamine-functionalized $SwCNT_{COOH}$ (Scheme 1 and Scheme S2). Fortunately, a reproducible and high immobilization yield (95.5±1.5% of the *Pc*PAL bound to $SwCNT_{COOH}$), a high activity yield (61±1.3%, determined by L-Phe at 30 °C, for 10 min in Tris buffer at pH 8.8[42i]), and stable covalent bonds (see TEM data after recycling in Figure S2) were characteristic of this product ($SwCNT_{COOH}$-PAL^{II}).

After the PAL adsorbed on $SwCNT_{COOH}$ was washed with distilled water, no *Pc*PAL activity remained (Section 2.1 and Figure S2 in the Supporting Information), which is indirect support for the presence of a durable bond in $SwCNT_{COOH}$-PAL^{II}.

Biocatalytic behavior of SwCNT-PALs in stereoselective biotransformations of 1 and 2 in batch mode

Next, the SwCNT-PALs (Scheme 1) were tested as biocatalysts in ammonia elimination from **1** and ammonia addition to **2** (Scheme 2) in batch mode at room temperature.

$SwCNT_{COOH}$-PALs showed high conversions (in the kinetic resolution from racemic **1** close to the theoretically possible 50% conversion) and could be reused several times without significant loss of their initial activity (Table 1). Not surprisingly, under the much harsher conditions required for ammonia addition to **2** (6 M ammonia, at pH 10) the SwCNT-PALs were much less durable (Table 1). Among the two forms, $SwCNT_{COOH}$-PAL^{II} enabled the most recycling and retained more than 85% of the initial activity even after four cycles (Table 1 and Figure 1).

The initial conditions of ammonia addition to **2** (6 M ammonia, at pH 10) were too harsh even for the durable $SwCNT_{COOH}$-PAL^{II}. As earlier studies indicated that ammonia addition can be performed at lower ammonia concentrations,[39,41] the concentration was decreased to 3 and 2 M. $SwCNT_{COOH}$-PAL^{II} exhibited a significantly higher stability at lower ammonia concentrations. In 2 M ammonia more than 80% of the initial activity was retained even after 12 cycles (Figure 1).

In contrast to non-immobilized *Pc*PAL, the activity of which in ammonia addition to **2** in 2 M NH_3 was approximately 50% of that in 6 M ammonia solution, a significantly lower decrease of activity (only to 77%) was observed with $SwCNT_{COOH}$-PAL^{II} if the ammonia concentration was decreased from 6 to 2 M. As the conversion of **2** into enantiopure (*S*)-**1** in the reaction with 2 M ammonia (23% conversion, after 6 h) was comparable with that in 6 M ammonia (30% conversion, after 6 h), further studies were conducted in 2 M ammonia.

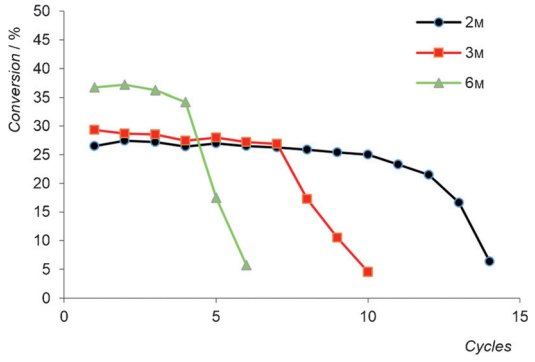


Figure 1. Recyclability of $SwCNT_{COOH}$-PAL^{II} in batch-mode ammonia addition to **2** (4.5 mM) at various ammonia concentrations (2, 3, and 6 M; pH 10.0; 25 °C; 17 h).

Investigation of the ammonia addition reaction to 2 catalyzed by SwCNT$_{COOH}$-PAL^{II} in a continuous-flow packed-bed microreactor

Continuous-flow ammonia addition to **2** was studied in a microreactor system (SynBioCart) that included a biocompatible packed-bed polytetrafluoroethylene (PTFE) column filled with SwCNT$_{COOH}$-PAL^{II}. The bioreactor unit was placed in the thermostatted column compartment of an HPLC system with full control of pressure and temperature (Figure 2).

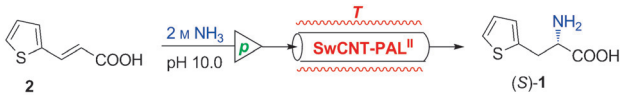


Figure 2. Ammonia addition to **2** (4.5 mM) catalyzed by SwCNT$_{COOH}$-PAL^{II} in a continuous-flow packed-bed microreactor (column: 30 mm × 3 mm ID; flow rate: 0.1 mL min^{-1}).

Our first goal was to compare the specific reaction rate of ammonia addition to **2** with our most durable SwCNT$_{COOH}$-PAL^{II} biocatalyst at 30 °C in batch and continuous-flow modes.

Initial studies on ammonia addition to **2** in continuous-flow mode without backpressure indicated a rapid apparent deactivation of the enzyme (probably because of bubble formation), which could be restored by washing the reactor with distilled water (Figure S3). This could be avoided by using backpressure in further experiments (15 bar, no apparent PAL deactivation was observed as a result of the pressure).

In a continuous-flow reactor the specific reaction rate (r_{flow}) is a possible measure of the biocatalyst productivity. This can be calculated from the product concentration ([*P*], μmol ml^{-1}), the flow rate (*f*, mL min^{-1}), and the mass of the biocatalyst (m_e, g) according to $r_{flow} = ([P] \times f)/m_e$.[32a]

A stirred (or shake flask) batch reaction can also be characterized by the specific reaction rate (r_{batch}) that can be calculated from the amount of product (n_p, μmol), the reaction time (*t*, min), and the mass of the biocatalyst (m_e, g) according to $r_{batch} = n_p/(t \times m_e)$.[32a]

Although these specific reaction rates can be calculated at any degree of conversion (*c*), rigorous comparisons between the productivity of an immobilized biocatalyst in continuous-flow reaction and its batch-mode counterpart using their *r* values can only be made at the same degree of conversion because the rate of product formation is not a linear function of *c* in many cases.[32a] Thus, the batch- and continuous-flow-mode reactions in 2 M ammonia (pH 10, at 30 °C) were compared at 58% conversion (which is far enough from the >90% equilibrium conversion of the ammonia addition to **2**[40d] if the ammonia concentration is above 1 M). As expected, the specific reaction rate with SwCNT$_{COOH}$-PAL^{II} in the continuous-flow reactor ($r_{flow} = 2.39$ μmol min^{-1} g^{-1}) was significantly higher than that in the batch reaction ($r_{batch} = 1.34$ μmol min^{-1} g^{-1}). If we take the known protein content of SwCNT$_{COOH}$-PAL^{II} into account, the productivity of *Pc*PAL (in 2 M ammonia, pH 10.0, 30 °C, 58% conversion) was 27.5 μmol min^{-1} g^{-1} in continuous-flow mode and 15.4 μmol min^{-1} g^{-1} in batch mode. As a result of the beneficial mass transfer situation, the productivity of PAL in SwCNT$_{COOH}$-PAL^{II} in the continuous-flow system was even higher than the specific activity of the native *Pc*PAL (25.8 μmol min^{-1} g^{-1}) with the same substrate (**2**) in batch mode under the same conditions.

Next, the influence of the temperature on ammonia addition to **2** was investigated in the temperature range of 30–80 °C (Figure 3).

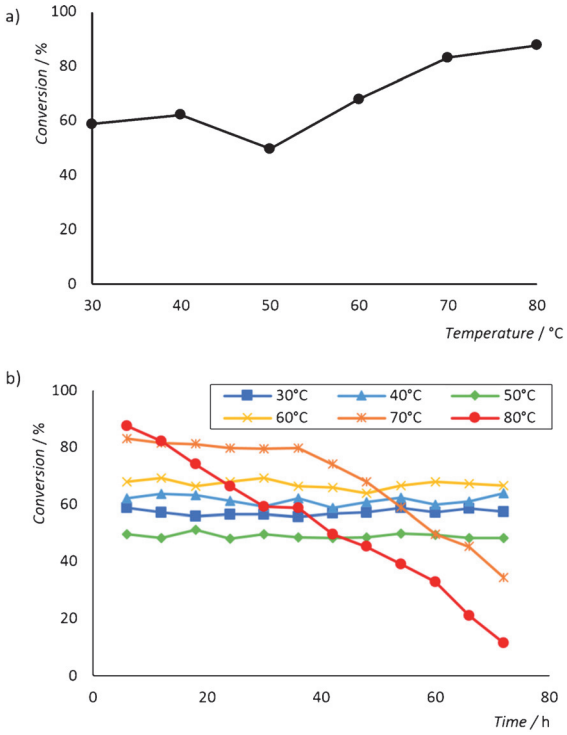


Figure 3. Effect of temperature between 30–80 °C on a) the initial conversion (stationary state, 2 h after starting the run) and b) the long-term stability in ammonia addition to **2** (4.5 mM) catalyzed by SwCNT$_{COOH}$-PAL^{II} in a continuous-flow microreactor (2 M ammonia, pH 10, 30 °C, under 15 bar backpressure).

Notably, also in a continuous-flow reactor, the conversion remained constant even after 72 h of operation and at temperatures up to 60 °C, which indicates the remarkably improved durability of SwCNT$_{COOH}$-PAL^{II} in 2 M ammonia (Figure 3b).

As the conversions depend primarily on the activity of the biocatalyst, a maximum value of the conversions at a so-called "optimum temperature" was expected. Accordingly, the activity of immobilized PAL increased if the temperature of the continuous-flow system was increased from 30 to 40 °C (Figure 3a). Unexpectedly, if the temperature was increased beyond 40 °C, a local minimum of the initial conversion was observed at around 50 °C (Figure 3a). This is why the conversion was significantly lower at 50 °C than at 40 or 30 °C (Figure 3b). This apparent decrease of PAL activity at 50 °C cannot be attributed to irreversible thermal inactivation because the level of activity at 50 °C remained stable over 72 h. This was also indicated by the run at 60 °C in which the conversion remained stable over 72 h and was higher than that observed in runs between 30

and 50 °C. At 70 and 80 °C, however, although the initial activity of the biocatalyst increased with temperature, the enzyme was deactivated rapidly and irreversibly over time.

To correlate temperature-dependent activity changes with the conformational behavior of *Pc*PAL, the soluble form of *Pc*PAL was investigated by circular dichroism (CD) spectroscopy (Figure 4).

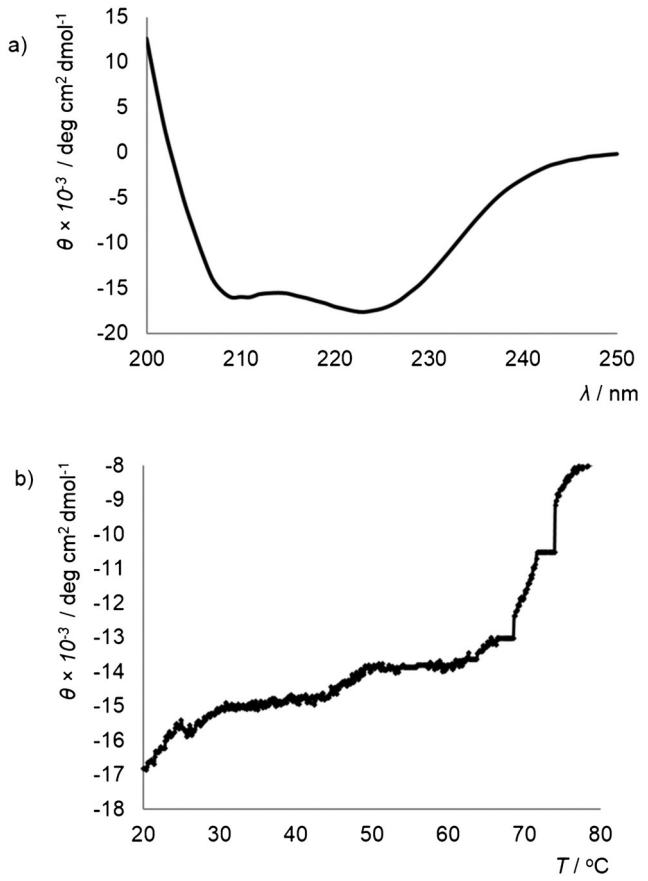


Figure 4. a) CD spectrum of *Pc*PAL at 25 °C and b) temperature dependence of the signal at 210 nm, which is sensitive to the α-helical structure between 30 and 80 °C.

A temperature-dependent CD study revealed a change of conformation between 45 and 50 °C (Figure 4b). This change can be related to the decreased reaction rate of $SwCNT_{COOH}$-PAL^{II} in the continuous-flow reactions at around 50 °C (Figure 3). However, if we take into account the fact that the activity of *Pc*PAL was stable over several days at 50 °C or even at 60 °C in continuous-flow mode, the slight conformational change in *Pc*PAL between 45 and 50 °C is not detrimental to the stability.

Differential scanning fluorimetry (DSF, also known as Thermofluor) measurements of native *Pc*PAL in the absence (melting temperature, $T_m = 73.5$ °C) and presence of **2** ($T_m = 75.0$ °C; Figure S4) were in agreement with the CD results, which indicate that the native enzyme is thermally stable up to 60 °C.

Conclusions

Our study demonstrated that carboxy-functionalized single-walled carbon nanotubes ($SwCNTs_{COOH}$) can be applied efficiently as a nanostructured support for the covalent immobilization of phenylalanine ammonia-lyase (PAL) from *Petroselinum crispum*. The most durable biocatalysts were obtained using carboxy-functionalized SwCNT as the support and glycerol diglycidyl ether as a linker molecule for PAL immobilization. The biocatalyst prepared in this way ($SwCNT_{COOH}$-PAL^{II}) was efficient in the preparation of (*R*)-2-amino-3-(thiophen-2-yl)propanoic acid [(*R*)-**1**] by the kinetic resolution of racemic **1** or its enantiomer (*S*)-**1** by the enantioselective ammonia addition to (*E*)-3-(thiophen-2-yl)acrylic acid (**2**). Recycling studies revealed that $SwCNT_{COOH}$-PAL^{II} retained more than 80% of its original activity after seven cycles in ammonia elimination from **1** and 12 cycles in ammonia addition to **2** (in 2 M NH_3). Studies with a $SwCNT_{COOH}$-PAL^{II}-filled packed-bed continuous-flow microreactor in the 30–80 °C temperature range showed an apparent decrease of PAL activity at 50 °C, which could be rationalized by a conformational change of PAL. Unaltered PAL activity for ammonia addition to **2** could be maintained for at least 72 h and at up to 60 °C in 2 M NH_3 in a packed-bed continuous-flow microreactor. The major advantage of this new type of immobilization is not a large enhancement of the thermostability of native PAL but the easy recovery of the PAL biocatalyst from batch reactions and the usefulness of this form as a biocatalyst packing in columns for biotransformations in the continuous-flow mode.

Experimental Section

The sources of materials and enzymes and details for PAL immobilization on $SwCNT_{COOH}$ are provided as Supporting Information.

Ammonia elimination from 1 with SwCNT-PALs in batch mode

$SwCNT_{COOH}$-PAL (6 mg) was added to a solution of racemic **1** (0.8 mg, 4.7 mM) in Tris buffer (0.1 M, pH 8.8, 1 mL). The resulting mixture was shaken (1250 rpm) at RT for 17 h. The conversion of the reactions was calculated from the ^{1}H NMR spectra of the residue after the removal of the solvent. After 17 h, the product was filtered, the filtrate was concentrated, and the immobilized enzyme was washed with aqueous buffer and reused under the same conditions.

Ammonia addition to 2 with SwCNT-PALs in batch mode

SwCNT-PAL (6 mg) was added to a solution of **2** (0.7 mg, 4.5 mM) in ammonia solution (6 M, 1 mL, pH set to 10 with CO_2), and the mixture shaken (1350 rpm) for 17 h at RT. The conversion of the reactions was calculated from the ^{1}H NMR spectra of the residue after the removal of the solvent. After 17 h, the product was filtered, the filtrate was concentrated, and the immobilized enzyme was washed with aqueous buffer and reused under the same conditions.

Reactions with the SwCNT-PALs in 3 and 2 M ammonia solutions (1 mL, pH set to 10 with CO_2) were performed similarly.

Ammonia addition to 2 with $SwCNT_{COOH}$-PAL^{II} in continuous-flow packed-bed microreactor

Continuous-flow experiments were performed in a thermostatted SynBioCart system (30 mm × 3 mm ID PTFE SynBioCart columns filled with 110 mg of $SwCNT_{COOH}$-PAL^{II} in a polyether ether ketone (PEEK) SynBioCart column holder). The SynBioCart system was attached to the pump module of an Agilent LC 1150 HPLC system and a backpressure regulator valve (VICI AG, JR-BPR1). Before the reaction, the columns were washed with 2 M ammonia solution (pH set to 10 with CO_2) at 0.5 mL min^{-1} for 20 min.

A solution of **2** (4.5 mM) in 2 M ammonia solution (pH set to 10 with CO_2) was pumped through the $SwCNT_{COOH}$-PAL^{II}-filled column at various temperatures (25–80 °C) at a flow rate of 0.1 mL min^{-1}. After reaching a stationary state (usually 30 min), 10 μL samples were withdrawn every 10 min and analyzed by using a UV spectrophotometer (in 990 μL 2 M ammonia solution, at 290 nm). In the first series of experiments at RT (25 °C), no backpressure was applied. In the temperature effect studies between 30 and 80 °C, the backpressure was set to 15 bar.

After the experiments between 25–60 °C, the $SwCNT_{COOH}$-PAL^{II}-filled column was washed with distilled water (0.5 mL min^{-1}, 30 min) and stored at 4 °C. For the experiments at 70 and 80 °C, a separate $SwCNT_{COOH}$-PAL^{II}-filled column was applied at each temperature.

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Keywords: biotransformations · enzyme catalysis · immobilization · nanotubes · supported catalysts

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