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Subtilisin integrated artificial plant cell walls as heterogeneous catalysts for asymmetric synthesis of (*S*)-amides†

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Subtilisin integrated artificial plant-cell walls (APCWs) were fabricated by self-assembly using cellulose or nanocellulose as the main component. The resulting **APCW** catalysts are excellent heterogeneous catalysts for the asymmetric synthesis of (*S*)-amides. This was demonstrated by the **APCW**-catalyzed kinetic resolution of several racemic primary amines to give the corresponding (*S*)-amides in high yields with excellent enantioselectivity. The **APCW** catalyst can be recycled for multiple reaction cycles without loss of enantioselectivity. The assembled **APCW** catalyst was also able to cooperate with a homogeneous organoruthenium complex, which allowed for the co-catalytic dynamic kinetic resolution (DKR) of a racemic primary amine to give the corresponding (*S*)-amide in high yield. The **APCW**/Ru co-catalysis constitutes the first examples of DKR of chiral primary amines when subtilisin is used as a co-catalyst.

1 Introduction

Chiral compounds have a key role in the chemical industry. Most of the active substances in drugs and natural products are optically active.[1] Although they have the same chemical structure, enantiomers of chiral drugs can exhibit marked differences in biological activity. This is due to the fact that biomolecules (*e.g.* amino acids, sugars, nucleosides, nucleotides, *etc.*) of the biosynthetic apparatus, which produce our proteins, receptors and nucleic acids, are assembled as single enantiomers. During the past four decades many strategies were developed to direct the outcome of organic reactions toward enantiomerically pure products. Enzymes are well known to be powerful tools for the synthesis of chiral molecules with an extremely high degree of stereoselectivity.[2] Amides are important functional groups in organic synthesis and they occur in around 35% of all drugs.[3] An important way to get access to enantiopure amines and amides is the enzyme-mediated kinetic resolution (KR) of racemic primary amines. The stability, activity and selectivity of the enzyme *Candida antarctica* lipase B makes it one of the most common enzymes employed for this purpose in industry and academia. Lipases are usually *R*-selective, directing the stereoselectivity outcome of the amidation reactions only toward the *R*-enantiomer. Extensive studies were done by Bäckvall regarding the use of CALB in the synthesis of hybrid heterogeneous enzyme-metal catalysts for the dynamic kinetic resolution (DKR) of primary amines.[4,5] Some significant examples are the cross-linked enzyme aggregate (CLEA), where CALB itself constitutes the heterogeneous support used as a matrix to incorporate palladium metal nanoparticles[4b,c] and the immobilization of CALB and palladium nanoparticles on the cavities of siliceous mesocellular foam.[4a] Subtilisin[6] provides a complementary stereoselectivity to the lipase-catalyzed kinetic resolution since the stereospecificity of subtilisin is opposite to that of lipases.[7] One of the drawbacks for the use of this enzyme was shown to be the decrease in activity and selectivity in organic solvents compared to the much more stable lipases. Due to this intrinsic structural problem, the use of subtilisin as catalyst was mainly limited to the kinetic resolution of secondary alcohols.[7] More recently, alcohols were used as substrates for the subtilisin co-catalyzed DKR of alcohols.[8] However, subtilisin has so far not been used for the DKR of primary amines. With respect to the kinetic resolution of chiral amines, only few papers were published until now. In 1989 Klibanov, in his seminal work, showed the possibility to use subtilisin for the stereoselective amidation of secondary amines.[9] It was found that the enantioselectivity factor was strongly influenced by the nature of the reaction medium.[9] Inspired by the Klibanov article, Gutman subsequently reported a continuous-flow bioreactor, where subtilisin was immobilized on glass beads, for the kinetic resolution of 1-(L-naphthy1)ethylamine.[10] In 2009, a large screening on different classes of proteases, to determine the best acyl donor for the kinetic resolution of 3-amino-1-phenylbutane, was made.[11] The results were then correlated to an automated

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docking determination of the binding affinity for subtilisin.[11] In 2016, the construction of a continuous flow cascade reactor for the dynamic kinetic resolution of racemic *N*-Boc-phenylalanine ethyl thioester with benzylamine using subtilisin supported on macroporous silica gels was disclosed.[12] To circumvent the decrease in reactivity of biomolecules in non-aqueous media a large variety of organic and inorganic materials were tested as heterogeneous solid supports.

The greatest advantage of immobilization is the significant improvement of the stability of the enzyme by protecting it from undergoing denaturation at elevated temperatures and harsh reaction conditions. Another significant advantage is the ease of isolation and the reusability of the catalyst over successive catalytic cycles. Many different materials such as silica,[13] inorganic oxides,[14] resins,[15] bio-[16] and synthetic[17] polymers were taken in consideration as scaffolds. Various immobilization techniques have been also developed, including adsorption, covalent binding, entrapment, encapsulation and cross-linking.[18] The transition from petrochemical-based raw materials to biomass-based materials have attracted the interest of the scientific community during the last decades.[19] Natural polymers received considerable attention because of their abundance, biocompatibility, bio-degradability and non-toxicity.[20] Cellulose is the most abundant biopolymer in the world. Its use as a solid support, in the field of heterogeneous catalysis, offers great advantages such as great absorption capacity, good chemical stability, and insolubility in common solvents.[21] We have been involved in the valorization of biomass, heterogeneous catalysis and on the construction of functional cellulose-based materials.[22–24] Recently, we disclosed a concept for how to assemble artificial plant cell walls (**APCW**) containing lipase or lipase and Pd nanoparticles within a polysaccharide-based matrix (cellulose) with a multilayer architecture.[24] Driven by the successful asymmetric synthesis of (*R*)-amides by this concept, we became intrigued if we could assemble **APCW** with activity for the asymmetric synthesis of chiral (*S*)-amides using subtilisin as enzyme and cellulose as the polysaccharide carrier component. Here we disclose the self-assembly of recyclable APCWs that operates as a highly enantioselective catalyst for the asymmetric synthesis of (*S*)-amides (Fig. 1). The APCW's co-catalysis with a Ru-complex allowed for the first examples of DKR of primary amines with a catalyst containing a protease.

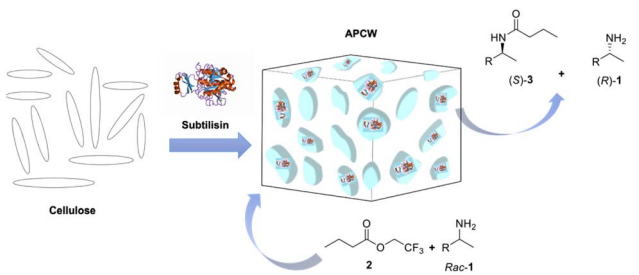


Fig. 1 Simplified scheme of self-assembly of **APCW** and kinetic resolution of racemic amines **1**.

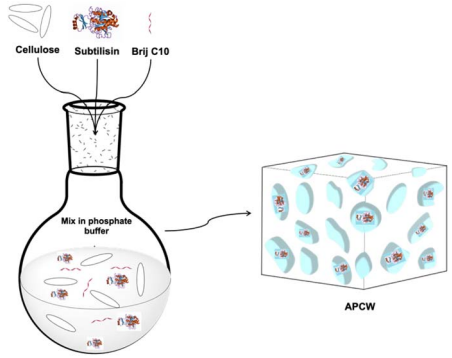


Fig. 2 Self-assembly of **APCW**.

2 Experimental

2.1 Self-assembling of APCW catalyst

APCW was self-assembled as shown in Fig. 2. To cellulose (60 mg), dispersed in sodium phosphate buffer (6 mL, 0.1 M, pH 7.2), Brij C10 (20 mg) was added. Brij was selected as the surfactant since it has a beneficial effect for enhancing subtilisin's and CALBs activity in kinetic resolutions of secondary alcohols and primary amines.[8,24] The suspension was stirred with a spatula until completely solubilization of Brij. Next subtilisin (20 mg) was added, the mixture was stirred with a spatula until completely solubilization of the enzyme and rapidly frozen in liquid nitrogen. The catalyst was lyophilized for 70 hours to give **APCW** as a solid white foam.

2.2 General procedure for the KR of *rac*-1

In a microwave vial, was added **APCW**, solvent (1 mL), amine *rac*-**1** (0.25 mmol, 1 equiv.) and 2,2,2-trifluoroethyl butyrate **2** (85 mg, 0.5 mmol, 2 equiv.). The vial was sealed and flushed with nitrogen. The reaction was stirred at room temperature for the time reported in the table. The reaction mixture was directly purified by column chromatography (hexane : ethylacetate) to afford the desired product (*S*)-**3** in the reported yield and ee.

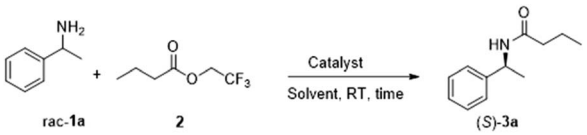
2.3 General procedure for the DKR of *rac*-1 catalyzed by APCW4 and SHVO catalyst

In a microwave vial was added the **APCW4**, 4-dimethyl-3-pentanol (1 mL), Shvo catalyst, amine *rac*-**1** (0.25 mmol, 1 equiv.), 2,2,2-trifluoroethyl butyrate **2** (85 mg, 0.5 mmol, 2 equiv.) and Na_2CO_3. The vial was sealed and an argon balloon was connected. The reaction was stirred at 90 °C for the time reported in the table. The reaction mixture was directly purified by column chromatography (hexane/ethyl acetate 1 : 1) to afford the corresponding amide (*S*)-**3** in the reported yield and ee.

3 Results and discussion

We began our research by screening the influence of the different components of **APCW** and tuning their ratio to find an optimal system for the kinetic resolution of amine **1a**. As shown in the screening table, subtilisin itself is almost inactive in 3-

Table 1 Screening of the **APCW**-catalyzed kinetic resolution[a]



Entry	**APCW** (assembled components)	Solvent	Time [h]	Yield **3a** [b] [%]	ee **3a** [c] [%]
1[d]	—	3-Methyl 3-pentanol	168	0	ND
2[d,e]	Subtilisin	3-Methyl 3-pentanol	168	8.5	ND
3	Sub/Brij (1 : 1)	3-Methyl 3-pentanol	23	42	85
4	**APCW1** CNC/Sub (1 : 1)	3-Methyl 3-pentanol	42	46	82
5	**APCW1**	THF	46	37	45
6[f]	**APCW2** CNC/Sub/Brij (1 : 1:1)	THF	23	55	51
7	**APCW2**	3-Methyl 3-pentanol	19	47	82
8	**APCW2**	Amyl alcohol	42	40	75
9	**APCW2**	Toluene	90	26	17
10	**APCW2**	MTBE	46	35	35
11	**APCW2**	Dioxane	17	42	64
12	**APCW3** MCC/Sub/Brij (1 : 1:1)	3-Methyl 3-pentanol	22	39	87
13	**APCW4** MCC/Sub/Brij (3 : 1:1)	3-Methyl 3-pentanol	23	44	86
14[g]	**APCW4**	3-Methyl 3-pentanol	70	35	90
15[h]	**APCW4**	3-Methyl 3-pentanol	45	43	86
16[i]	**APCW4**	3-Methyl 3-pentanol	45	37	88
17	**APCW4**	2,4-Dimethyl-3-pentanol	24	48	90
18	**APCW5** NFC/Sub/Brij (3 : 1:1)	3-Methyl 3-pentanol	27	44	85
19	**APCW6** MCC/Sub/Brij (3 : 1:3)	3-Methyl 3-pentanol	21	46	84
20	**APCW6**	2-Ethylhexanol	20	0	ND
21[j]	**APCW6**	3-Methyl 3-pentanol	72	5	ND
22	MCC/CNC or NFC	3-Methyl 3-pentanol	48	0	ND

[a] Reaction conditions: **1a** (0.25 mmol, 1 equiv.), **2** (0.5 mmol, 2 equiv.), catalyst (9 mg), solvent (1 mL), room temperature. [b] Isolated yield. [c] Determined by chiral HPLC. [d] Yield determined by ^{1}H-NMR. [e] Lyophilized subtilisin 2.2 mg used. [f] Catalyst 18 mg used. [g] Reaction performed at 4 °C. [h] Na_2CO_3 was added (0.26 mmol). [i] NaOAc was added (0.26 mmol). [j] Ethyl butyrate used as acyl donor. ND stands for not determined. **APCW** = artificial cell wall, MCC = microcrystalline cellulose, CNC = cellulose nanocrystals, NFC = nanofibrilated cellulose, Brij = polyethylene glycol hexadecyl ether (Mn = 683).

methyl-3-pentanol (Table 1, entry 2) confirming what was reported in the literature and by the kinetic experiments (Fig. 3). Modifying subtilisin with a surfactant polyethylene glycol hexadecyl ether (Brij) led to an increased activity of the enzyme (Table 1, entry 3). The main drawback of this system is that it cannot be recycled and used for multiple reactions. The use of cellulose nanocrystals (CNC) as a support material also led to a similar increase of the activity (Table 1, entry 4). The results show that the nanocellulose alone can activate the subtilisin (entries 4 and 5). This is interesting since Park and Kim have demonstrated that dextrin does not improve the activity of subtilisin in kinetic resolutions of secondary alcohols.[8d] The assembly of **APCW2** (CNC, subtilisin, Brij) was successful and turned out to be better than the previously tested systems. Different solvents were screened for the kinetic resolution of amine 1a (entries 6–11) and 3-methyl 3-pentanol appeared to be the best reaction medium (entry 7).

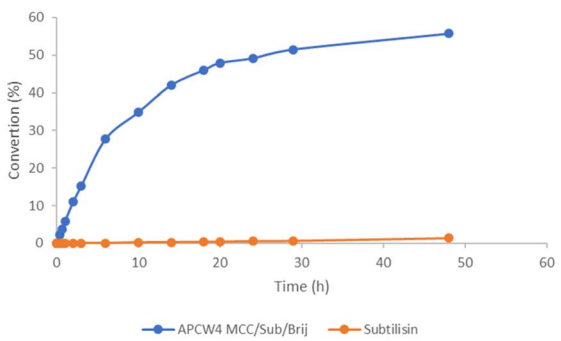


Fig. 3 Monitoring the formation of amide (*S*)-**3a** as a function of time for the amidation of *rac*-**1a** using subtilisin and modified subtilisin with structural components in 2,4-dimethyl-3-pentanol.

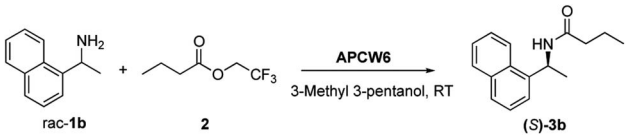
Switching the cellulose source from CNC to MCC (microcrystalline cellulose) for the assembly of **APCW3** gave a better result in terms of enantioselectivity. This could be due to the more robust structure of the microcrystalline cellulose that offers an extra degree of protection for the enzyme (entry 12). The tuning of the ratio between the components for the self-assembly of **APCW4** led to an increase in the yield (entry 13). Lowering the temperature from 20 °C to 4 °C resulted in a slower reaction and required an increased reaction time (entry 14). Adding Na_2CO_3 or NaOAc as additives did not show any significant improvement (entries 15–16). Switching to 2,4-

dimethyl-3-pentanol as solvent gave the best result in terms of yield and enantioselectivity (entry 17). NFC (nanofibilated cellulose) was also investigated as a polysaccharide component for the self-assembly of **APCW5** (entry 18). The APCW 5-catalyzed KR of **1a** gave results similar to when CNC and MCC were used as components (entries 7 and 13), which demonstrates the broad scope of the artificial plant cell wall concept.

Once the reaction condition had been optimized, the kinetic resolution of different racemic secondary amines was investigated (Scheme 1). Benzylic amines **1a–1e** gave the corresponding *S*-amides **3a–3e** in high yield and good ee. The absolute stereochemistry of the products was determined by comparison with the literature.[9] Amines with an electron-withdrawing group (**1c**) or a methyl group (**1d**) in the *p*-position of the aromatic ring were well tolerated. Even rigid system such as **1e** and aliphatic amine **1f** gave the corresponding (*S*)-amide **3** with high ee. Bulky amine **1b** bearing a naphthyl ring gave the best yield and ee. Since lifetime and recycling of heterogeneous catalysts have a key role for practical applications, we evaluated the efficiency of **APCW** conducting recycling experiments on the kinetic resolution of racemic 1-(1-naphthyl) ethylamine **1b**. For example, **APW6** was recycled 8 times without any significant loss in activity giving the corresponding (*S*)-**3b** in high yield and enantiomeric excess (Table 2).

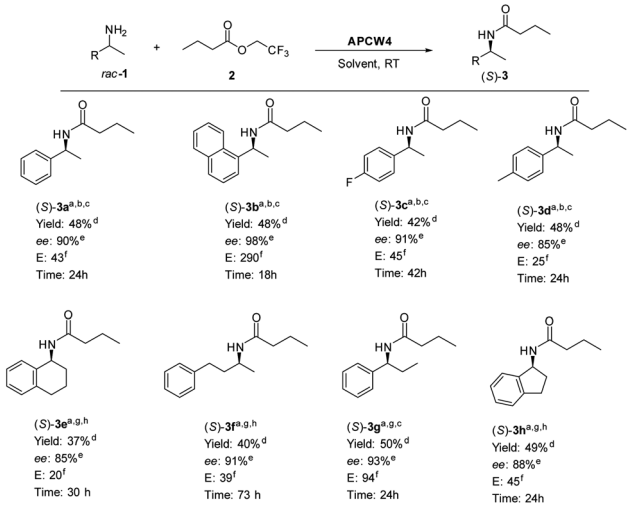
Dynamic kinetic resolution is an elegant way to overcome the yield limit of 50% imposed by the kinetic resolution. For this to be achieved with great success, selective racemization of the primary amine substrate has to occur during the reaction. However, palladium-catalyzed racemization of benzylic primary amines, in the presence of hydrogen under harsh conditions, can lead to the destructive formation of side products.[26] In 2005, Bäckvall disclosed that the ruthenium Shvo catalyst is able to promote *in situ* racemization of various types of amines under inert atmosphere.[27] Bearing this in mind, we tested the racemization of enantiopure (*S*)-amines **1** in an unusual solvent for this transformation such as 2,4-dimethyl-3-pentanol. We found that the Shvo catalyst slowly promoted the racemization of (*S*)-1-(1-naphthyl) ethylamine (**1b**) and (*S*)-1-phenylethan-1-amine (**1a**), respectively (Table 3, entries 2–3). However, a high reaction temperature not optimal for subtilisin was required. Despite this inconvenience we decided to investigate the DKR using the Shvo catalyst from a conceptual standpoint. The **APCW4**/Shvo co-catalyzed system was able to catalyze a DKR in a synergistic fashion (Table 4). For example, (*S*)-amide **3b** was synthesized in 75% yield and 76% ee using combined **APCW4**/Shvo co-catalysis at 90 °C (Table 4, entry 6). Thus, a DKR can be achieved but needs further development. In this context,

Table 2 Recycling of APCW6 for the catalytic KR of racemic **1b**[a]



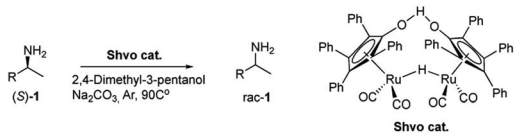
Cycle	Time [h]	Yield **3b** [b] [%]	ee **3b** [c] [%]	E^d
1	22	39	96	259
2	22	41	95	206
3	22	51	94	170
4	22	42	97	235
5	22	46	96	194
6	23	45	96	175
7	22	42	96	133
8	22	38	98	208
9	27	38	98	203
10	43	40	97	151

[a] Reaction conditions: **1b** (0.5 mmol, 1 equiv.), **2** (1.0 mmol, 2 equiv.), 3-methyl 3-pentanol (2 mL), **APCW6** (54 mg, 9 wt% (4.9 mg) subtilisin), room temperature. [b] Isolated yield. [c] Determined by chiral HPLC. [d] *E* = enantiomeric ratio, selectivity factor as determined by Chen *et al.*



Scheme 1 APCW4-catalyzed deracemization of chiral amines[a]. [a]Reaction conditions: *rac*-**1** (0.25 mmol, 1 equiv.), **2** (0.5 mmol, 2 equiv.), solvent (1 mL), **APCW4**, room temperature. [b]**APCW4** (9 mg, 11 wt% (1 mg) subtilisin), used. [c]2,4-Dimethyl-3-pentanol used as solvent. [d]Isolated yield. [e]Determined by chiral HPLC. [f]*E* = enantiomeric ratio (selectivity factor as determined by Chen *et al.*[25]). [g]**APCW4** (18 mg, 11 wt% (2 mg) subtilisin) used. [h]3-Methyl 3-pentanol used as solvent.

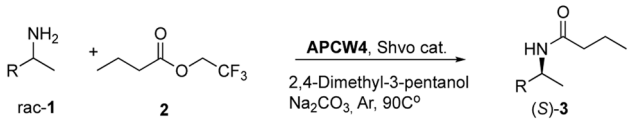
Table 3 Screening for the racemization catalyzed by Shvo catalyst[a]



Entry	R	SHVO cat. [mol%]	Time [h]	ee[b] [%]
1	1-Naft	4.4	22	94
2	1-Naft	18.4	23	74
3	Ph	4.4	24	67

[a] Reaction conditions: **1** (0.25 mmol, 1 equiv.), 2,4-dimethyl-3-pentanol (1 mL), Shvo catalyst, Na_2CO_3 (0.275 mmol, 1.1 equiv.), 90 °C, Ar. [b] Determined by chiral HPLC.

Table 4 Screening of APCW4/Shvo co-catalyzed dynamic kinetic resolution[a]



Entry	R	SHVO catalyst [mol%]	APCW4 [mg]	Time [h]	Yield 3 [b] [%]	ee 3 [c] [%]
1	Ph	4.4	9	120	52	43
2	Ph	8.8	9	92	44	44
3	Ph	16.6	18	48	61	57
4	Ph	—	9	29	44	40
5	1-Nph	18.4	18	71	69	70
6[d]	1-Nph	24	18	71	75	76
7[d]	1-Nph	—	18	28	40	62

[a] Reaction conditions: **1** (0.25 mmol, 1 equiv.), **2** (0.5 mmol, 2 equiv.) 2,4-dimethyl-3-pentanol (1 mL), Shvo catalyst, **APCW4**, Na_2CO_3 (0.275 mmol, 1.1 equiv.), 90 °C, Ar. [b] Isolated yield. [c] Determined by chiral HPLC. [d] Na_2CO_3 (0.75 mmol, 3 equiv.).

a suitable racemization catalyst operating at lower temperatures in solvents such as 2,4-dimethyl-3-pentanol is desirable. Another option would be to improve the subtilisin catalysts enantioselectivity and thermal stability in a solvent optimal for the Shvo co-catalyst by directed evolution or protein engineering.[28]

4 Conclusions

We have disclosed that the concept of artificial plant-cell wall (**APCW**) assembly can dramatically improve the catalytic activity of proteases for organic reactions in non-aqueous media. This was demonstrated by the kinetic resolution of several racemic primary amines to give the corresponding (*S*)-amides in high yields and ees. The heterogeneous **APCW** catalyst showed a remarkable recyclability and was used in 10 cycles without decreasing the high ee of the synthesized product amide. The combination of the **APCW** catalyst with a ruthenium metal catalyst allowed for tandem catalysis and for the first example of an (*S*)-selective dynamic kinetic resolution of a racemic primary amine with a heterogeneous hybrid enzyme/metal catalyst. Further investigation of **APCW** and other self-assembled heterogeneous catalyst and multi-catalyst systems based on materials from renewable resources for organic transformations and enzyme catalysis are ongoing in our laboratories.

Conflicts of interest

There are no conflicts of interest.

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