ARTICLE

OBC
www.rsc.org/obc

Engineered phenylalanine dehydrogenase in organic solvents: homogeneous and biphasic enzymatic reactions

Gianfranco Cainelli,*[a] Paul C. Engel,*[b] Paola Galletti,[a] Daria Giacomini,[a] Andrea Gualandi[a] and Francesca Paradisi[b]

[a] *Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, I-40126, Bologna, Italy. E-mail: gianfranco.cainelli@unibo.it; Fax: +39.015.2099456; Tel: +39.051.2099510*

[b] *Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin, 4, Ireland. E-mail: paul.engel@ucd.ie; Fax: (+353) 1 283 7211; Tel: (+353) 1 716 6764*

Received 1st August 2005, Accepted 10th October 2005
First published as an Advance Article on the web 14th November 2005

The use of engineered phenylalanine dehydrogenase N145A supported on Celite for the reductive amination of phenylpyruvic acid in homogeneous and biphasic aqueous–organic solvents is reported. The results indicate that the immobilised biocatalyst is remarkably robust, even in the presence of high concentrations of polar or non-polar organic solvents such as acetone, methanol, *n*-hexane, toluene and methylene chloride. Cofactor regeneration with alcohol dehydrogenase from *Saccharomyces cerevisiae* and ethanol was successfully explored. Application to the non-natural poorly water-soluble 2-oxo acid *p*-NO_2-phenylpyruvic acid was successfully performed, resulting in the biocatalytic synthesis of *p*-NO_2-phenylalanine. In all cases 100% stereoselectivity for the production of the amino acid was retained.

Introduction

Enzymatic synthesis in non-aqueous media has emerged as an attractive alternative to chemical synthesis. Numerous strategies have been developed for carrying out enzymatic reactions in organic media including multiphase aqueous–organic media, reverse micelles and monophasic media.[1]

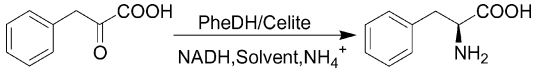
Most synthetic uses of enzymes to date, including those in non-aqueous solvents, have involved hydrolases such as lipases. The effects of organic media are particularly straightforward in such cases, especially in terms of the stereoselectivity.[2,3] It is known, in fact, that organic solvents can alter the enzymatic selectivity of hydrolases thus allowing a modulation of the stereoselectivity control through the solvent.[4] Several examples of solvent-controlled enantioselection have been reported in resolution of racemates with lipases.[5] Furthermore, in the presence of organic solvents lipases show an increased stability to temperature, an improved control of unwanted side-reactions (hydrolysis) and the possibility of using highly hydrophobic substrates insoluble in aqueous phases.

In the production of enantiomerically pure compounds, redox enzymes have a significant advantage over lipases because they may permit substrate conversion of up to 100%. In this field many alcohol dehydrogenases display high stereoselectivity and a broad substrate range.[6] However, dehydrogenases have been used less often in organic solvents,[7] the main drawback being their limited stability in non-aqueous solvents and the requirement for a co-factor which is sparingly soluble and unstable in some organic solvents (*e.g.* nicotinamide adenine dinucleotide species are almost insoluble in many lipophilic media).[8] Notwithstanding these limitations, interesting contributions have recently appeared in the literature on alcohol dehydrogenases.[9]

Phenylalanine dehydrogenase (PheDH) interconverts phenylalanine and phenylpyruvate plus ammonia *via* a NAD^+-dependent oxidoreduction. The wild-type enzyme from *Bacillus sphaericus* accepts various non-natural amino substrates in place of phenylalanine.[10] We have recently reported a successful study based on this specific phenylalanine dehydrogenase in which single point mutations greatly affected the substrate specificity of the enzyme, allowing the new biocatalysts to accept non-natural substrates (2-oxo-acids), yielding the corresponding L-amino acids with total retention of the enantioselectivity.[11,12]

Among the different mutations, the N145A PheDH, in which asparagines at position 145 is replaced by alanine, proved to be the most versatile and efficient biocatalyst with several synthetic 2-oxo-acids. Unfortunately, substituted phenylpyruvic acids (*e.g.* *p*-MeO-, *p*-NO_2- and *p*-CF_3-phenylpyruvic acid) have dramatically diminished solubility in water (10- to 20- fold relative to phenylpyruvic acid), casting a substantial doubt on the possibility of scaling-up those reactions.

To overcome this problem, performing the enzymatic reductive amination in the presence of organic solvents would be the ideal solution. Toleration of the organic solvents by the biocatalyst can be judged in two senses. First of all, there is the basic question of whether the enzyme catalyst is active under these conditions. Secondly, even where there is a substantial level of catalytic activity, there is also the issue of longer-term stability. To our knowledge, even though there have been several reports of enantioselective reduction of ketones to alcohols, *via* alcohol dehydrogenases for example,[13,14] there are no previous studies on amino acid dehydrogenases in organic solvents, and here we report our latest results on the use of the N145A mutant immobilised by deposition on Celite with regard to stability, efficiency and yields in mono- and biphasic systems in presence of organic solvents. The commercially available phenylpyruvic acid is used as the substrate in all the screening reactions (Scheme 1).



Scheme 1

Finally, we report the practical application of our system in the synthesis of a more hydrophobic non-natural amino acid, *p*-NO_2-phenylalanine, starting from the poorly water-soluble *p*-NO_2-phenylpyruvic acid.

DOI:10.1039/b510816k

Results and discussion

Enzyme immobilisation

The PheDH used in this work is not commercially available but cultured and purified as previously reported,[15] and usually stored as an ammonium sulfate precipitate at 4 °C. For synthetic application, however, a much more practical way to handle the biocatalyst is to have it in a solid form.

Free enzyme powder continues to be used even in nearly anhydrous media, but immobilization offers an enhanced stabilization of enzymes in organic solvents.[16] We have tried two different preparations: lyophilised and immobilised enzyme. The lyophilised PheDH lost almost 10% activity with respect to the free enzyme under standard reaction conditions,[15] while when the purified enzyme was immobilised by deposition on Celite® 521 as reported by Persson *et al.*[17] the retention of activity was excellent. The supported enzyme stored at 4 °C appeared to be stable for several months. Also, when the freshly prepared material was redissolved in buffer and centrifuged to remove the Celite and other solid debris, over 90% of the enzyme could be recovered in a fully active state. Although in our case extensive experiments showed that the unsupported enzyme is extremely stable (results not shown), the augmented volume of the immobilised biocatalyst simplified the preparation of the reaction mixture with a more accurate quantification.

Homogeneous organic co-solvents

We first examined organic solvents which form homogeneous solutions with the aqueous buffer. In the absence of a recycling system for the cofactor, an excess of NADH, with respect to the substrate, is necessary to push the reaction to completion. Reaction time-courses were therefore followed in homogeneous co-solvent systems with THF, acetonitrile, acetone and methanol as 10, 50, and 90% (*v*/*v*) components of the liquid phase. Aliquot samples were filtered and directly analysed by HPLC using a chiral column. This allowed a check on both the yield and the enantiopurity of the product. In all cases the enantioselectivity of the enzyme afforded exclusively L-phenylalanine and no traces of D-phenylalanine were ever detected. Results, reported in Fig. 1 and Table 1, are expressed as % conversion. The reactions with various amount of co-solvents were compared with reactions in purely aqueous buffered solution, which were efficient and quantitative in 1 h.

In THF, at only 10% the reaction proceeded more slowly than in Tris buffer alone, but negligible reaction was seen with 50 or 90% THF, which forms a biphasic system at these higher concentrations. The enzyme was also found to be very sensitive to the purity of this solvent: reactions performed in THF other than freshly distilled failed to yield any product. The breakdown products of THF, such as butyraldehyde, 4-hydroxybutyraldehyde and several others as reported in the literature[18] seem to be responsible for destroying the enzyme activity. When the THF is freshly distilled the reaction proceeds up to 99.5% conversion.

The reaction in acetonitrile was slow, but nevertheless proceeded even in 50% organic solvent. In 10% acetone, reaction

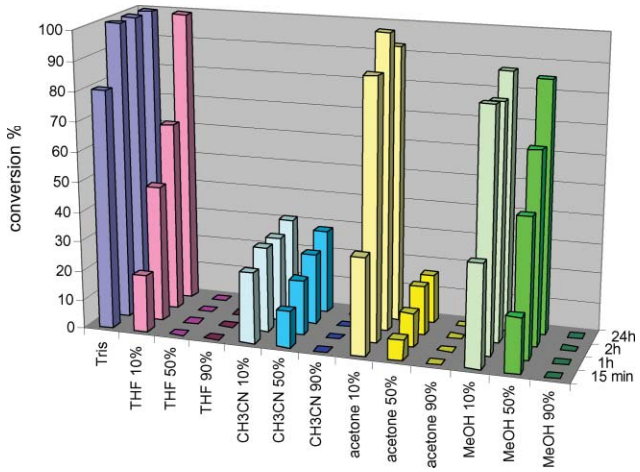


Fig. 1

was somewhat slower than in purely aqueous solution, but still reached almost complete conversion within 2 h. In 50% acetone, the extent of conversion was much less and the results suggest that after 2 h the catalytic activity was lost. In methanol, conversion did not exceed 90%, but nevertheless even in 50% solvent reaction was clearly proceeding steadily well beyond the second hour.

In the presence of large amounts of organic co-solvents, KCl and NH_4Cl precipitated, so that for the main aqueous protocol KCl was omitted, and NH_4OAc replaced NH_4Cl with good results in terms of recovery of L-phenylalanine (Table 2).

KCl is usually added to the reaction mixture to improve the stability of the free enzyme; in this case it seems that the supported enzyme shows excellent stability and optimum efficiency even without the salt. However, when pure organic solvents (such as ethyl ether or hexane), previously saturated for 24 h with a 50 mM Tris solution (pH 8.5), were used, the reaction did not proceed at all with or without KCl or with or without NH_4OAc replacing NH_4Cl. Finally, replacing ammonium chloride with hydrochlorides of organic amines, (such as methylamine hydrochloride or benzylamine hydrochloride, which are soluble in organic solvents) failed to support any reaction.

Homogeneous organic co-solvents with cofactor regeneration

Since the design of economically useful enzymatic synthesis requires cofactor regeneration, the possibility of recycling

Table 2 Conversion (%) of phenylpyruvic acid into L-phenylalanine using Celite-supported enzyme in aqueous Tris buffer solution (pH 8.5), no co-solvents and different ammonia source

Time	Tris–NH_4Cl	Tris–NH_4OAc–KCl	Tris–NH_4OAc
15 min	51.7	66.4	45.7
1 h	60.8	71.2	75.1
2 h	92.0	82.8	93.2
3 h	97.0	94.1	97.6

Table 1 Conversion (%) of phenylpyruvic acid into L-phenylalanine using Celite-supported enzyme in a homogeneous system of aqueous Tris buffer solution (pH 8.5) and different amounts of co-solvents

Time/min	Tris	THF 10%	THF 50%	THF 90%[a]	CH_3CN 10%	CH_3CN 50%	CH_3CN 90%[a]	Acetone 10%	Acetone 50%	Acetone 90%[a]	MeOH 10%	MeOH 50%	MeOH 90%[a]
15	80.3	19.2	—	—	24.0	12.3	—	32.9	6.8	—	34.5	18.5	—
60	100	45.9	—	—	28.7	18.7	—	88.1	11.6	—	81.7	47.2	—
120	100	64.2	—	—	28.4	23.6	—	99.7	16.7	—	79.9	65.3	—
1440	100	99.5	—	—	30.9	28.2	—	93.0	16.4	—	87.1	85.2	—

[a] With a 90 : 10 mixture *vs.* Tris, the conversion reached 1% after 1 week.

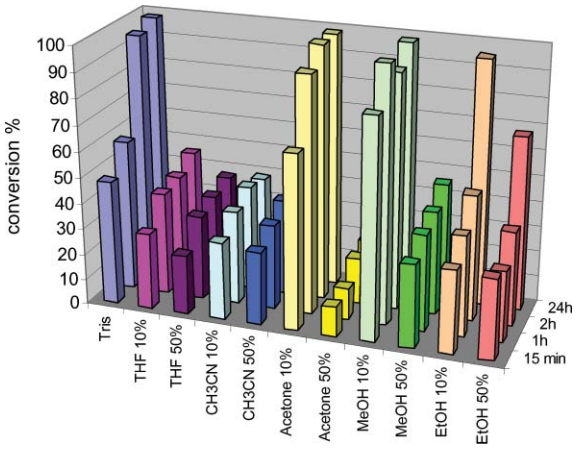


Fig. 2

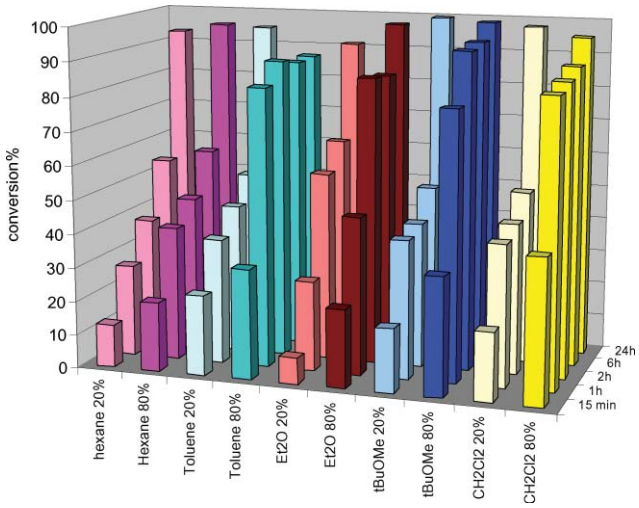


Fig. 3

with alcohol dehydrogenase from *Saccharomyces cerevisiae* and ethanol was explored (Fig. 2).

Under these circumstances, despite the sub-stoichiometric (20%) NADH concentration relative to phenylpyruvate, virtually complete conversion to L-phenylalanine was again accomplished in the aqueous system, showing that the recycling system with ethanol and alcohol dehydrogenase is effective. In general, the results in the presence of organic solvents are not dissimilar from those of Table 1 (NADH in excess and no recycling) and in fact show somewhat higher conversion in most cases (Table 3). Fig. 2 also includes results for ethanol, with near complete conversion in 10% ethanol and almost 70% conversion even in 50% ethanol.

Biphasic organic co-solvents

A number of solvents immiscible with water were tested in biphasic systems in a ratio of 20% organic phase added to 80% aqueous reaction mixture. These included *tert*-butylmethyl ether, dichloromethane, toluene, *n*-hexane, and diethyl ether as members of different classes: oxygenated and halogenated solvents, aromatic and aliphatic hydrocarbons (Fig. 3).

The organic and the aqueous phases were separately analysed. The former contained phenylpyruvic acid and a small amount of NADH while the L-phenylalanine was in the aqueous phase with most of the NADH. Although reaction was slower under these conditions (50–60% after 6 h), the enzymatic system appears to tolerate the presence of an immiscible organic solvent well, with over 90% conversion in every case (Table 4) and virtual completion with methylene dichloride and *t*-butyl methyl ether. Strict enantioselectivity is retained in all cases as only L-phenylalanine was detected in the crude reaction mixture.

The trial of immiscible solvents was extended by reversing the ratio and using 80% of organic phase with only 20% aqueous. The results show that conversion rates were faster, reaching 50–60% conversion in most cases in the first hour, possibly because of the higher coenzyme concentrations in the 20% aqueous phase where the reaction takes place, or more efficient inter-phase transfer of the substrate. With the possible exception of toluene, high final extents of conversion were achieved even with these 4 : 1 organic–aqueous systems.

Biphasic system with co-factor regeneration

In experiments to test whether the enzymatic recycling system for the cofactor would also tolerate the organic solvents, in the case of 20 : 80 organic–aqueous mixtures all incubations gave a high yield of product, the lowest being 93% after 24 h with hexane (Fig. 4).

On the other hand, when the organic : aqueous ratio was reversed, the yields in the biphasic systems after 24 h were in all cases less than 90% (Table 5). Comparison with the results in Table 4 suggests that the poorer result here probably reflects sensitivity of the recycling system (alcohol dehydrogenase) to the high concentrations of organic solvent.

Table 3 Conversion (%) of phenylpyruvic acid into L-phenylalanine using Celite-supported enzyme in homogeneous system of aqueous Tris buffer solution (pH 8.5) with different co-solvents and co-factor regeneration

Time	Tris	THF 10%	THF 50%	CH_3CN 10%	CH_3CN 50%	Acetone 10%	Acetone 50%	MeOH 10%	MeOH 50%	EtOH 10%	EtOH 50%
15 min	47.9	29.5	22.9	30.0	28.4	67.8	11.5	84.6	32.2	32.2	31.0
1 h	58.5	39.8	32.6	36.3	33.0	92.1	12.4	98.6	37.3	39.0	27.6
2 h	95.6	41.3	35.2	40.9	37.1	98.7	18.0	91.5	40.3	48.7	36.7
24 h	98.4	45.8	37.6	38.7	40.5	98.6	19.4	98.5	45.7	95.1	67.6

Table 4 Conversion (%) of phenylpyruvic acid into L-phenylalanine using Celite-supported enzyme in biphasic systems of aqueous Tris buffer solution (pH 8.5), 20 and 80% of different co-solvents

Time	Hexane 20%	Hexane 80%	Toluene 20%	Toluene 80%	Et_2O 20%	Et_2O 80%	*t*BuOMe 20%	*t*BuOMe 80%	CH_2Cl_2 20%	CH_2Cl_2 80%
15 min	12.6	20.4	23.6	32.4	7.7	23	18.7	34.7	20.2	42.2
1 h	27	39.5	37	82.5	26.5	46.5	40.8	79	41.7	84
2 h	38.1	45.6	44.1	88.4	55.6	84.6	42.6	93.4	44.5	85.9
6 h	54.2	57.9	51.3	86.5	63.1	83.4	50.5	94.4	50.9	88.5
24 h	93.1	95.5	95	86.7	90.9	97.3	99.7	98.8	97.9	95.2

Table 5 Conversion (%) of phenylpyruvic acid into L-phenylalanine with co-factor regeneration using Celite-supported enzyme in biphasic systems of aqueous Tris buffer solution (pH 8.5), 20 and 80% of different co-solvents

Time	Tris	Hexane 20%	Hexane 80%	Toluene 20%	Toluene 80%	Et_2O 20%	Et_2O 80%	*t*BuOMe 20%	*t*BuOMe 80%	CH_2Cl_2 20%	CH_2Cl_2 80%
15 min	47.9	18.5	18.4	24.5	38.7	20.3	48.4	18.9	25.7	18.6	25.2
1 h	58.5	28.1	23.6	30.1	50.7	33.7	54.1	33.7	51.1	27.5	38.5
2 h	95.6	34.3	35.9	31.9	46.7	37.1	61.9	46.1	81.7	28.1	49.1
24 h	98.4	93.3	89.9	96.7	70.6	96.9	71.3	98.1	85.1	96.5	85.5

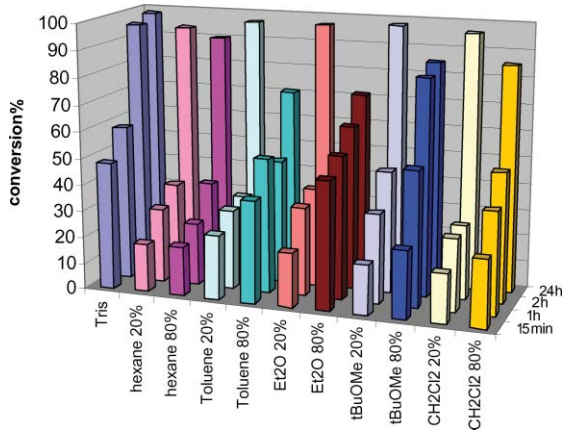


Fig. 4

Application to a non-natural oxo-acid: synthesis of *p*-NO_2-phenylalanine

As mentioned above, our mutated PheDH is efficient with several non-natural 2-oxoacids. However, there were aqueous solubility concerns in many cases. To further explore the improved performance of the supported N145A PheDH in organic solvents, several experiments were carried out to convert the commercially available *p*-NO_2-phenylpyruvic acid into the corresponding amino acid. The solubility of such substrates in fact, is greatly improved by the addition of organic solvents.† Two different reactions were compared: (A) the reductive amination of 10 mg *p*-NO_2-phenylpyruvic acid in 3 mL 50 mM Tris buffer (pH 8.5) with 5% EtOH and (B) of 15 mg of the same substrate in 3 mL 50 mM Tris buffer (pH 8.5) with 5% EtOH and 10% MeOH (same reaction conditions as reported for cofactor regeneration in homogeneous systems). Owing to the formation of a precipitate, samples were centrifuged and supernatant and precipitate were analysed separately. The product concentration in the supernatant is reported in Table 6.

In both cases, the precipitate was redissolved in fresh buffer and it was confirmed by HPLC to be the amino acid product. Concentration of *p*-NO_2-phenylalanine in solution was 14.2 mM (89.3%) in reaction A (5% EtOH) and 16 mM (67%) in reaction B (5% EtOH and 10% MeOH). Injections repeated after 43 h did not show increased yields and the apparent shortfall from 100% may be attributed to the separation of a portion of the total product as precipitate. Analysis of the crude reaction mixture by ^{1}H-NMR did not show any traces of the starting material in reaction A, confirming total conversion, while 5–10% unreacted oxo-acid was still detectable in the spectrum of reaction B.

Table 6 Conversion of *p*-NO_2-phenylpyruvic acid into *p*-NO_2-L-phenylalanine using Celite-supported enzyme in a homogeneous system of aqueous Tris buffer solution (pH 8.5), 10% MeOH with co-factor regeneration. Values are expressed as % of the total expected conversion and also as product concentration (mM)

Time/h	Tris	MeOH 10%
18	89.3% (14.2 mM)	67% (16.0 mM)
24	89.3% (14.2 mM)	67% (16.0 mM)
43	89.3% (14.2 mM)	67% (16.0 mM)

† In pure Tris buffer we estimated a maximum solubility of 1.8 mg mL^{-1} (*ca.* 9 mM). Simply by adding 5% EtOH necessary to use the recycling system, the solubility improved by almost 2 fold (3.3 mg mL^{-1}, *ca.* 16 mM). MeOH was selected among the miscible organic solvents for this experiment at a 10% ratio. Under these conditions the solubility of the substrate was increased to 5 mg mL^{-1} (*ca.* 24 mM).

Conclusion

Overall, the results indicate that the immobilised biocatalyst is remarkably robust, even in the presence of high concentrations of non-polar organic solvents, offering a good prospect for its use even with poorly water-soluble substrates. It has been shown elsewhere[12,19] that the range of substrate specificity of phenylalanine dehydrogenase may be greatly extended by site-directed mutagenesis. However, the utility of such novel biocatalysts could, in principle, be limited by the insolubility of some of the target substrates. It is therefore of considerable practical importance that in the immobilised state, as used here, phenylalanine dehydrogenase will tolerate concentrations of organic solvent in homogeneous systems that will greatly extend the solubility of such substrates. In fact, we have successfully exemplified how the synthesis of the non-natural amino acid *p*-NO_2-phenylalanine can be scaled-up in the presence of organic solvent: our results show that it is possible to work at much higher concentrations, with correspondingly higher yields. This favourable outcome is reinforced by the results obtained with biphasic systems where the enzyme remains in the predominantly aqueous phase and the organic phase serves as a reservoir for the substrate. As expected, in no case was the strict enantioselectivity of the catalyst compromised. Observation of the reactions over several hours confirms that the catalytic capacity of the enzyme persists, although there are indications of declining activity in some cases.

Experimental

Materials

All organic solvents, L-phenylalanine and phenylpyruvic acid were analytical grade purchased from Sigma-Aldrich, Celite® 521 was purchased from Sigma-Aldrich, NADH was obtained from Roche Diagnostic, and *Saccharomyces cerevisiae* alcohol dehydrogenase from Sigma. *p*-NO_2-phenylpyruvic acid was purchased from Frinton Laboratories, Inc., while *p*-NO_2-phenylalanine was obtained from Bachem. Mutant phenylalanine dehydrogenase N145A was prepared as described elsewhere.[15]

Immobilisation of the enzyme

The purified enzyme (0.6 mg mL^{-1}) was taken directly from the eluate of the Procion Red P3BN chromatography column in potassium phosphate buffer (20 mM), pH 7.9, containing NaCl (0.5 M),[15] without precipitating with ammonium sulfate. 2 mL of this protein solution was added to Celite (1 mg). The preparation was dried for approx. 18 h under vacuum and then stored at 4 °C.

Reactions in homogeneous co-solvents

For these experiments a freshly prepared stock reaction mixture was used consisting of KCl (200 mM), NH_4Cl (800 mM), NADH (2 mM) and phenylpyruvic acid (1 mM) in Tris (50 mM, pH 8.5). To 3 mL of this solution different amounts of homogeneous organic co-solvents: THF, CH_3CN, acetone and MeOH were added. The reactions were initiated with the enzyme-impregnated Celite (3 mg) and reaction mixtures were magnetically stirred in capped vials at room temperature. When cofactor regeneration was employed, the reaction mixture differed from that above in the addition of ethanol (5%) and EDTA (1 mM) and also the use of a lower NADH concentration (0.2 mM). When reaction was initiated as above, yeast alcohol dehydrogenase (1 mg) was also added.

Reactions in biphasic systems

The freshly prepared stock reaction mixture was as for the experiments of Table 1. In the first set of experiments (20%) organic solvent (0.75 mL) (*t*BuOMe, CH_2Cl_2, toluene, hexane or diethyl ether) was added to this solution (3 mL). To minimize changes in the phase volumes, the organic solvent was previously saturated with the Tris solution before use. The reactions were initiated as before with immobilised enzyme and the mixtures were efficiently magnetically stirred in capped vials at room temperature. In a second set of experiments the organic : aqueous ratio was reversed. To maintain the same total amounts of reactants in the combined volumes of both phases, the stock solution in Tris (50 mM, pH 8.5) was modified as follows: KCl (1600 mM), NH_4Cl (3200 mM), phenylpyruvic acid (4 mM) and NADH (8 mM): (80%) of the organic solvent (6 mL) and the supported enzyme (6 mg) were added to the stock solution (1.5 mL).

The recycling system for cofactor was also tested as follows: the supported enzyme (3 mg) and yeast alcohol dehydrogenase (1 mg) were added to the aqueous Tris buffer stock solution (3 mL), followed by (20%) of *t*BuOMe, CH_2Cl_2, toluene or diethyl ether (0.75 mL), and in this case the stock solution, freshly prepared in Tris buffer (50 mM, pH 8.5), contained: KCl (200 mM), NH_4Cl (800 mM), phenylpyruvic acid (1 mM), NADH (0.2 mM), EDTA (1 mM) and 5% EtOH.

Moving to the 80 : 20 organic: aqueous ratio we changed the stock solution as follows: freshly prepared stock solution in Tris buffer (50 mM, pH 8.5) contained KCl (1600 mM), NH_4Cl (3200 mM), phenylpyruvic acid (4 mM), NADH (0.8 mM), EDTA (4 mM) and 5% EtOH. To the Tris stock solution (1.5 mL) the supported enzyme (6 mg), alcohol dehydrogenase from *Saccharomyces cerevisiae* (2 mg) and (80%) of one of the following solvents *t*BuOMe, CH_2Cl_2, toluene, and diethyl ether (6 mL) were added.

Synthesis of *p*-NO_2-phenylalanine

The buffers for these reactions were prepared as described above for the reaction with homogeneous co-solvent and cofactor regeneration. The substrate concentration differed in the two experiments as follows: when no co-solvent other than 5% EtOH was added to the reaction mixture, *p*-NO_2-phenylpyruvic acid was 16 mM, while when 10% MeOH is present, the substrate concentration was 24 mM. Reactions were started as described above. As the reactions proceeded, the formation of an insoluble product was observed. In a different study (unpublished results), we observed that *p*-NO_2-phenylalanine was extremely insoluble in aqueous medium (2 mM) and that in presence of 10% MeOH we could achieve a concentration of 10 mM. Thus, it may be deduced that, in the present experiment, because of the high concentration of substrate, part of the product precipitated. After 24 h, samples of the reactions were centrifuged and supernatants were injected into the HPLC column to assess enantiopurity, and product concentration. The precipitated product was also analysed by HPLC. The crude reaction mixture was then evaporated and analysed by ^{1}H-MNR.

Quantitative product analysis

Formation of L-phenylalanine and *p*-NO_2-phenylalanine was monitored by HPLC analysis on a chiral column CHIROBIOTIC T (250 × 4.6 mm, eluent H_2O–EtOH 1 : 1, flow rate 1 mL min^{-1}, $l = 208$ nm). At different times, aliquot samples were filtered on a PTFE filter (0.20 m), diluted and directly injected. Calibration curves obtained with commercial L-phenylalanine and *p*-NO_2-phenylalanine were used for quantitative analysis.

Acknowledgements

This work was supported by MURST (ex-60% and FIRB), University of Bologna (Funds for selected topics) and in part by a grant from the Advanced Technology Research Programme of Enterprise Ireland.

References

1 A. Klibanov, *Nature*, 2001, **409**, 241–246.
2 G. Cainelli, V. De Matteis, P. Galletti, D. Giacomini and P. Orioli, *Chem. Commun.*, 2000, **23**, 2351–2352.
3 G. Cainelli, P. Galletti, D. Giacomini, A. Gualandi and A. Quintavalla, *Helv. Chim. Acta*, 2003, **86**, 3548–3559.
4 G. Carrea and S. Riva, *Angew. Chem., Int. Ed.*, 2000, **112**, 2226–2254.
5 A. Ghanem and H. Y. Aboul-Enein, *Tetrahedron: Asymmetry*, 2004, **15**, 3331–3351.
6 W. Hummel, *Trends Biotechnol.*, 1999, **17**, 487–492.
7 A. M. Klibanov, *Curr. Opin. Biotechnol.*, 2003, **14**, 427–431.
8 D. Metrangolo-Ruìz de Teminio, W. Hartmeier and M. B. Ansorge-Schumacher, *Enzyme Microb. Technol.*, 2005, **36**, 3–9.
9 H. Gröger, W. Hummel, S. Buchholz, K. Drauz, T. Van Nguyen, C. Rollmann, H. Husken and K. Abokitse, *Org. Lett.*, 2003, **5**, 173–176.
10 Y. Asano, A. Nakazawa and K. Endo, *J. Biol. Chem.*, 1987, **21**, 10346–10354.
11 S. Y. K. Seah, K. L. Britton, D. W. Rice, Y. Asano and P. C. Engel, *Biochemistry*, 2002, **41**, 11390.
12 P. Busca, F. Paradisi, E. Moynihan, A. R. Maguire and P. C. Engel, *Org. Biomol. Chem.*, 2004, **2**, 2684–2691.
13 J. Grunwald, B. Wirz, M. P. Scollar and A. M. Klibanov, *J. Am. Chem. Soc.*, 1986, **108**, 6732–6734.
14 J. B. Jones and T. Takemura, *Can. J. Chem.*, 1984, **62**, 77–80.
15 Y. K. Seah, K. L. Britton, P. J. Baker, D. W. Rice, Y. Asano and P. C. Engel, *FEBS Lett.*, 1995, **370**, 93–96.
16 M. Persson, E. Wehtje and P. Adlercreutz, *ChemBioChem*, 2002, **3**, 566–571.
17 M. Persson, E. Wehtje and P. Adlercreutz, *Biotechnol. Lett.*, 2000, **22**, 1571–1575.
18 T. Morikawa and K. Yoshida, *Kagaku to Kogyo*, 1963, **37**, 107.
19 P. C. Engel, A. R. Maguire, F. Paradisi, O. McCrohan, D. Giacomini, P. Galletti, *Ir. Pat.* S2004/0547, 2004.