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Cross-Linked Aggregates of the Hydroxynitrile Lyase from *Manihot esculenta*: Highly Active and Robust Biocatalysts

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Abstract: The precipitation and cross-linking into CLEAs of the hydroxynitrile lyase (E.C. 4.1.2.10) from *Manihot esculenta* was investigated and an optimized procedure, which involved precipitation with $(NH_4)_2SO_4$, was developed. It was found that a fast (photometric) assay severely underestimated the activity recovery in the CLEA due to rate-limiting diffusion, whereas in a synthetic assay the activity recovery was up to 93% of the starting activity. The CLEA was applied in the hydrocyanation of various aldehydes and ketones in microaqueous medium, conditions that rendered the non-enzymatic background reaction insignificant, even with unreactive substrates.

Keywords: cross-linked enzyme aggregate; cyanohydrin synthesis; hydrocyanation; hydroxynitrile lyase; *Manihot esculenta*; microaqueous medium

Introduction

The (*R*)- and (*S*)-selective oxynitrilases (hydroxynitrile lyases, E.C. 4.1.2.10), which catalyze the addition of HCN to aldehydes, are undergoing a rapid transition from laboratory curiosities into industrial biocatalysts, in particular since the (*S*)-selective oxynitrilases from *Manihot esculenta*[1] and *Hevea brasiliensis*[2] have become available *via* recombinant expression.

The enantiomeric purity of the product is critically dependent on the competition of the enantioselective enzymatic hydrocyanation and the non-selective chemical reaction. It is common practice nowadays to suppress the chemical reaction by maintaining an acidic environment. A biphasic, aqueous-organic medium, with an aqueous working phase and an organic extractive phase further reduces the contribution of chemical hydrocyanation.

The non-enzymatic reaction is reduced even further in a micro-aqueous reaction medium,[3] in which the amount of aqueous phase is the minimum consistent with maintaining enzymatic activity. We have shown that this approach is particularly effective with a slow-reacting aldehyde, such as 2-chlorobenzaldehyde.[4] Quite recently, we have shown that a cross-linked enzyme aggregate (CLEA) of the (*R*)-specific oxynitrilase from *Prunus amygdalus* efficiently catalyzed hydrocyanation in the presence of only 2% of added buffer,[5] conditions that are difficult to reconcile with the use of free enzyme. A particular advantage of CLEAs, in comparison with carrier-fixed preparations,[6] is that a CLEA consists of nearly pure protein and requires only a minute amount of water to maintain full hydration.[5]

We now report an optimization study, complimentary to recent results from our laboratory,[7] of the preparation of CLEAs of the oxynitrilase from *M. esculenta* (*Me*HnL) and the use of the latter as a hydrocyanation catalyst.

Results and Discussion

Precipitation Study of *Me*HnL

The preparation of a CLEA involves the precipitation/aggregation of the protein followed by cross-linking.[8] We studied the precipitation step in isolation by treating a *Me*HnL solution with various amounts of organic solvents and salts. The enzymatic activity in the precipitate – upon redissolution – and in the supernatant were measured using a standard UV assay. A precipitant threshold concentration was often observed (see Figure 1, panel A for an example). The activity recoveries at the optimum precipitant concentration, which varied widely, are listed in Table 1. Pre-

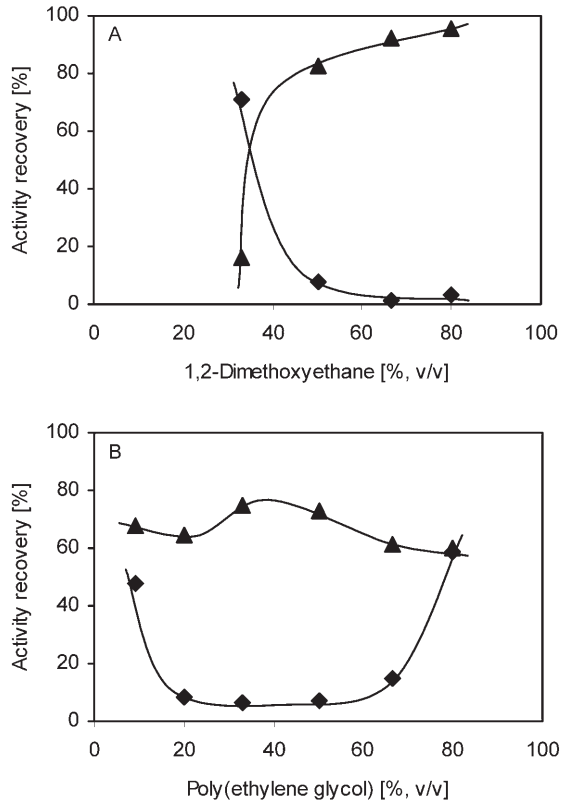

Figure 1. Precipitation of *Me*HnL with 1,2-dimethoxyethane (**A**) and poly(ethylene glycol) (**B**); activities recovered from the precipitate (▲) and the supernatant (◆).

cipitation with poly(ethylene glycol), which was very efficient with penicillin acylase,[8] gave an erratic result with *Me*HnL (see Figure 1, panel B) and was not investigated further.

Preparation of *Me*HnL CLEAs

Cross-linking of the precipitated *Me*HnL with glutaraldehyde resulted in the formation of insoluble CLEAs. These were rinsed and subsequently subjected to a dissolution test. The amount of soluble enzyme that could be recovered from the CLEAs was low, typically 0.3–0.5 % of the starting activity. The recovered activity of the CLEAs, as measured in the UV test, was low and showed no obvious correlation with the results of the precipitation-redissolution test (see Table 1). The activity recoveries in a subsequent synthetic assay, which were much higher, also did not reveal a relationship with the precipitation-redissolution test. This latter observation indicates that structural changes of the protein upon exposure to the precipitant are fixed by subsequent cross-linking, whereas the precipitation-redissolution test is only an indicator of irreversible structural changes. The precipitants of choice for the preparation of *Me*HnL CLEAs are 1,2-dimethoxyethane and ammonium sulfate with activity recoveries of 87 and 93 %, respectively. The morphology of the CLEA particles was investigated by scanning electron microscopy (Figure 2), which revealed the existence of micropores of 50–100 nm size.

The discrepancy between the results of the UV and synthetic assays reasserts the importance of diffusion effects on immobilized enzymes in a fast assay. Hence, a meaningful assay should be based on a reaction that is not diffusion limited. A hydrocyanation reaction, such as used above, is quite suited although the necessity to monitor its progress by HPLC makes it somewhat less convenient than a spectrophotometric assay. In the following, synthetic, part of the paper we have quantified amounts of *Me*HnL in mandelonitrile synthesis units (MSU).

Table 1. Effects of the precipitant on the activity recovery.

Precipitant	Conc. [%, v/v][a]	Activity in precipitate [%][b,c]	Glutaraldehyde [%, *v/v*]	CLEA UV activity [%][b]	CLEA synthetic activity [%][b,d]
Acetone	50	100	1.0	11	6
2-Propanol	50	100	1.0	22	38
1,2-Dimethoxyethane	80	96	0.5	11	87
1-Propanol	67	94	0.5	14	32
Ammonium sulfate	80	84	0.5	23	93
tert-Butyl alcohol	80	80	1.0	35	38
Acetonitrile	50	74	2.0	15	41
Ethanol	80	69	0.5	14	49
Poly(ethylene glycol)	50	61	n.d.	n.d.	n.d.

[a] Optimized for recovery from the precipitate.
[b] Activity in % recovery of the starting activity.
[c] UV assay of the redissolved precipitate
[d] Determined in the hydrocyanation of cinnamic aldehyde.

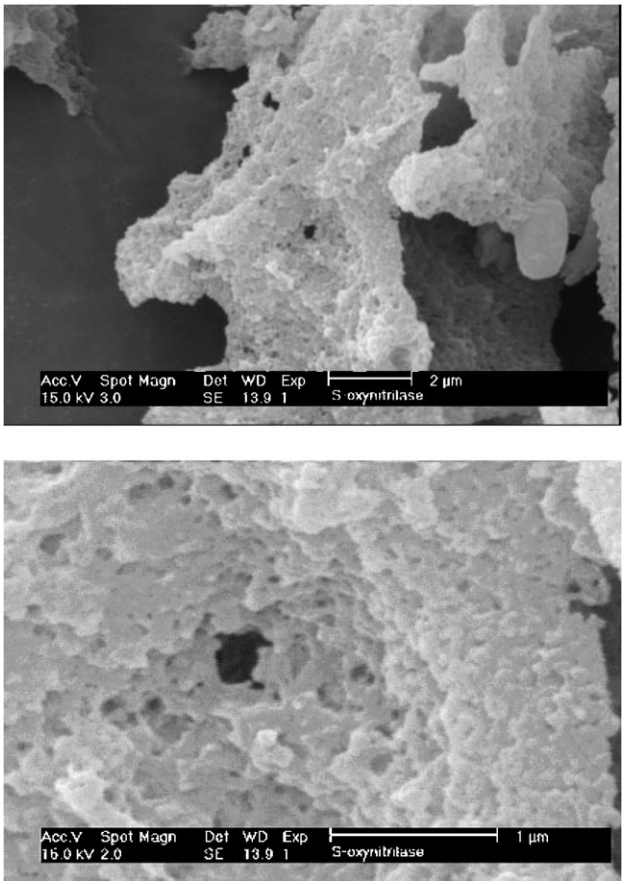

Figure 2. SEM photographs of an *Me*HnL CLEA at $5 \cdot 10^3$ (top) and $20 \cdot 10^3$ (bottom) magnification.

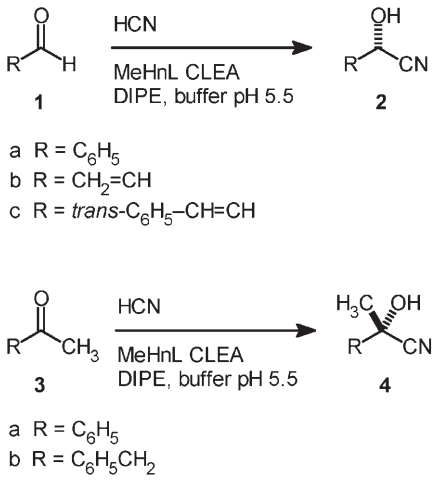

Figure 3. Enzymatic hydrocyanation of aldehydes and ketones.

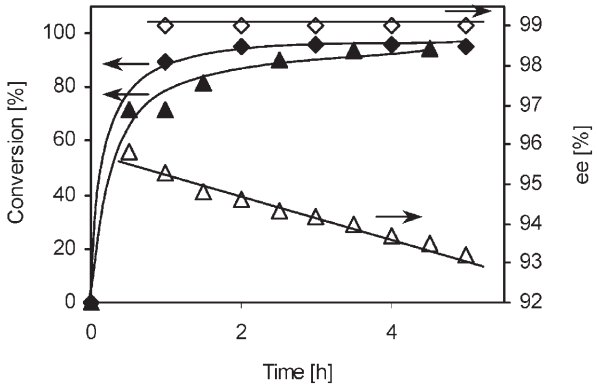

Figure 4. Hydrocyanation of **1a** (100 mM) in DIPE-citrate buffer pH 5.5; *Me*HnL CLEA (22 MSU mL^{-1}), 10% buffer, 500 mM HCN, progress (▲), *ee* (△); *Me*HnL CLEA (7 MSU mL^{-1}), 2% buffer, 700 mM HCN, progress (◆), *ee* (◇).

Synthetic Application in Microaqeous Medium

The *Me*HnL CLEA prepared by precipitation with ammonium sulfate was selected for further study in cyanohydrin synthesis. A major advantage of the CLEA methodology is the possibility to perform reactions in microaqueous (<2% buffer) diisopropyl ether (DIPE), to restrict the uncatalyzed hydrocyanation.

The hydrocyanation of benzaldehyde (**1a**, see Figure 3), which is a common test-bed for enantioselective HCN addition, was investigated first. The enantiomeric purity of the produced (*S*)-mandelonitrile (**2a**) was only 96% initially when the reaction was performed in the presence of 10% (v/v) buffer solution. This modest enantioselectivity, which is lower than previously reported, is attributed to a competing uncatalyzed reaction in the pH 5.5 buffer fraction. A slight erosion of the *ee* became manifest at the approach of equilibrium (see Figure 4). We ascribe this latter phenomenon to equilibrium-related cycling of reactant and product, which is bound to result in the accumulation of (*R*)-**2a** because the dehydrocyanation is more (*S*)-selective than the HCN addition. Non-enzymatic cycling of reactant and product may also play a role in the loss of *ee*.

Enantiomerically pure **2a** was formed in 96% yield when the amount of buffer was reduced to 2%. Presumably because of more efficient interphase mass-transfer – the reaction mixture became monophasic as all of the buffer was absorbed by the biocatalyst – the amount of biocatalyst could be reduced three-fold without sacrificing reaction rate (see Figure 4); the excess of HCN was increased from three- to seven-fold to delay the enzymatic dehydrocyanation of (*S*)-**2a**. The product *ee* stayed high in the course of this latter reaction, presumably because, due to the suppression of the non-enzymatic reaction, there was much less (*R*)-**2a** formed.

The procedure was subsequently scaled up, with minimum change, to a 25 g scale and afforded (*S*)-**2a** in near-quantitative yield and *ee*. Reducing the amount of buffer to 0.2% did not result in further im-

Table 2. Hydrocyanation of aldehydes and ketones in the presence of *Me*HnL CLEA.

Reactant	Enzyme [MSU mL^{-1}]	Buffer [%]	HCN [equivs.]	Time [h]	Conversion [%]	*ee* [%]
1a	22	10	5	4.0	94	94
	7	2.0	7	3.0	96	99
	7	0.2	7	5.5	95	99
1b	3	0.1	6	1.0	96	49
	9	0.0	5	1.0	96	42
0 °C	12	0.1	12	2.0	99	55
1c	400	2.5	6	6.2	86	87
	400	2.5	12	6.2	90	92
3a	32	0.2	12	3.8	7	>99
	32	0.2	12	23	12	91
0 °C	32	0.2	12	23	11	98
3b	17	0.5	12	2.5	90	>96

provement (see Table 2). This latter result attests, however, to the extreme stability of the *Me*HnL CLEA, as is also noted in an accompanying study;[7] a CLEA of the (*R*)-specific oxynitrilase from *P. amygdalus*, in contrast, required 2% of water.[5]

The reduction of the buffer phase that the CLEA methodology makes possible is particularly attractive with a reactant such as 2-propenal (**1b**), which readily dissolves in water. It is also noteworthy that the enantioselectivities that have been obtained in the hydrocyanation of **1b** in the presence of two very similar (*S*)-oxynitrilases (*Me*HnL: 56%;[1] *Hb*HnL: 98%[9]) do rather diverge and the question arises whether the discrepancy is inherent or caused by an uncontrolled background reaction. We found that **1b** reacted rapidly in microaqueous DIPE although, in contrast with a previous report,[10] at least a three-fold excess of HCN was required to obtain >95% conversion. The enantiomeric purity of the product (**2b**) was a modest 55–56% initially and decreased when the reaction proceeded towards equilibrium conversion (see Figure 5). This low *ee* is not caused by a non-enzymatic background reaction, because neither a decrease of the pH to 4 nor reducing the amount of buffer had any effect (data not shown). The *Me*HNL CLEA even maintained its activity in completely dry DIPE (Table 2), but no beneficial effect on the product *ee* became apparent. This latter experiment attests to the extreme robustness of the CLEA.[7]

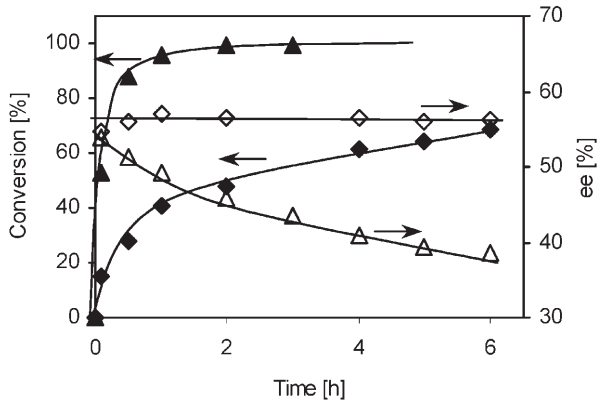

Figure 5. Hydrocyanation of **1b** (100 mM) in DIPE-citrate buffer pH 5.5; *Me*HnL CLEA (3 MSU mL^{-1}), 0.1% buffer, 600 mM HCN, progress (▲), *ee* (△); *Me*HnL CLEA (1.8 MSU mL^{-1}), 500 mM HCN, progress (◆), *ee* (◇).

An experiment with a reduced enzyme load (Figure 5), in which equilibrium was not reached, demonstrated that *Me*HnL effects the hydrocyanation of **1b** with an inherent *ee* of 56–57%. We conclude that the erosion of the *ee* of **2b** noted above is caused by (*S*)-selective enzymatic dehydrocyanation upon the approach of equilibrium.

A much improved result was obtained when the hydrocyanation of **1b** was performed at 0 °C. The enzyme load was doubled to compensate for the loss in activity and the excess of HCN was increased to 12-fold to retard the enzymatic dehydrocyanation. Full conversion (>99%) into **2b** with 55% *ee* was reached in 2 h under these conditions.

The hydrocyanation of cinnamic aldehyde (**1c**) is notoriously slow and suffers from an unfavorable equilibrium.[11] We accordingly increased the amount of enzyme (see Table 2), but obtained only 84% conversion with 87% *ee*. Eventually, upon the application of a very large (12-fold) excess of HCN, **2c** was obtained in 90% yield and 93% *ee*, which compares favorably with a reported result (80% yield, 95% *ee*).[12] The question arises whether the somewhat modest product *ee* is caused by a non-enzymatic background reaction, or is inherent to the *Me*HnL. An experiment with a reduced amount of enzyme, to prolong the reaction over time (see Figure 6) showed the absence of a background reaction. It also became clear that the enantiopreference of the enzyme is high but that erosion of the product *ee* already starts at < 50% conversion, presumably because enzymatic dehydrocyanation already makes itself felt.

The conclusions from the hydrocyanation of **1a–c** in the presence of the CLEA biocatalyst are two-fold.

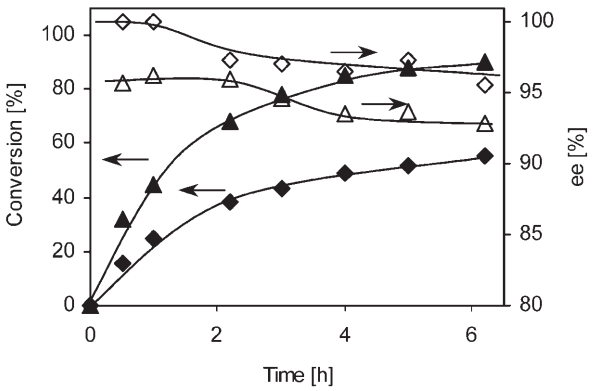

Figure 6. Hydrocyanation of **1c** (100 mM) in DIPE-citrate buffer pH 5.5, 1.2 M HCN; *Me*HnL CLEA (400 MSU mL^{-1}), 0.25 % buffer, progress (▲), *ee* (△); *Me*HnL CLEA (200 MSU mL^{-1}), progress (◆), *ee* (◇).

First, the severe reduction of the buffer phase that the CLEA tolerates renders the non-enzymatic background reaction insignificant. Second, the erosion of the product *ee* upon the approach of equilibrium, which should be expected on theoretical grounds but previously was obscured by the non-enzymatic hydrocyanation, is now a major limiting factor whenever the intrinsic enantiomer preference of the biocatalyst is short of absolute.

The high enzyme concentrations that become possible upon adopting the CLEA methodology are particularly advantageous in the hydrocyanation of recalcitrant reactants, such as ketones. Thus, the hydrocyanation of acetophenone (**3a**, see Figure 6) is known to be incomplete and the *ee* of the adduct (**4a**) is modest (up to 87 %)[1,12] when the reaction is performed in the presence of *Me*HnL in biphasic medium.

We performed the hydrocyanation of **3a** at 0.2 % buffer concentration and found that the reaction did not proceed beyond 8–11 % conversion, depending on the reactant concentrations. Because adding extra enzyme had no effect – which attests that the stagnation is not caused by biocatalyst deactivation – we conclude that equilibrium is already reached at these conversions. The non-catalytic hydrocyanation was negligible (approx. 1 % over 24 h) at 0.2 % of buffer. The product *ee* was initially > 99 % but slowly decreased at the approach of equilibrium (see Table 2), as before. The enantiomeric purity of the product was improved to 98 % (at 11 % conversion, see Table 2) by performing the reaction at 0 °C.

1-Phenylpropanone (**3b**) reacted much faster than **3a** and the equilibrium conversion was much higher as well. Its HCN adduct (**4b**) was obtained in 90 % yield and > 96 % *ee*.

Conclusions

An optimized procedure affords a cross-linked enzyme aggregate (CLEA) of the hydroxynitrile lyase from *Manihot esculenta* with up to 93 % recovery of the synthetic activity. A fast (photometric) assay severely underestimated the activity recovery in the CLEA due to rate-limiting diffusion.

The CLEA is a very stable biocatalyst that is very suitable for hydrocyanation reactions in microaqueous and even anhydrous media. Such reaction conditions render the non-enzymatic hydrocyanation insignificant. The synthetic potential of the CLEA has been demonstrated in the enantioselective hydrocyanation of a number of aldehydes and ketones. A decay of the product *ee* was observed upon the approach of equilibrium conversion and was ascribed to enzymatic cycling of reactant and product.

Experimental Section

Materials

Semi-purified (*S*)-hydroxynitrile lyase from *Manihot esculenta* (3000 U mL^{-1}) was obtained from Jülich Fine Chemicals (Jülich, Germany). A CLEA of *Me*HnL [batch number CLEAMEHNL-S03–150–03512, 78 mandelonitrile synthesis units (MSU, see below) mg^{-1}] from CLEA Technologies (Delft, The Netherlands) was used in the synthetic hydrocyanation experiments.

Acetophenone, acrolein, benzaldehyde, cinnamic aldehyde, diisopropyl ether (DIPE), 1,3-dimethoxybenzene and 1-phenyl-2-propanone were obtained from Acros, Aldrich or Fluka and were used as received. Racemic mandelonitrile was obtained from Fluka. Racemic 2-hydroxy-3-butenenitrile,[13] 2-hydroxy-2-methyl-3-phenylpropionitrile[11b] and 2-hydroxy-4-phenyl-*trans*-3-butenenitrile[11b] were prepared according to literature procedures.

A 2 M solution of hydrogen cyanide in DIPE was prepared from sodium cyanide as described.[4] **Warning:** sodium cyanide and HCN are highly poisonous. They should be handled in a fume cupboard with a good draught. It is strongly advised to keep an HCN alarm switched on.

Analytical Procedures

The progress of the reactions was measured by HPLC; enantiomeric purities were measured by chiral HPLC or GC as described. HPLC analyses were carried out using a Waters 510 pump and a Waters 468 variable wavelength UV detector at 215 nm. GC analyses were performed on a Shimadzu GC-17 instrument equipped with a FID detector and a Varian Inc. 25 m × 0.32 mm Chirasil-Dex CB column; the carrier gas was N_2. Samples (10 μL) were treated with a derivatization reagent (300 μL) that contained acetic anhydride (0.8 mL) and pyridine (0.8 mL) in dichloromethane (10 mL) and analyzed after standing for 1 h. Further details are given below.

Photometric Assay

To citrate buffer pH 5.5 (3 mL, 20 mM) in a quartz cuvette was added a solution of mandelonitrile in 1,2-dimethoxyethane (0.4%, v/v, 20 μL) and the uncatalyzed dehydrocyanation was monitored spectrometrically (Cary 3 bio UV spectrophotometer) at 250 nm and 25 °C. Then an appropriate amount of *Me*HnL was added and the enzymatic dehydrocyanation was monitored as above.

The activity recovery from precipitation and cross-linking experiments was measured with a 20 μL sample from the 10 times diluted pellet or supernatant solutions. One unit (U) of *Me*HnL will liberate 1 μmol of benzaldehyde per min under these conditions.

Kinetic Assay

2-Hydroxy-4-phenyl-trans-*3-butenenitrile:* To citrate buffer pH 5.5 (2 mL, 20 mM) were added *Me*HnL solution (250 μL) or an equivalent amount of CLEA, DIPE (0.4 mL), cinnamic aldehyde solution in DIPE (2 M, 0.4 mL) and HCN solution in DIPE (2 M, 1.2 mL). The mixture was stirred and a temperature of 0 °C was maintained. The reaction vessel was kept closed to prevent escape of HCN.

A 20 μL sample was taken every h from the organic phase for up to 3 h and dissolved into hexane containing 2% TFA (1 mL). The remaining water was removed by adding some Na_2SO_4. The sample was centrifuged and analyzed by HPLC.

Mandelonitrile: To citrate buffer pH 5.5 (2 mL) in a 10 mL screw-cap vessel were added an appropriate amount of enzyme preparation, HCN solution in DIPE (2 M, 1.2 mL), and DIPE (700 μL). The mixture was stirred magnetically, chilled to 0 °C in ice/water and benzaldehyde (100 μL) was added. After 10 min (< 30% conversion) a sample (10 μL) was taken from the organic phase, diluted with eluant (990 μL), dried over Na_2SO_4 and analyzed by HPLC. One mandelonitrile synthesis unit (MSU) will synthesize one μmol of mandelonitrile per min under these conditions.

Precipitation Experiments

A solution of *Me*HnL (250 μL, 3000 U mL^{-1}) in an Eppendorf tube was mixed with phosphate buffer pH 8 (50 μL, 0.1 M), an appropriate amount of precipitant was added and the mixture was mechanically shaken at 0 °C for 4 min, then centrifuged for 3 min. To the supernatant citrate buffer pH 5.5 (20 mM) was added to a total volume of 1 mL; the pellet was dissolved in citrate buffer pH 5.5 (1 mL, 20 mM). The enzymatic activity in both fractions was measured photometrically as described above.

CLEA Preparation

Precipitates of *Me*HnL were prepared using the solvents and concentrations listed in Table 1. Glutaraldehyde (25% in water) was added in amounts of 0.5, 1.0 and 2.0% (v/v) with respect to the total volume and the resulting suspensions were mechanically shaken at 0 °C for 5 min. The CLEA was separated by centrifugation, resuspended in 20 mM citrate buffer pH 5.5 and centrifuged. The enzymatic activity in the supernatant and the pellet (resuspended in citrate buffer) were assayed photometrically. The optimum amounts of glutaraldehyde, as regards activity recovery in the CLEA, are listed in Table 1. The residual activity in the supernatant was < 0.5% of the starting activity in all experiments.

A leakage test was performed by keeping CLEAs for 3 d in 20 mM citrate buffer pH 5.5. The activity recovery in the supernatant was < 0.5%.

The *Me*HnL CLEA for use in the hydrocyanation experiments was prepared by precipitation with ammonium sulfate and cross-linking with 0.5% (v/v) of glutaraldehyde solution, washed with citrate buffer as described above and lyophilized.

Enzymatic Hydrocyanation; General Procedure

The reagents and internal standard (1,3-dimethoxybenzene) were taken from 2 M stock solutions in DIPE. DIPE was kept saturated with 20 mM citrate buffer pH 5.5 (unless noted otherwise). The *Me*HnL CLEA biocatalyst (from CLEA Technologies, equivalent to the one described above) was kept as a suspension in DIPE and appropriate amounts were pipetted into the reaction mixtures.

The hydrocyanation experiments were carried out in 2 mL glass reactors equipped with a PTFE-lined screw cap. The appropriate amounts of buffer, DIPE, MeHnL CLEA suspension, HCN solution and internal standard were introduced into the reactor, which was magnetically stirred and thermostatted using a water bath (25 °C) or a cryostat (0 °C). The reaction was started by adding the aldehyde or ketone (100 mM with respect to the total volume).

Samples (10 μL) for HPLC analysis were taken periodically, diluted with eluant (990 μL), dried over sodium sulfate and centrifuged prior to injection.

Benzaldehyde (**1a**): The stock solution of **1a** was kept over saturated $NaHCO_3$ to prevent accumulation of benzoic acid. The hydrocyanation reactions of **1a** were carried out as described in the general procedure. Progress and *ee* were measured by HPLC [4.6 × 250 mm 5 μ Chiralcel OB-H column (Daicel), eluant hexane/2-propanol (90:10, v/v) at 0.5 mL min^{-1} at 35 °C]. Retention times of mandelonitrile: 17.3 min (*R*), 18.3 min (*S*).

Acrolein (**1b**): The hydrocyanation reactions of **1b** were carried out as described in the general procedure. The reaction progress was measured by HPLC (4.6 × 250 mm 5 μ Chiralcel OB-H column (Daicel), eluant hexane/2-propanol (90:10, v/v) at 0.5 mL min^{-1}). Chiral analysis was carried out by GC as described above; temperature program: 10 min at 100 °C, then a gradient of 13 °C min^{-1} to 180 °C. Retention times of 2-hydroxy-3-butenenitrile: 2.2 min (*R*), 2.8 min (*S*).

Cinnamic aldehyde (**1c**): The hydrocyanation experiments of **1c** were carried out as described in the general procedure. The reaction progress was measured by HPLC [4.6 × 250 mm 5 μ Chiralcel OB-H column (Daicel), eluant hexane/2-propanol (90:10, v/v) at 0.5 mL min^{-1}]. Chiral analysis was carried out by GC as described above at 160 °C column temperature. Retention times of 2-hydroxy-4-phenyl-*trans*-3-butenenitrile: 7.3 min (*R*), 8.1 min (*S*).

Acetophenone (**3a**): The hydrocyanation reactions of **3a** were carried out as described in the general procedure. Progress and *ee* were measured by HPLC [4.6 × 250 mm 5 μ Chiralcel OB-H column (Daicel), eluant hexane-2-propanol

(90:10, v/v) at 0.5 mL min^{-1} at 35 °C]. Retention times of 2-hydroxy-2-phenylpropionitrile: 14.0 (*R*), 14.6 (*S*).

1-Phenylpropanone (**3b**): The hydrocyanation reactions of **3b** were carried out as described in the general procedure but at 6 mL scale in a 10 mL reaction vessel, to accommodate the increased sample volume. The reaction progress was monitored by HPLC {4.6×250 mm 5 μ Chiralcel OD-H column (Daicel), eluant hexane/2-propanol (90:10, v/v) at 0.5 mL min^{-1}].

Samples for chiral analysis (200 μL) were mixed with a derivatization reagent composed of butyric anhydride (40 μL), *N*,*N*-dimethylaminopyridine (40 mg) in dioxane (400 μL), stirred for 2 h and washed with dilute HCl. A sample of the organic phase was taken, diluted with eluant and analyzed by HPLC [4.6×250 mm 5 μ Chiralcel OB-H column (Daicel), eluant hexane/2-propanol (90:10, v/v) at 0.5 mL min^{-1} at 35 °C]. Retention times of 2-hydroxy-2-methyl-3-phenylpropionitrile: 22.1 min (*R*), 33.0 min (*S*).

Large-Scale Synthesis of (*S*)-mandelonitrile (2a)

To DIPE (0.25 L) in a 1 L round-bottomed flask were added *Me*MnL CLEA (0.25 g, 19.6 kMSU) and 20 mM citrate buffer pH 5.5 (2 mL). The mixture was stirred vigorously in an ice-water batch until a fine suspension was obtained. HCN was added as a 2 M solution in DIPE (200 mL), followed by a solution of benzaldehyde (25 g, 0.24 mol) in DIPE (250 mL). The mixture was stirred at room temperature.

The progress and product *ee* were monitored as described above. Complete conversion was obtained in 2 h. The reaction mixture was filtered to remove the biocatalyst, dried over Na_2SO_4 and concentrated under vacuum at 50 °C until the last traces of HCN had been removed. Compound **2a** was obtained as a colorless oil in 99 % yield and 99.6 % *ee*.

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