A continuous-flow cascade reactor system for Alcalase-catalyzed dynamic kinetic resolution of *N*-Boc-phenylalanine ethyl thioester with benzylamine

Péter Falus[a], Lorenzo Cerioli[b], Gabriella Bajnóczi[a], Zoltán Boros[a,c], Diána Weiser[a], József Nagy[a,c], Davide Tessaro[b,d], Stefano Servi[b,d] and László Poppe[a,c]*

[a] Department of Organic Chemistry and Technology, Budapest University of Technology and Economics, H-1111, Műegyetem rkp. 3. Budapest, Hungary
Tel: +36-1-463-3299; fax: +36-1-463-3697; e-mail: poppe@mail.bme.hu

[b] Department of Chemistry, Materials, and Chemical Engineering "Giulio Natta", Politecnico di Milano, Plaza L. da Vinci 32, 20131 Milano, Italy

[c] SynBiocat LLC., H-1173 Budapest, Lázár deák u. 4/1., Hungary

[d] Centro Interuniversitario di Ricerca in Biotecnologie Proteiche "The Protein Factory", Politecnico di Milano and Università degli Studi dell'Insubria, Via Mancinelli 7, 20131 Milano, Italy

Abstract. Subtilisin A (Alcalase) was immobilized by simple hydrophobic adsorption onto various surface grafted macroporous silica-gels resulting in easy-to-prepare and stable biocatalysts enabling efficient kinetic resolution (KR) and dynamic kinetic resolution (DKR) of racemic *N*-Boc-phenylalanine ethyl thioester *rac*-**1** with benzylamine. The performance of the immobilized Alcalase biocatalysts in enzymatic aminolysis was tested in batch and continuous-flow KRs resulting in (*S*)-*N*-Boc-phenylalanine benzylamide (*S*)-**2** in high enantiomeric purity. In KR of thioester *rac*-**1** by Alcalase-catalyzed aminolysis in continuous-flow reactor, the productivity (specific reaction rate, r_{flow}) and enantiomeric ratio (*E*) were studied in the 0–100°C range. The effect of the temperature on base-catalyzed racemization of the non-transformed (*R*)-thioester [(*R*)-**1**] in continuous-flow reactor was also investigated in the 0–150°C range.

The continuous-mode DKR of the thioester *rac*-**1** in a serial cascade system of six biocatalyst-filled columns at 50°C for KR and five grafted silica gel-filled columns at 150°C for racemization resulted in formation of (*S*)-**2** in 79% conversion and 98% *ee*. This is the first example of a dynamic kinetic resolution of an amino acid derivative in continuous-flow mode using an alternating cascade of packed-bed enzyme reactors and racemization reactors kept at different temperatures.

Keywords: amino acid amides; biocatalysis; continuous-flow biotransformation; dynamic kinetic resolution; enantioselectivity; subtilisin; supported catalysts

Introduction

Amides are valuable functional groups in small as well as in more complex synthetic and natural compounds. The amide function is indispensable for almost all biological and many pharmaceutical processes.[1] The amide functional group occurs in around 25–35% of known drugs.[2,3] The amide function is chemically stable, neutral and can play either a hydrogen-acceptor or a hydrogen-donor role in a hydrogen bond. These features can explain its abundance in drugs.

The formation of an amide bond requires activation of a carboxylic acid group.[4] Recently, special attention was given to thioacids and thioesters as reagents in peptide and amide bond formations.[5-7] Thioacids have increased acidity compared to the corresponding carboxylic acid; the thiocarboxylate anion is more nucleophilic than the carboxylate anion;

thioesters are more reactive toward amines than simple esters; and moreover thioesters are excellent acyl donors in lipase or protease catalyzed kinetic resolutions (hydrolysis and transesterification reactions).[8-14]

There is a continuously increasing demand of the pharmaceutical and fine chemistry industry for enantiomerically pure compounds.[15] The use of biocatalysts follows this expansion[16-19] since biocatalysts are especially useful in enantioselective synthesis due to their inherent chirality and their reactivity under mild conditions.

Selectivity, specificity, catalytic activity, and enzyme stability are key factors affecting the efficiency of biocatalysts.[16-18,20-26] Immobilization can often improve these key properties.[27-32] Immobilized biocatalysts are recyclable, storable, and easy to handle[27-32] which can enhance their synthetic applicability.

The vast majority of biocatalytic reactions, however, have been performed in batch mode so far.[16-18,20-26,33-35] Often the operational parameters reported for them seem to be rather suboptimal. Therefore, promoting faster transfer to an industrial scale, there is still much room for optimization. Continuous processes offer several advantages – such as facile automation, reproducibility, and safety – during optimization, upscaling and production.[36-42] Immobilization of the biocatalysts also enable their use under continuous-flow conditions at both industrial and analytical scale.[19,43-46]

Among the factors influencing selectivity, catalytic activity and stability of the biocatalysts under continuous-flow conditions, temperature effects are the most important.[19] Although biotransformations in continuous-flow systems could enhance the efficiency of the hydrolase-catalyzed processes,[19] the effect of temperature on continuous-flow biotransformations was investigated in only a few cases for lipase-catalyzed kinetic resolutions (KRs).[19,42, 46-48]

KR is a common way to prepare pure enantiomers of chiral compounds from their racemates. However, a serious disadvantage of this method is that the yield of the desired enantiomer cannot exceed 50%. Dynamic kinetic resolution (DKR) involving an *in situ* racemization of the non-reacting enantiomer parallel to the enantiomer selective reaction represents a way to overcome this limitation.[49-51]

The two elementary parts of a DKR process (*i.e.* kinetic resolution and simultaneous racemization that could consist of numerous other elementary reactions) may have different operation optima. When a DKR is performed in a stirred tank reactor, the two different reactions required for the DKR can only be optimized at once.

Although a multitude of realizations of DKR were developed,[52-57] hardly any of them were carried out in a continuous-flow manner.[58-68] Because the enantiomer selective reaction and the racemization in the DKR process are two separate reactions, the common optimum is obviously suboptimal for both of them. In the vast majority of cases the two elementary parts of the DKR were not separated in space and therefore the working parameters were suboptimal for both the KR and the racemization.[52-64]

This issue could be solved if the two reactions of DKR are separated in space. Only a few studies realized the KR spaced apart from racemization allowing separate optimization of the two parts of the DKR process.[65-68] Two of these DKRs were batch mode processes with flow-through recirculation[65,66] and only two other cases were real continuous-flow DKRs.[67, 68] In the latter two cases, the elementary parts were still connected but the temperature optima for the two reactions could be set separately even in a continuous-flow system applying the same flow rate.[67] It was shown that this principle could be applied for lipase-catalyzed DKRs of secondary alcohols[67,68] and amines.[67]

Herein, a study for immobilization of Subtilisin A (Alcalase) enabling KR and DKR of *N*-Boc-protected rac-phenylalanine thioethyl ester (*rac-N*-Boc-Phe-SEt, *rac*-**1**)[8] in batch and continuous-flow mode is reported. In a real continuous-flow DKR of *rac*-**1** the KR and racemization parts were optimized separately, allowing to reach the real optima of the two elementary reactions. According to our best knowledge this is the first reported DKR of *rac*-**1** performed in real continuous-flow mode.

Results and Discussion

For this study aiming a real continuous-flow DKR process, the thioethyl ester *rac*-**1** was chosen as substrate due to its relatively high reactivity.[8] The substrate *N*-Boc-phenylalanine thioethyl ester (*rac*-**1**) was synthesized as described earlier.[8]

Immobilization of Alcalase on surface grafted silica for continuous-flow applications

To create a form of Alcalase suitable for continuous-flow applications and to extend its lifetime, enzyme immobilization[27-32] offered an obvious solution.

Although Alcalase was immobilized by sol-gel entrapment,[69] as cross-linked enzyme crystals (CLECs)[70] or as cross-linked enzyme aggregates (CLEAs),[8] these forms were considered not optimal for continuous-flow applications. The sol-gel Alcalase was a stable biocatalysts but with low specific activity, while Alcalase CLECs and CLEAs were complicated to produce and not possible to fill into columns. On the other hand, adsorption to surface-modified silica gels proved to be an easy and cheap way to immobilize hydrolases enabling the use of the enzyme preparation in columns for the continuous-flow processes.[19,48b]

Based on these considerations, immobilization of Alcalase was performed by adsorption onto 24 differently surface-modified silica gels to select the suitable biocatalyst for our designed DKR.

Screening the immobilized Alcalases in kinetic resolution of racemic *N*-Boc-Phe thioethyl ester (*rac*-**1**) in batch mode

The biocatalytic properties of the resulting immobilized Alcalase biocatalysts were tested on analytical scale by KRs of *rac*-**1** (24 mM in *tert*-butyl alcohol) by amidation with benzylamine (29 mM) at 30°C for 24 h (Table S1 and Scheme S1). Due to the good enantiomer selectivity (*E* ›100) of the most biocatalysts, enantiomeric excess of the product (*S*)-**2** was always above 96%. Seven of the adsorbed Alcalase preparations could result in (*S*)-**2** with excellent 98% *ee* with conversions exceeding 35% (Entries 2-8 in Table S1). Alc-Dv250-Et biocatalyst (Alcalase adsorbed on ethyl-grafted macroporous silica gel) proved to be the most suitable catalyst with high activity and excellent enantiomer selectivity (*E* ›200; *c*= 41.3% and *ee*= 98%, see Entry 2 in Table S1).

Noteworthy, ~~that~~ grafting of the macroporous silica with moderately apolar functions proved to be most beneficial for Alcalase adsorption immobilization. Grafting with highly apolar functions (e.g. Entries 20, 21 or 24 in Table S1) or amino functions (e.g. Entries 13, 16 or 18 in Table S1) resulted in the least selective Alcalase preparations (*ee* ‹98%). The activity and the selectivity of the best four Alcalase biocatalysts (*c*= 41–43% with *ee*= 98%, Entries 2-5 in Table S1) surpassed the performance of the basic Alcalase biocatalyst adsorbed onto non-grafted silica (Alc-Dv250, *c*= 33.9% with *ee*= 97%, Entry 1 in Table S1).

It is widely known that proteases gradually lose their activity as a result of self-hydrolysis reducing their storage time.[71] For instance, the commercially available aqueous solution of Alcalase loses its activity in six month which is one of the most serious limitation of its applicability as biocatalysts. Since the long term storability of adsorbed Alcalase biocatalysts have never been tested before, we investigated the shelf life of our preparations. Thus, after storing at 4°C under argon for 12 months, the Alcalase preparations were tested again in the same KR of *rac*-**1** (*tert*-butyl alcohol, 1.2 equiv. benzylamine, 30°C, 24 h) as the freshly made biocatalysts (Figure 1). As expected, all Alcalase biocatalysts have lost some of their activity and selectivity but not to the same degree (Figure 1). Eleven out of the 23 preparations preserved their activity with less than 5 percentage point (*pp*) reduction in conversion. More than 15 *pp* of reduction in conversion was experienced in only 3 cases. Twelve biocatalysts remained almost as selective as they were a year earlier (less than 2 *pp* of reduction in *ee*). Alc-Dv250-ClPr (which was not the most active and selective biocatalyst originally) could best preserve its

selectivity and activity (*ee* remained 96% and *c* decreased only marginally from 41.6% to 41.0%) after 12 months. The least stable Alcalase preparations were the ones adsorbed onto octyl- and isobutyl-grafted silica gels (Alc-Dv250-Oct and Alc-Dv250-iBu).

Kinetic resolution of racemic *N*-Boc-Phe thioethyl ester (*rac*-1) in Alcalase-filled continuous-flow reactor

Alcalase-catalyzed KRs of racemic *N*-Boc-Phe thioethyl ester (*rac*-**1**) in continuous-flow mode were carried out in a multicolumn reactor system (Figure S1). The system comprised stainless steel packed bed columns (CatCart™) filled with the immobilized Alcalase in a multicolumn metal block reactor holder with an external cooling–heating thermostat, a temperature control unit and an isocratic HPLC pump (Figure S1A).

To perform a thorough temperature dependence test in the 0–100°C range, *tert*-butyl alcohol, which is solid below room temperature, should be replaced by *tert*-amyl alcohol having lower melting point but similar properties. To test the suitability of *tert*-amyl alcohol as solvent, KRs of *rac*-**1** with the best four Alcalase biocatalysts [Alc-Dv250-Et, Alc-Dv250-DiMe, Alc-Dv250-MePhe, Alc-Dv250-Dodec (Table S1, Entries 2-5)] were performed in batch mode in both alcohols. Since no significant differences were experienced either in activity or in selectivity (data not shown), *tert*-amyl alcohol proved to be solvent of choice for the KRs of *rac*-**1** in continuous-flow mode.

The continuous-flow KR of *rac*-**1** with benzylamine was investigated using the selected four immobilized Alcalase preparations. Thus, a solution of *rac*-**1** (5
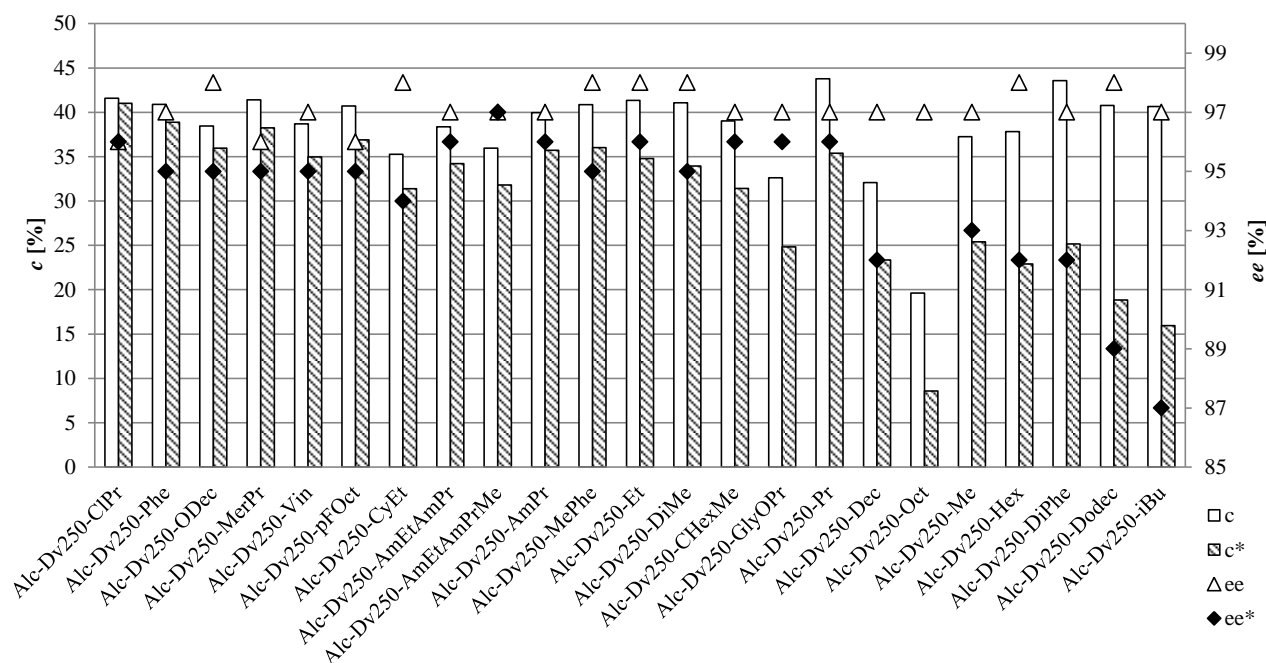

Figure 1. Effect of one year storage on activity and the selectivity of 23 different Alcalase preparations in the kinetic resolution of *N*-Boc-Phe-SEt (*rac*-**1**) with benzyl amine (batch mode, 24 h at 30°C). Conversion (*c*) and enantiomeric excess (*ee*) of the freshly immobilized Alcalase are compared to the conversion (*c**) and enantiomeric excess (*ee**) of the preparations stored at 4°C for 12 months. (For the abbreviations of the biocatalysts, see Experimental Section.)

mg mL^{-1}) and benzylamine (1.2 equiv.) in *tert*-amyl alcohol was pumped through the columns filled with the selected immobilized Alcalase biocatalyst. The Alcalase-filled columns were thermostated to various temperature values (first to 30°C, then to values from 0°C to 100°C, with 10°C steps) at a flow rate of 0.2 mL min^{-1} (for further details see Sections 1.6 and 2.4 of SI and Figure S1).

First, the immobilized biocatalysts were tested at 30°C in the KR of *rac*-**1** both in batch and continuous-flow mode to investigate the differences between the two operation modes (Table 1). To characterize the productivity of the biocatalysts, specific reaction rates in batch reactions (r_{batch}) were calculated using the equation $r_{batch} = n_P/(t \times m_B)$ (where n_P [μmol] is the amount of the product, t [h] is the reaction time and m_B [g] is the mass of the applied biocatalyst).[47] Specific reaction rates in continuous-flow systems (r_{flow}) were calculated using the equation $r_{flow} = [P] \times v/m_B$ (where [P] [μmol mL^{-1}] is the molar concentration of the product, v [mL h^{-1}] is the flow rate and m_B [g] is the mass of the applied biocatalyst).[47] Because the rate of product formation is not a linear function of c, rigorous comparisons between the productivity of a continuous-flow reaction and its batch mode counterpart using their r values can only be made at comparable degrees of conversions.[47] This comparison caring the comparable conversions revealed – in agreement with our previous studies[42,47,48] – that the productivity (specific reaction rate, r) of each Alcalase biocatalysts in continuous-flow system surpassed the corresponding value for the batch mode (Table 1).
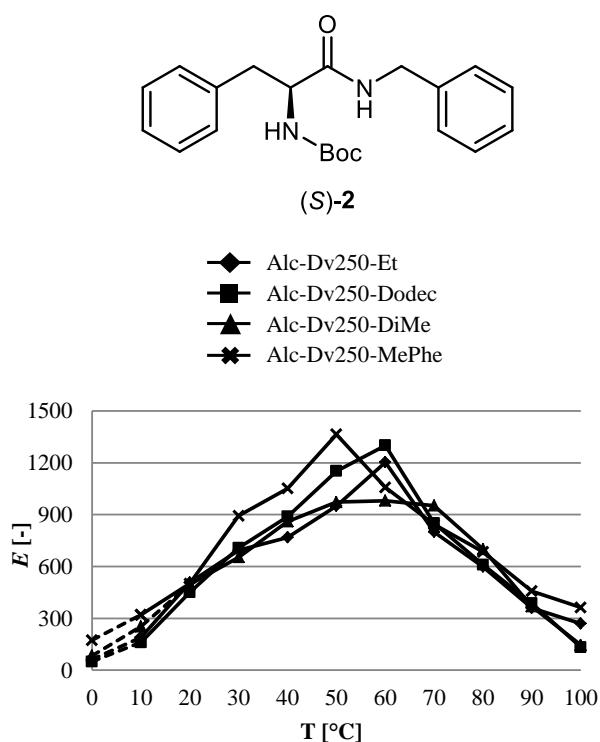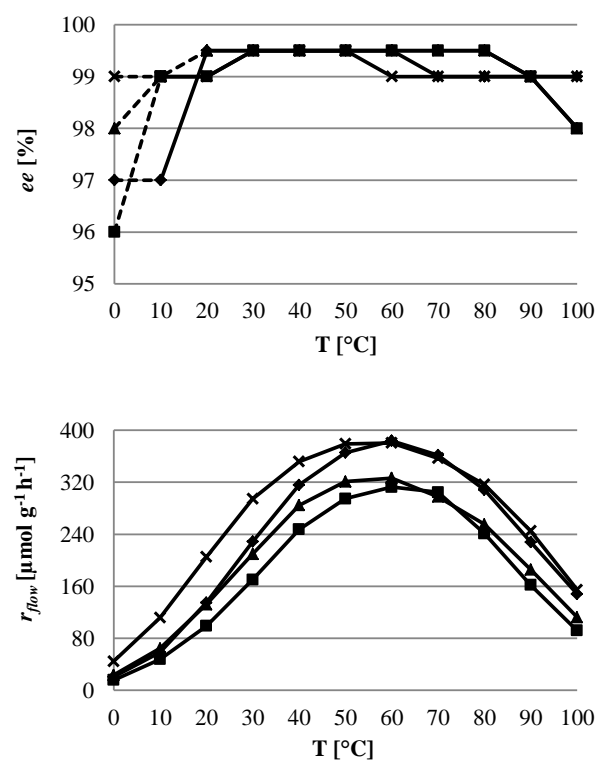
Table 1. Comparison of the specific reaction rates of immobilized Alcalases in KRs of *rac*-**1** at 30°C in batch mode and in continuous-flow mode (flow rate 0.2 mL min^{-1})

Biocatalyst	r_{batch} [μmol g^{-1} h^{-1}]	r_{flow} [μmol g^{-1} h^{-1}]
Alc-Dv250-Et	82.3[a]	228.7[a]
Alc-Dv250-Dodec	86.4[b]	170.0[b]
Alc-Dv250-DiMe	83.1[c]	209.5[c]
Alc-Dv250-MePhe	63.8[d]	294.4[d]

[a] c= 24% and *ee*= 98% after 12 h in batch, c= 25% and *ee*= ›99% in flow. [b] c= 21% and *ee*= 98% after 8 h in batch, c= 20% and *ee*= ›99% in flow. [c] c= 23% and *ee*= 98% after 10 h in batch, c= 23% and *ee*= ›99% in flow. [d] c= 36% and *ee*= 98% after 20 h in batch, c= 35% and *ee*= ›99% in flow.

Next, the temperature-dependent properties of the selected four Alcalase biocatalysts were investigated in the 0–100°C temperature range. The results of KRs of *rac*-**1** with the four Alcalase biocatalysts in continuous-flow mode are shown in Figure 2. Expectedly, since the mode of immobilization of Alcalase was the same, the selected four biocatalysts behaved similarly. Enantiomer selectivity (*E*) increased up to optima at around 50–60°C with *E* »200. These optima fortuitously coincide with the optima of specific reaction rate (r_{flow}) at 60°C. At high temperatures (from optima to 100°C) the enantiomer selectivity (*E*) decreased along with the specific reaction rate (r_{flow}).

Figure 2. Temperature-dependent biocatalytic properties (*E*, *ee* and r_{flow}) of the best four Alcalase biocatalysts in kinetic resolution of *rac*-**1** by amidation with benzylamine in a continuous-flow reactor. Dashed lines are from HPLC measurements with the minor enantiomers under detection limits (for experimental details, see SI).

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The progress of the r_{flow}–temperature curves followed the classic behavior with an increase until the optima at around 50–60°C due to the Arrhenius law, and then, due to the denaturation of the enzyme, decreased towards 100°C. All the four Alcalase biocatalysts proved to be significantly more selective under the continuous-flow conditions than in the batch mode. This result could be explained by taking the concurrent non-selective chemical amidation into account. Reaction of the nucleophilic amino moiety of benzylamine with the starting thioester, rac-**1** without enzyme catalysis is a non-negligible by-reaction in the batch mode.[8] This side-reaction could be repressed by utilizing continuous-flow mode due to the much shorter reaction time (several minutes of residence time in flow mode versus the 24 h reaction time in batch mode). Thus, in all reactions with the four Alcalase preparations the enantiomeric excess of the product ($ee_{(S)\text{-}2}$) exceeded the excellent 99% in a wide temperature range. Alc-Dv250-Et resulted in excellent $ee_{(S)\text{-}2}$ (›99%) between 20 and 60°C with high productivity (›380 µmol g^{-1} h^{-1} at 60°C). Alc-Dv250-MePhe was the most dissimilar biocatalyst having an enantiomer selectivity (E) maximum at 50°C and keeping $ee_{(S)\text{-}2}$ at least 99% even at 0 and 100°C, while all the other three Alcalase biocatalysts had an E optimum at 60°C.

Since the highest productivity and selectivity were observed with Alc-Dv250-MePhe and Alc-Dv250-Et, the further experiments towards a DKR process were carried out with these two biocatalysts. The next question to be answered was how long these enzyme preparations could be used under the desired reaction conditions as an active and selective catalyst. To answer this question, a solution of rac-**1** and benzylamine in tert-amyl alcohol was pumped through the Alcalase-filled column at 50°C at a flow rate of 0.2 mL min^{-1}. This temperature was selected for the operational stability tests because this was the common temperature optima for productivity and selectivity of the two selected Alcalase preparations. The results of the long run KRs under these continuous-flow operation conditions are shown in Figure 3 (the slight differences between the values in Figure 2 and Figure 3 are due to the different enzyme-filled columns used for the two experimental series). This experiment demonstrated that high productivity and enantiomeric excess were maintained even after 5 days of continuous running at 50°C. As expected from the previous temperature dependence study indicated about 10°C higher temperature optimum for Alc-Dv250-Et than for Alc-Dv250-MePhe, the Alcalase on ethyl-grafted surface (Alc-Dv250-Et) could better preserve its original selectivity during the long term run. Since slightly higher average value of productivity and selectivity was achieved with Alc-Dv250-Et in the operational stability tests (r_{flow}= 414 µmol g^{-1} h^{-1}, ee= 99.5%) than with Alc-Dv250-MePhe (r_{flow}= 403 µmol g^{-1} h^{-1}, ee= 99.0%), Alc-Dv250-Et was selected as biocatalyst for the DKR.
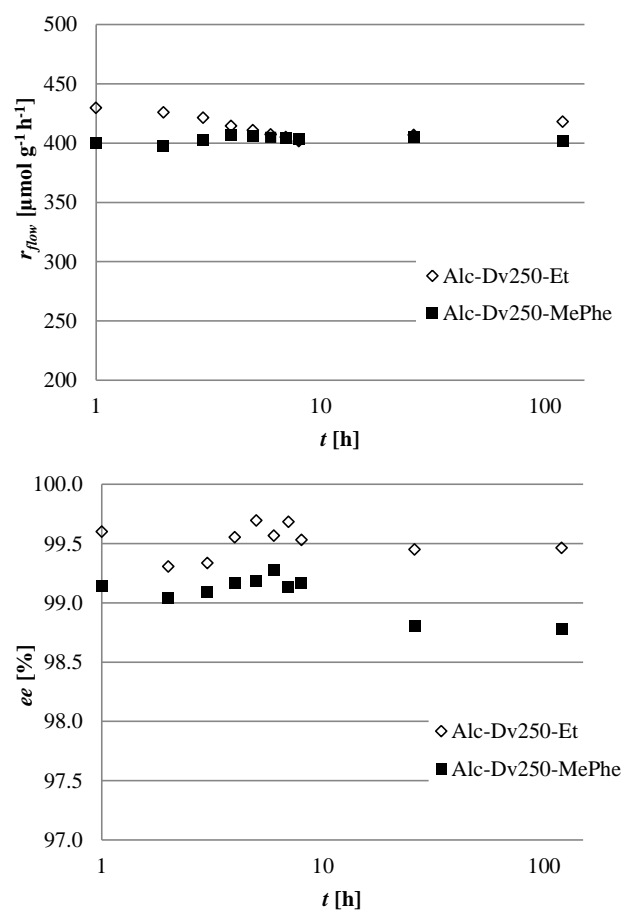

Figure 3. Operational stability of the Alc-Dv250-Et and Alc-Dv250-Et biocatalysts in the kinetic resolution of rac-**1** in continuous-flow reactor (rac-**1**, 5 mg mL^{-1} and benzylamine, 1.2 equiv. in tert-amyl alcohol at 50°C, 0.2 mL min^{-1}).

Racemization of the residual thioethyl ester enantiomer (R)-1 in continuous-flow mode

To carry out DKR of rac-**1** in continuous-flow mode, the racemization of (R)-**1** was optimized next. As a proper non-nucleophilic base for racemization 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) proved to be suitable in accordance with the previous studies[8,72]. For the racemization tests, a solution containing enantioenriched (R)-**1** (ee= 85%) and (S)-**2** (ee= 92%) in a molar ratio of 1:1, 3 equiv. of DBU and 1.2 equiv. of benzylamine in tert-amyl alcohol was pumped through a column filled with Dv250-Et (ethyl-grafted silica gel without enzyme) and thermostated to various temperatures in the 0°C to 150°C range (in 10°C steps). After reaching the stationary state at each temperature, samples were analyzed by HPLC (Figure 4). As the temperature increased, the enantiomeric excess of (R)-**1** followed a decreasing sigmoid curve with an inflexion point at around 90°C. Since enantiomeric excess of (S)-**2** remained unaltered at the high temperature regime ($ee_{(S)\text{-}2}$ remained 92% even at 150°C) it was obvious to choose 150°C resulting in $ee_{(R)\text{-}1}$= 4% as the optimal racemization temperature.
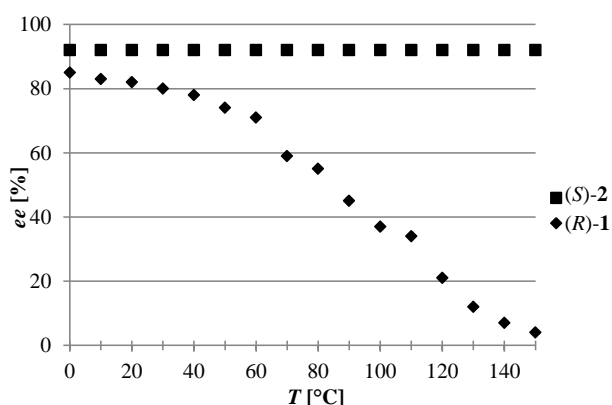
Figure 4. Effect of the temperature on the racemization of (*R*)-**1** with DBU (3 equiv.) and benzylamine (1.2 equiv.) in the presence of equimolar amount of (*S*)-**2** in a DV-250-Et-packed reactor (*tert*-amyl alcohol, 0.2 mL min⁻¹, under continuous-flow conditions)

Dynamic kinetic resolution of racemic thioethyl ester (*rac*-**1**) in continuous-flow mode

The DKR of *rac*-**1** in continuous-flow mode was carried out in a system contained two separate metal multicolumn reactor blocks (a multicolumn reactor block used for KRs is depicted in Figure S1) equipped with a temperature control unit and with external cooling-heating thermostats and an isocratic HPLC pump. The reactions were performed in stainless steel CatCart™ packed-bed columns [6 columns packed with Alc-Dv250-Et for KR and 5 columns packed with Dv250-Et for racemization (Rac)] placed in the two reactor blocks thermostated to different temperatures (50°C for KR and 150°C for Rac). The Alcalase-filled and the ethyl-grafted silica gel-filled columns followed each other in an alternating sequence starting with an Alcalase-filled column. A solution of *rac*-**1** (5 mg mL⁻¹), benzylamine (1.2 equiv.) and DBU (3 equiv.) in *tert*-amyl alcohol was pumped through the eleven columns thermostated to different temperatures (50°C for KR and 150°C for Rac) at a flow rate of 0.2 mL min⁻¹ (Figure 5 and Table 2).

Table 2. The realized results of the DKR of *rac*-**1** in a continuous-flow system of alternating KR and Rac columns operating at significantly different temperature compared to the theoretical, technical and kinetics controlled limits

Entry[a]		c [%]	ee [%]
1	Result achieved	79	98
2	Theoretical limit[b]	98.4	100
3	Technical limit[c]	97.0	99.5
4	Kinetics controlled limit[d]	77.1	99.5

[a] In a system of alternating KR and Rac columns [*rac*-**1**, 5 mg mL⁻¹ and benzylamine, 1.2 equiv. in *tert*-amyl alcohol at 50°C for KR (6×) and 150°C for Rac (5×), 0.2 mL min⁻¹]. [b] The c and $ee_{(S)\text{-}2}$ for the DKR system assuming full KR and full Rac. [c] The c and $ee_{(S)\text{-}2}$ for the DKR system assuming each corresponding units achieving the performance of the single column KR (c= 45.9% and $ee_{(S)\text{-}2}$= 99.5%) and the single column Rac ($ee_{(R)\text{-}1}$= 4%) steps. [d] The c and $ee_{(S)\text{-}2}$ for the DKR system assuming decrease of c in the KR steps proportional with the decrease of the (*S*)-**1** concentration but retaining selectivity ($ee_{(S)\text{-}2}$= 99.5%) and reaching $ee_{(R)\text{-}1}$= 4% in all racemization steps.

After reaching the stationary state (3 h), HPLC analysis indicated that the continuous-flow DKR of *rac*-**1** performed in good conversion and with high selectivity (Entry 1 in Table 2: c= 79% and $ee_{(S)\text{-}2}$= 98%) approaching the kinetics controlled limits (Entry 4 of Table 2: c= 77.1% and $ee_{(S)\text{-}2}$= 99.5%). The product (*S*)-**2** was isolated from the collected effluent in good yield (71%, based on *rac*-**1**) as a white solid.

The results indicated that the decrease of the concentration of the fast reacting enantiomer [(*R*)-**1**] resulted in a proportional decrease of the rate of the reaction, and thus the conversion (in accordance with the Michaelis-Menten kinetics in the low substrate concentration regime). The slightly higher conversion (c= 79% *vs.* c= 76.9%) but lower selectivity ($ee_{(S)\text{-}2}$=
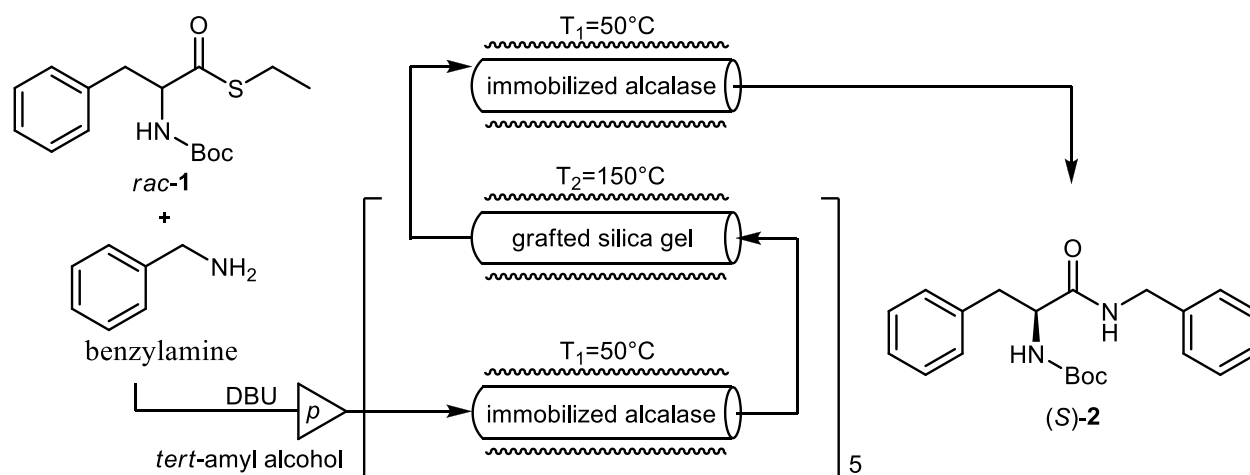

Figure 5. Dynamic kinetic resolution of racemic *N*-Boc-phenylalanine ethyl thioester (*rac*-**1**) in continuous-flow mode using an alternating cascade of packed-bed enzyme reactors and racemization reactors kept at different temperatures.

98%ee *vs. ee*$_{(S)-2}$= 99.5%) achieved in the real DKR process (Entry 1 in Table 2) as compared to the kinetics controlled case (Entry 4 in Table 2) could be rationalized by assuming about ~2% of the conversion by non-selective chemical amidation. Comparing the results to the technical limit (assuming no dependence of the conversion on the substrate concentration in KRs, see Entry 3 of Table 2: *c*= 97.0% and *ee*$_{(S)-2}$= 99.5%) indicated that this kinetics related effect influenced most seriously the final conversion of the process.

Conclusion

To our best knowledge this is the very first continuous-flow chemo-enzymatic dynamic kinetic resolution (DKR) of the racemic *N*-Boc-phenylalanine thioethyl ester (*rac*-**1**). The kinetic resolution (KR) part of the system was catalyzed by an Alcalase preparations immobilized onto surface grafted silica gels. The adsorbed Alcalase preparations were stable, selective and active biocatalyst with a shelf life at 4°C as long as one year. The best two Alcalase biocatalysts (Alc-Dv250-Et and Alc-Dv250-MePhe) preserved their activity and selectivity even after 120 h of continuous run in continuous-flow mode KR of *rac*-**1**. 1,8-Diazabicycloundec-7-ene (DBU, 3 equiv.) at 150°C in ethyl-grafted silica gel-filled reactor proved to be suitable for racemization of the non-reacting enantiomer [(*S*)-**1**] of the substrate. The DKR process was accomplished in an alternating cascade of packed-bed enzyme reactors and racemization reactors kept at significantly different temperature (50°C for KR and 150°C for racemization). The DKR yielded the product amide (*R*)-**2** in good yield (71%, from a reaction mixture of *c*= 79%) with excellent enantiomeric excess (98%). The DKR system presented here offers a generalizable possibility to combine KR and racemization steps requiring significantly different optimal temperatures.

Experimental Section

Materials

All chemicals and starting materials were purchased from Sigma-Aldrich (Saint Louis, MO, USA) or Alfa Aesar Europe (Karlsruhe, Germany) and used without further purification. Solvents from Merck KGaA (Darmstadt, Germany) were dried and/or freshly distilled prior to use.

Surface modified silica gels (Dv250-series: irregular, 30–70 μm particle size, 250 Å pore diameter) were the products of SynBiocat LLC (Hungary, Budapest). The grafting functions on Dv250 were the following: none (-), methyl- (Me), ethyl- (Et), propyl- (Pr), isobutyl- (iBu), hexyl- (Hex), octyl- (Oct), decyl- (Dec), dodecyl- (Dodec), octadecyl- (ODec), phenyl- (Phe), (1H,1H,2H,2H)-perfluorooctyl- (pFOct), vinyl- (Vin), 2-cyanoethyl- (CyEt), 3-chloropropyl- (ClPr), 3-mercaptopropyl- (MerPr), dimethyl- (DiMe), methyl − phenyl- (MePhe), diphenyl- (DiPhe), cyclohexyl − methyl- (CHexMe), 3-aminopropyl- (AmPr), 3-(2-aminoethyl-amino)propyl- (AmEtAmPr), 3-(2-aminoethylamino)-propyl − methyl- (AmEtAmPrMe), 3-glycidyloxypropyl- (GlyOPr). Subtilisin A (Alcalase or Alc, from *Bacillus licheniformis*) was purchased from Merck KGaA (Darmstadt, Germany).

Immobilization of Subtilisin A by adsorption on surface grafted macroporous silica gel supports

The surface-grafted silica gel (200 mg) was added to Subtilisin A solution (1 mL) in TRIS buffer (9 mL, pH=8.0; 50 mM, ionic strength controlled with NaCl to 150 mM) and the resulting suspension was shaken at 450 rpm at 4°C. After 24 h, the immobilized biocatalyst was filtered off and washed with isopropanol (2×10 mL) and hexane (1×10 mL) and dried at room temperature for 8 h. The Alcalase biocatalysts adsorbed onto surface-grafted silica gels [abbreviations were composed from abbreviation of the enzyme-support-grafting (e.g. Alc-Dv250-Et, Alcalase adsorbed on Davisil with ethyl grafting)] were stored in amber screw cap vials at 4°C. Activity and selectivity of the freshly prepared Alcalase biocatalysts in KR of *rac*-**1** are shown in Table S1. Data for the Alcalase preparations after one year storage are shown in Figure 1.

Dynamic kinetic resolution of racemic *N*-Boc-Phe-SEt (*rac*-**1**) in continuous-flow bioreactor

Eleven CatCart™ columns [six columns filled with Alc-Dv250-Et (total filling weight: 1269.2 mg) and five columns filled with Dv250-Et (total filling weight: 1049.8 mg)] were connected in alternating series starting with an Alc-Dv250-Et-filled one (Scheme 1).

A solution of racemic *N*-Boc-Phe-SEt (*rac*-**1**, 5 mg mL^{-1}), benzylamine (1.2 equiv.) and 1,8-diazabicycloundec-7-ene (DBU, 3 equiv.) in *tert*-amyl alcohol was pumped through the columns thermostatted to different temperatures (50°C for KR and 150°C for racemization) at a flow rate of 0.2 mL min^{-1}. Samples (sample size: 200 μL, diluted with hexane/isopropanol 98:2 to 500 μL) were collected after stationary operation has been established (3 h after starting the operation) and analyzed by HPLC.

The product solution from the stationary phase of the DKR process was collected (24 mL) and the solvent was removed by vacuum rotary evaporator. The residue was dissolved in MeOBu^t (10 mL) and washed with 0.1 M HCl (2×5 mL). The organic phase was dried over MgSO$_4$ and the solvent was removed under vacuum. Further purification was performed by column chromatography on silica gel (eluent: hexane–ethyl acetate 3:1) yielding after removal of the solvent the pure amide [(*S*)-**2**] as white crystals.

Yield: 71% (98 mg, 0.28 mmol); Mp: 126.1–127.3°C (lit.[8]: 128°C); $[\alpha]_D^{25}$= −3.1 (*c* 1, methanol), (*ee*= 98% by HPLC, see Section 2.4) {lit.[8]: $[\alpha]_D^{25}$= −2.7 (*c* 1, methanol), (*ee*= 98% by HPLC)}.

Details on analytical methods, biocatalyst immobilization, kinetic resolutions in batch mode, the reactor setup and KRs in continuous-flow mode are included as Supporting Information.

Acknowledgements

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A continuous-flow cascade reactor system for Alcalase-catalyzed dynamic kinetic resolution of *N*-Boc-phenylalanine ethyl thioester with benzylamine

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