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Xylella fastidiosa Esterase Rather than Hydroxynitrile Lyase

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In 2009, we reported that the product of the gene SCJ21.16 (XFa0032) from *Xylella fastidiosa*, a xylem-restricted plant pathogen that causes a range of diseases in several important crops, encodes a protein (*Xf*HNL) with putative hydroxynitrile lyase activity. Sequence analysis and activity tests indicated that *Xf*HNL exhibits an α/β-hydrolase fold and could be classified as a member of the family of FAD-independent HNLs. Here we provide a more detailed sequence analysis and new experimental data. Using pure heterologously expressed *Xf*HNL we show that this enzyme cannot catalyse the cleavage/synthesis of mandelonitrile and that this protein is in fact a non-enantioselective esterase. Homology modelling and ligand docking simulations were used to study the active site and support these results. This finding could help elucidate the common ancestor of esterases and hydroxynitrile lyases with an α/β -hydrolase fold.

Introduction

Sequence alignment and database search tools have been used in various life science fields to find alternate or missing enzymes in metabolic pathways, as well as in drug discovery and in toxicity studies.[1] Additionally, they can shed light on the evolutionary aspects of enzyme mechanisms. These tools have improved over time,[2] and currently they constitute a powerful set of methods for analysing relationships among sequences in protein (super)families and developing hypotheses about structure–function relationships in families and superfamilies.

In 2000, the genome of *Xylella fastidiosa*, the first plant phytopathogen to be completely sequenced, was unravelled.[3] In a search to elucidate the plant–pathogen interaction, a sequence similarity search assigned the gene product XFa0032 (a 251 amino acids protein) as an FAD-independent hydroxynitrile lyase (HNL) with an α/β-hydrolase fold.[4]

HNLs form an interesting group of enzymes, and are used for the formation of enantiopure cyanohydrins. Over the last two decades, the enzymes have been investigated extensively, especially regarding their potential as catalysts in organic chemistry,[5] as cyanohydrins constitute important building blocks for fine chemicals.[6] Within this group of enzymes, which belong to different protein families with no significant sequence similarity, some of the best characterised HNLs are those having an α/β-hydrolase fold. They are related to the esterase/lipase superfamily: the *S*-selective enzymes from *Hevea brasiliensis* (*Hb*HNL), *Manihot esculenta* (*Me*HNL), *Sorghum bicolor* (*Sb*HNL) and *Baliospermum montanum* (*Bm*HNL) and the *R*-selective enzyme from *Arabidopsis thaliana* (*At*HNL).[7] Most HNLs have been discovered in plants, and several have been cloned successfully and expressed in *Escherichia coli* or yeasts such as *Pichia pastoris*.[8]

The amino acid sequence of *Xf*HNL was compared with HNLs of the esterase/lipase superfamily, and the sequence alignment showed an overall identity of about 30% between all sequences, with greater similarity to the *R*-selective *At*HNL, although in the work of Caruso et al., the enantioselectivity of XFa0032 (*Xf*HNL) was not described and the crystal structure was not available.[4]

*Xf*HNL is the first example of a bacterial HNL, although recently HNLs showing sequence similarity to proteins of the cupin superfamily have been described in various bacteria.[9] Our findings show that *Xf*HNL is able to hydrolyse esterase substrates and, despite the high sequence homology and the results of a previous report,[4] no hydroxynitrile lyase activity could be detected.

Results and Discussion

HNL activity of *Xf*HNL

As mentioned above, a sequence similarity search can help to determine the activity of an unknown protein, though this is a first step. Subsequently, the protein has to be purified and an activity test has to be carried out with natural substrates for each class of enzyme (oxidoreductase, hydrolase, lyase, etc.) in order to identify the enzymatic activity. The cleavage of mandelonitrile is the common reaction to test for HNL activity (Scheme 1).[10] This substrate has the disadvantage that it is not stable above pH 5; it rapidly decomposes to benzaldehyde, so spontaneous decomposition has to be subtracted from the

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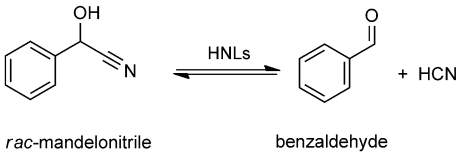

Scheme 1. Cleavage of mandelonitrile catalysed by HNLs.

enzymatic reaction in an activity test. Another difficulty is that a change of 1 °C in the cuvette of the UV spectrophotometer results in a measuring error of approximately 10%. Finally, the mandelonitrile purity should be high enough to give an initial absorption of 0.4–0.8 (at 280 nm) to obtain reliable results.

HNL activity of 42.9 U mg^{-1} at pH 7 was reported for *Xf*HNL by Caruso et al.[4] The authors mentioned that the enzymatic reaction at pH 8.0 (where *Xf*HNL is more stable) was not evaluated, because of the significant degradation of mandelonitrile. In our experience, even at pH 7 spontaneous degradation of mandelonitrile is quite fast. This makes it difficult to detect an enzymatic reaction. Caruso et al. also reported 5.1 U mg^{-1} at pH 5; this is a low but acceptable activity.

Here, in order to elucidate the enantioselectivity of this HNL, we synthesised the gene based on gene accession number NP_061688 according to the method of Caruso et al.[4] (Supporting Information). The synthetic gene was cloned into the expression vector pET28a. *E. coli* TOP10 competent cells were used for plasmid propagation, then the plasmid was transformed into *E. coli* BL21(DE3) for expression and purification of the full-length protein. All experiments were also performed with the originally published clone of the enzyme,[4] with as expected identical results.

Surprisingly, during the preliminary activity assays, no activity was observed at pH 5. Additionally, at pH 5.5, 6, 6.5 and 7, the same results were obtained for the controls as for the reactions with enzyme (Figure 1). It is important to note that as the pH increases, so does the rate of decomposition of mandelonitrile. Control reactions for background subtractions must be carried out carefully. Additionally, mandelonitrile synthesis reactions at pH 5 and 6.5 were carried out in order to confirm the total absence of HNL activity in both directions of the reaction. A biphasic system using an excess of HCN in MTBE was used (see Experimental Section). As in the case of the cleavage of mandelonitrile, no activity was observed.

Amino acid sequence analysis of *Xf*HNL

In view of these results, a second analysis of the sequence alignment of *Xf*HNL was performed with the HNLs of the α/β-hydrolase fold family for which X-ray data are available (*Me*HNL, *Hb*HNL, *At*HNL, and *Bm*HNL; Figure 2). *Sb*HNL was not included because this enzyme has a completely different active-site architecture: the conventional classic triad is not present.[11] The sequence analysis showed that *Xf*HNL

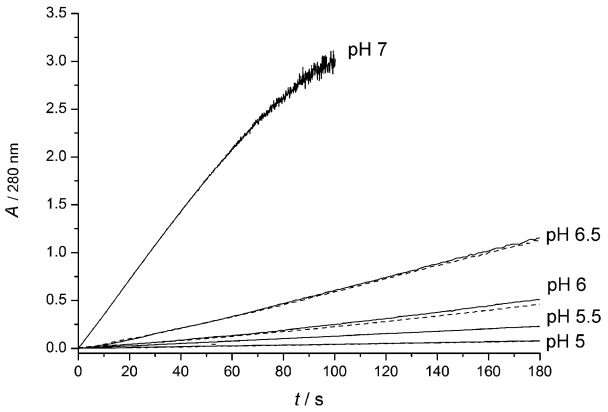

Figure 1. Absorbance (280 nm) comparison between the reaction activity tests with enzyme (-----) and the control reactions without enzyme (——) at pH 5, 5.5, 6, 6.5 and 7.

shares about 25% amino acid identity with the HNLs and that Ser87 is one of the catalytically active residue. The small amino acids flanking the serine allow the formation of the sharp bend that is typical for the "nucleophile elbow" of α/β-hydrolase-fold proteins.[12] The two other residues of the catalytic triad are also found at conserved positions in *Xf*HNL (Asp200 and His226).

Interestingly, members of the α/β-hydrolase fold show both *R* and *S* selectivity.[7,13] For the *S*-selective HNLs (*Hb*HNL, *Me*HNL, and *Bm*HNL), the enzymatic mechanism involves the Ser-His-Asp catalytic triad as a general base to deprotonate the cyanohydrin hydroxyl group. In this triad, the histidine residue

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XfHNL   -----MNAYRPIAFASSAASDKPTVLLKPTIVLVHGAFADGSTWNKVIHQLQAKGLNAVS 55
AtHNL   ------------------------MERKHHFVLVHNAYHGAWIWYKLKPLLESAGHRVTA 36
BmHNL   MNHKVHHHHHHHIEGRHMELGTLEGFMVSAHFILIHTICHGAWLWYKLIPLLQSAGHNATA 60
HbHNL   -------------------------MAFAHFVLIHTICHGAWIWHKLKPLLEALGHKVTA 35
MeHNL   -------------------------MVTAHFVLIHTICHGAWIWHKLKPALERAGHKVTA 35

                                                  #
XfHNL   VQ--------------NPLTSFEDDVAATRRVLALQTGPVVLVGHSYGGAVITEAGQD-- 99
AtHNL   VELAASGIDPRPIQAVETVDEVSKPLIETLKSLP-ENEEVILVGFSFGGINIALAADIFP 95
BmHNL   IDLVASGIDPRQLEQIGTWEQYSEPLFTLIESIP-EGKKVILVGESGGGINIALAAEKYP 119
HbHNL   LDLAASGVDPRQIEEIGSFDEYSEPLLTFLEALP-PGEKVILVGESCGGLNIAIAADKYC 94
MeHNL   LDMAASGIDPRQIEQINSFDEYSEPLLTFLEKLP-QGEKVIIVGESCAGLNIAIAADRYV 94

XfHNL   ERVKALVYIAAFAPSEGESVADLNKKY-PLPSG--------YNHLSSDKEGFLMLTPEGV 150
AtHNL   AKIKVLVFLNAFLPDTTHVPSHVLDKYMEMPGGLGDCEFSSHET-RNGTMSLLKMGPKFM 154
BmHNL   EKVSALVFHNALMPDIDHSPAFVYKKFSEVFTDWKDSIFSNYTY-GNDTVTAVELGDRTL 178
HbHNL   EKIAAAVFHNSVLPDTEHCPSYVVDKLMEVFPDWKDTTYFTYTK-DGKEITGLKLGFTLL 153
MeHNL   DKIAAGVFHNSLLPDTVHSPSYTVEKLLESFPDWRDTEYFTFTNITGETITTMKLGFVLL 154

                                                              #
XfHNL   EKYLAQDIPLEQTRLIIATQHPIRGANEEKKVSAAAWETKPSW------YLVSENDLMLQ 204
AtHNL   KARLYQNCPIEDYEL--AKMLHRQGSFTTEDLSKKEKFSEEGYGSVQRVYVMSSEDKAIP 212
BmHNL   AENIFSNSPIEDVEL--AKHLVRKGSFFEQDLDTLPNFTSEGYGSIRRVYVYGEEDQIFS 236
HbHNL   RENLYTLCGPEEYEL--AKMLTRKGSLFQNILAKRPFFTKEGYGSIKKIYVWTDQDEIFL 211
MeHNL   RENLFTKCTDGEYEL--AKMVMRKGSLFQNVLAQRPKFTEKGYGSIKKVYIWTDQDKIFL 212

                                  #
XfHNL   PALQKEMAQKIGAHIVNV--AASHVPHLSHAAEVEKIIMSAVNDVEVLE--- 251
AtHNL   CDFIRWMIDNFNVSKVYEIDGGDHMVMLSKPQKLFDSLSAIATDYM------ 258
BmHNL   RDFQLWQINNYKPDKVYCVPSADHKIQISKVNELAQILQEVANSASDLLAVA 288
HbHNL   PEFQLWQIENYKPDKVYKVEGGDHKLQLTKTKEIAEILQEVADTYN------ 257
MeHNL   PDFQRWQIANYKPDKVYQVQGGDHKLQLTKTEEVAHILQEVADAYA------ 258
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Figure 2. Multiple alignment of *Xf*HNL (NP_061688.1) with the plant hydroxynitrile lyases *Hb*HNL (7YAS_A), *Me*HNL (1DWP_A), *Bm*HNL (3WWP_A) and *At*HNL (3DQZ_A). Identical and similar amino acids are indicated by dark and pale grey backgrounds, respectively (the following amino acids were considered similar: A, S, T; D, E; F, Y, W; I, L, M, V; N, Q; R, K). The residues of the catalytic triad are marked #. The crucial amino acids involved in the enzymatic mechanism and enantioselectivity of HNLs are boxed.

acts as a base, the serine hydroxyl group acts as a mediator, and a lysine residue (Lys236) gives the positive charge that stabilises the negative charge evolving at the cyano group during the reaction together with Asp.[14] The Ser-His-Asp catalytic triad is conserved in the *R*-selective *At*HNL and is crucial for catalysis,[15] whereas *Hb*HNL and *At*HNL differ at several other residues surrounding the active site. Specifically, the lysine residue crucial for stabilising the negative charge on the cyanide (Lys236 in *Hb*HNL and *Bm*HNL, Lys237 in *Me*HNL) is replaced by methionine. In a recently proposed catalytic mechanism for *At*HNL,[7] His236 from the catalytic triad acts as a general base and the emerging negative charge on the cyano group is stabilised by main-chain amide groups (Ala13 and Phe82) and an α-helix dipole very similar to α/β-hydrolases. In both cases, deprotonation of the cyanohydrin is facilitated by hydrogen-bond interactions between the hydroxyl group and Asn12 (*At*HNL) or Thr11 (*Hb*HNL).

The analysis of the sequence alignment of the *Xf*HNL showed that these crucial residues involved in the enzymatic mechanism of HNLs (both for *S* and *R* enantioselectivity; boxed in Figure 2) are missing. Other amino acids are present. Thr11 and Lys236 involved in the catalytic mechanism of *S*-HNLs are different in *Xf*HNL. In place of Thr11 there is Gly31, an amino acid with quite different properties. Furthermore, there is Val237 instead of the charged Lys236 of *S*-HNLs. Valine is not a charged amino acid (it is a hydrophobic amino acid), therefore it cannot stabilise the negative charge on the cyanide group.

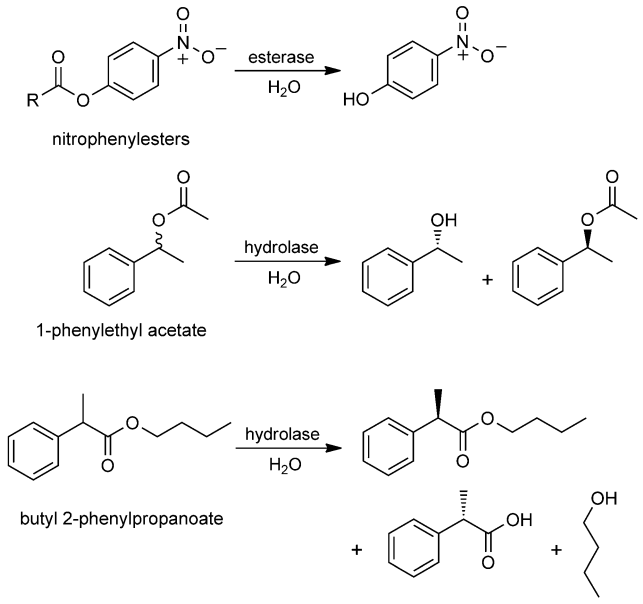
Regarding the amino acids involved in the catalytic mechanism of the *R*-enantioselective *At*HNL (Asn12, Ala13 and Phe82) there are fewer differences. Ala13 in the *At*HNL corresponds to Ala32 in *Xf*HNL, according to the homology model (see Homology model and docking simulations, below). Phe82 corresponds to Tyr88, which has similar properties. The difference lies in the important Asn12, which is a Gly31 in *Xf*HNL. Glycine is shorter than asparagine, and the backbone NH group, necessary to facilitate the deprotonation of the cyanohydrin, is in a different orientation and not in close proximity to the active site pocket. The positions of all these amino acids were confirmed in the homology model of *Xf*HNL.

Screening databases with the Jackhmmer server indicated that the amino acid sequence of *Xf*HNL has high homology (61% identity) with an esterase from *Ralstonia solanacearum* UW551 (UniProt accession: A3RYV8), as well as hydrolases from *Gallaecimonas xiamenensis* 3-C-1 (K2JM70), *Pseudomonas brassicacearum* (W8PX61) and *fluorescens* (U7DGE0), and a putative hydrolase from *Pseudomonas sp*. GM60 (J3B303). Unfortunately, these enzymes have not been studied, although, interestingly, there is an esterase that has been well characterised and shows significant homology to *Hb*HNL and *Me*HNL: EstC from *Burkholderia gladioli* (*Bg*EstC; accession number AAF66687.1).[16] *Xf*HNL shares about 41% sequence similarity with the *Bg*EstC, and a sequence alignment showed that both enzymes lack the crucial amino acids for HNL activity previously mentioned. Furthermore, the two enzymes share almost exactly the lipase motif around the catalytic serine (VVLVGHSXGG) and the His-Gly motif (VLVHGAXX) described by Reiter et al.[16]

Later, the Schwab group provided clear evidence for the conversion of the *Bg*EstC into an active hydroxynitrile lyase by the exchange of only few amino acids (conference poster, results not published).[18b] They exchanged Ser276 (corresponding to the Val237 in *Xf*HNL) in *Bg*EstC with the essential lysine. They also found two other positions that had an impact on the desired HNL activity. Gly24 (corresponding to Gly31 in *Xf*HNL, at the active site in the homology model) was exchanged to threonine, and His111 (His86 in *Xf*HNL), close to the catalytic serine, was exchanged to glutamic acid (which is present in *S*-HNLs). Activity assays based on the cleavage reaction provided evidence for successful conversion into an HNL. The activity was also confirmed for these mutants by using benzaldehyde as substrate. In 2010, the Kazlauskas group also reported the conversion of a plant esterase into an HNL with just two amino acid substitutions.[17] This analysis explains the absence of HNL activity for *Xf*HNL. At the same time, it strongly suggests an esterase activity.

Esterase activity

Purified *Xf*HNL protein was examined with different assays for esterase and lipase activity (Scheme 2, Table 1). As expected,



Scheme 2. Reactions used for determining esterase and lipase activity.

Table 1. Catalytic activity of *Xf*HNL at different pH values.

	Catalytic activity [μmol per min per μg of enzyme]	
	pH 7	pH 8
p-nitrophenyl acetate	10.9	66.9
p-nitrophenyl butyrate	3.1	4.2
tributyrin	0	0
1-phenyl ethyl acetate	0	0
butyl 2-phenyl propanoate	0	0

*Xf*HNL displayed good enzymatic activity in the hydrolysis of nitrophenyl esters, particularly for *p*-nitrophenyl acetate, which showed higher activity at pH 8 than at pH 7. With *p*-nitrophenyl butyrate, the pH difference was lower. Lipase activity was tested using tributyrin and two racemic substrates, 1-phenyl acetate and butyl 2-phenyl propanoate (substrates that are commonly used to check the enantioselectivity of these enzymes).[18] No significant lipase activity was detected with these substrates, as is the case for *Bg*EstC.[16]

Homology model and docking simulations

The X-ray structures of all HNLs in the sequence alignment are known (with the exception of *Xf*HNL). For this reason, a homology model of *Xf*HNL was made using the software YASARA. This allowed us to analyse the positions of the residues in the active site and carry out some docking simulations. The best result showed a protein (dimer) with an overall structure slightly similar to that of *Me*HNL and *At*HNL, with root mean square deviation (RMSD) values for C_α atoms of 17.137 Å between *Xf*HNL and *Me*HNL, and 18.140 Å between *Xf*HNL and *At*HNL. A superposition of these structures showed differences in the loop regions, and two β-sheets on the *Xf*HNL surface are shorter, probably because of the difference in length between these proteins (Supporting Information). An analysis of the catalytic triad (Ser-Asp-His) showed that these residues are in similar orientations in the active sites of these enzymes, thus highlighting the importance of other catalytic residues for HNLs (quite different in *Xf*HNL).

The *Me*HNL and *At*HNL crystal structures and the *Xf*HNL homology model were used to carry out docking studies. (A similar investigation with *Bg*EstC was not possible as no crystal structure is available.) Mandelonitrile was chosen as the ligand, and one monomer of the enzyme was used as the receptor. The docking of mandelonitrile was assayed 100 times by using the minimum-energy structures of the enzymes. In the case of *Me*HNL and *At*HNL, the 100 mandelonitrile molecules were docked to the active site in the same way (same orientation for each enzyme, but differences between them because of the different enantioselectivity). The 100 mandelonitrile molecules in the active site of *Xf*HNL had six different orientations, thus indicating unspecific binding (Figure 3 A). As explained above, the lack of some of the HNL catalytic residues can explain this unspecific binding, and hence the absence of HNL activity observed in the experiments.

Some authors have pointed out the importance of analysis of the hydrophobic residues surrounding the active site.[14b,19] These can play an important role in recognising various substrates and introducing the cyanide ion into the active site. An analysis of the *Xf*HNL residues corresponding to the nine hydrophobic residues in the active site of HNLs showed that only five are hydrophobic, and only four surround the active site. The rest are polar or are on the surface of the protein, far from the active site.

In order to validate the homology model and the docking results, a docking simulation using *p*-nitrophenyl acetate as ligand was carried out. The results (100 molecules; Figure 3 B) show that all the *p*-nitrophenyl acetate molecules were placed in the active site in the same orientation, as would be expected for a substrate for which the enzyme showed activity (Table 1).

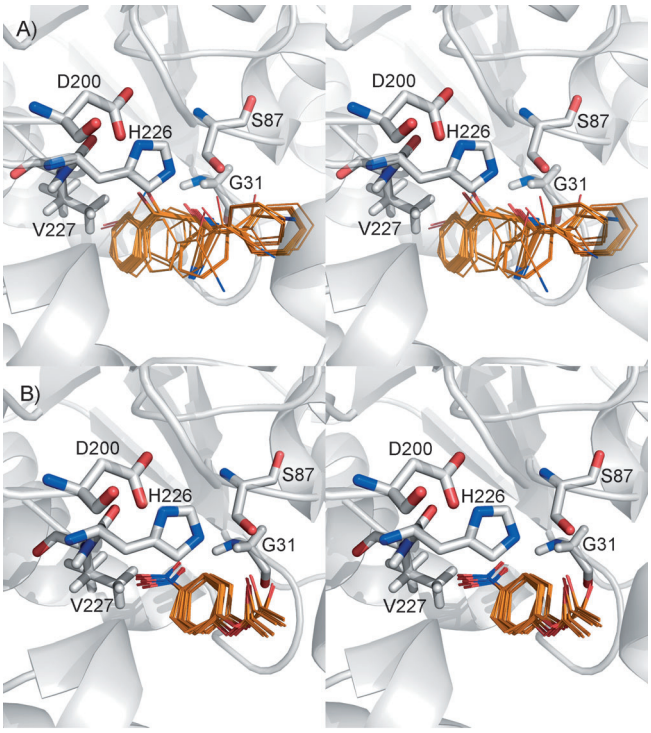


Figure 3. Stereo views (PyMOL) of the active-site structure of *Xf*HNL (homology model, YASARA). A) Docking simulation with 100 molecules of mandelonitrile (orange). B) Docking simulation with 100 molecules of *p*-nitrophenyl acetate (orange). Active-site residues (S87, D200, H226) and the corresponding residues for the Lys236 and Thr11/Asn12 in *S*- and *R*-HNLs (G31, V227) are shown as stick models.

Conclusions

In this study, it was demonstrated that the product of the gene SCJ21.16 (XFa0032) from *X. fastidiosa* encoded a protein with esterase activity. Despite the sequence homology, no hydroxynitrile lyases activity could be detected. Sequence alignment, docking simulations and activity assays support this conclusion. We think that our finding should support the renaming of the protein and could contribute to elucidating the common ancestor of esterases and hydroxynitrile lyases with an α/β-hydrolase fold.[20]

Experimental Section

CAUTION! All procedures involving hydrogen cyanide were performed in a well-ventilated fume hood equipped with a HCN detector. HCN-containing waste was neutralised by using commercial bleach and stored independently for disposal with a large excess of bleach.

Preparation and purification of *Xf*HNL: The synthetic gene (sequence deposited in the database under accession number NP_061688, without codon optimisation) cloned into the expression

vector pET28a was ordered from BaseClear (Leiden, The Netherlands). *E. coli* TOP10 competent cells were used for the plasmid propagation. The plasmid was transformed into *E. coli* BL21(DE3) for expression and purification of the full-length protein. Heterologous expression and purification of recombinant protein (*Xf*HNL) was performed according to Caruso et al.[4] A BC assay was used for protein quantification.[21]

Chemicals: (±)-Mandelonitrile (Sigma–Aldrich) was purified by column chromatography (PE/EtOAc 9:1→3:7) prior to use. Benzaldehyde (Acros Organics) was distilled prior to use and stored under nitrogen at 4 °C. Tributyrin, *p*-nitrophenylacetate, *p*-nitrophenylbutyrate, 2-phenylpropionic acid, *n*-butanol, 1-phenylethanol, isopropanol and heptane (HPLC grade) were purchased from Sigma–Aldrich. 1-phenylethyl acetate was purchased from Acros Organics. Petroleum ether and ethyl acetate (technical grade) were purchased from VWR. Methyl *tert*-butyl ether (MTBE, 99.9% extra pure, Acros Organics) was used without further treatment unless otherwise specified. Aqueous buffers were prepared from analytical grade salts.

Synthesis of butyl-2-phenyl propanoate: 2-Phenyl propionic acid (6 g, 40 mmol), *n*-butanol (5.92 g, 80 mmol), toluene (30 mL) and sulphuric acid (1 mL) were placed in a 100 mL round-bottom flask connected to a Dean–Stark-type trap. The mixture was heated to 120 °C, and the reaction was stopped when no more water formed. The mixture was washed three times with ice water (40 mL), saturated Na_2CO_3 (40 mL) and water (40 mL). The toluene layer was dried over $MgSO_4$, then the solvent was evaporated under vacuum to obtain the product (7.82 g, 95% yield). ^{1}H NMR (400 MHz, $CDCl_3$) butyl-2-phenyl propanoate: $\delta = 7.27$–7.33 (m, 5H, 5×CH, Ph), 4.07 (t, $J = 6.8$ Hz, 2H, OCH_2), 3.71 (q, $J = 7.2$ Hz, 1H, C*H*Me), 1.55–1.60 (m, 2H, CH_2), 1.51 (d, $J = 7.2$ Hz, 3H, CH*Me*), 1.27–1.32 (m, 2H, CH_2), 0.88 ppm (t, $J = 7.5$ Hz, 3H, Me); these data are in accordance with ref. [22].

Hydrogen cyanide 1.5–2 M in MTBE: HCN solution was prepared as described in ref. [21]; determination of HCN concentration was as in ref. [23].

Enzyme assays: HNL activity (cleavage of mandelonitrile) used purified *Xf*HNL and was according to reported procedures.[10a] Citrate-phosphate buffer (50 mM) was used for pH 5, 5.5 and 6; potassium phosphate buffer (50 mM) was used for pH 6.5 and 7, for both enzymatic and control reactions.

The reaction with benzaldehyde as substrate was carried out at two pH values. The enzyme sample (up to 50 μL) was added to citrate-phosphate buffer (50 mM, 450 μL, pH 5) or potassium phosphate buffer (50 mM, 450 μL, pH 6.5). Then, HCN (1.7 M in MTBE, 500 μL) with benzaldehyde (1 mmol) and 1,3,5-triisopropylbenzene as the internal standard (0.01 mmol), previously mixed under a nitrogen atmosphere, were added. The reaction was monitored by chiral HPLC[21] over 24 h while the reaction flask was stirred vigorously at room temperature (22 °C).

Esterase activity was determined using the generic esterase substrates *p*-nitrophenyl-acetate (two carbons) and *p*-nitrophenyl-butyrate (four carbons). Working solutions of *p*-nitrophenyl acetate and butyrate were prepared at 100 mM in acetonitrile. The reaction mixture consisted of enzyme (0.2 μg) and *p*-nitrophenyl ester (1 mM) in potassium phosphate buffer (50 mM, 1 mL, pH 7) or Tris·HCl buffer (50 mM, pH 8). The release of *p*-nitrophenol was monitored continuously at $OD_{405\ nm}$ over 5 min at 25 °C in a Cary 60 spectrophotometer (Agilent Technologies). A calibration curve of *p*-nitrophenol was performed under the same experimental conditions.

Lipase activity was assayed at pH 7 and 8 (buffers as above) by a pH-stat method with tributyrin, 1-phenylethyl acetate and butyl 2-phenyl propanoate as substrates.[24] Small-volume reactions with the last two substrates were also performed and checked by GC and HPLC in order to reduce the detection limit and rule out any activity. The reactions consisted of enzyme (0.5–10 μg) and 1-phenylethyl acetate (10–25 mg, 0.06–0.15 mmol) or butyl 2-phenyl propanoate (10–25 mg, 0.05–1.2 mmol) in citrate-phosphate buffer (1 mL, 50 mM, pH 5), potassium phosphate buffer (1 mL, 50 mM pH 7) or Tris·HCl buffer (50 mM, pH 8, 1 mL). The reaction mixture was vigorously stirred in a Thermomixer comfort (Eppendorf) at 25 °C for 24 h. The hydrolysis of 1-phenylethyl acetate was monitored by GC, and the hydrolysis of butyl 2-phenyl propanoate was monitored by HPLC.

All enzymatic assays and control reactions were carried out in at least duplicate.

GC method: Reaction mixtures were extracted with diethyl ether (2×), and the organic phase was dried with anhydrous $MgSO_4$. The final clear solution (1 μL) was injected into a model GC 2010 chromatograph (Shimadzu) equipped with a CP-Chirasil-Dex CB column (25 m×0.32 mm×0.25 μm; Agilent Technologies) with helium as the carrier gas: injector 250 °C, detector 270 °C, split 50, flow rate: 1.59 mL min^{-1}, maximum: 270 °C. The temperature program was 70 °C (0.1 min), increase to 110 °C (30 °C min^{-1}), hold (11 min), increase to 245 °C (30 °C min^{-1}), hold (1 min). Retention times: (*R*)-1-phenyl ethyl acetate, 9.1 min; (*S*)-1-phenyl ethyl acetate, 9.5 min: (*R*)-1-phenyl ethanol, 10.2 min; (*S*)-1-phenyl ethanol, 10.7 min.

HPLC method: Reaction mixtures were extracted with ethyl acetate and diethyl ether, and the combined organic phases were dried with anhydrous $MgSO_4$. The final clear solution (10 μL) was injected into an HPLC device (Waters). Analyses were performed on a Chiralpak AD-H column (4.6×250 mm, 5 μm; Daicel, Tokyo, Japan) coupled to an SpH 99 column thermostat (Chrompack), a 515 HPLC pump (Waters), a 717autosampler (Waters), and an SPD-10A UV/Vis detector (Shimadzu). The column temperature was maintained at 40 °C; mobile phase: heptane/isopropanol, 95:5 (0.1% TFA); flow rate: 1 mL min^{-1}; detection: 254 nm. Retention times: butyl 2-phenyl propanoate, 3.8 min; (*S*)-2-phenylpropionic acid, 6.3 min; (*R*)-2-phenylpropionic acid, 6.75 min,

Sequence alignment and database search tools: The structure-based multiple sequence alignment was constructed with MUSCLE,[25] provide by The European Bioinformatics Institute.[26] Homology searches were performed with BLAST[27] and Jackhmmer (HMMER suite).[28]

Homology modelling: The set of homology models was made using YASARA (version 14.7.17, http://www.YASARA.org)[29] with the FASTA sequence of *Xf*HNL. The best was selected based on the quality parameters of models estimated with ProSA.[30] The RMSD values for Cα atoms between *Me*HNL, *At*HNL and *Xf*HNL were calculated from structures previously overlaid by the MUSTANG algorithm[31] in YASARA.

Docking simulations: The HNL crystal structures (PDB IDs: 1DWP and 3DQZ) and the *Xf*HNL homology model were used. Initially, all the hydrogen atoms were shown, and the minimum energy structures were calculated. Active-site amino acids were identified based on the active sites in the crystal structures. After assignment of the substrate-binding site, mandelonitrile was docked to the structures by using AutoDock 4.2.3[32] (initial position, orientation

and torsions of the ligand molecules were set randomly, 100 runs) implemented in YASARA. Interactions at the active site were visualised with PyMol Molecular Graphics System (Version 1.4.1 Schrödinger). The same procedure was performed for the docking simulation of *Xf*HNL and *p*-nitrophenyl acetate.

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