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Expanding the Portfolio by a Novel Monomeric Oleate Hydratase from *Pediococcus parvulus*

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Oleate hydratases convert oleic acid into 10-hydroxy stearic acid, a valuable fine chemical, useful in lubricant and surfactant formulations. They are of large interest due to their high expression rates and solubility, however, they differ drastically by their overall stability and pH- and temperature ranges. To expand their portfolio, another oleate hydratase named OhyPp (originating from *Pediococcus parvulus*) was characterized. It is a close relative of the well-known oleate hydratase OhyRe from *Rhodococcus erythropolis*. OhyPp is only the second member of the monomeric oleate hydratase family with some surprising catalytic features. A distinct characteristic is OhyPp's higher affinity towards FAD compared to OhyRe's helping to understand and improve FAD binding in the future, which is a current drawback for the industrial application of oleate hydratases.

Introduction

Driven by climate change and legislative CO_2 capping measures, several industry sectors are developing sustainable process solutions based on renewable feedstocks. In this context enzymes substitute for chemical catalysts, as they can selectively convert renewable feedstocks at ambient temperatures. Specifically, the enzyme family of hydratases selectively catalyze the hydration of C=C bonds resulting in products, which are conventionally manufactured under high pressure and temperatures by chemical methods. Hydratases are usually well expressed in recombinant systems without the need of unstable cofactors, which would need to be recycled. Moreover, hydratases can catalyze sophisticated, asymmetric reactions.[1–3]

Recently, much attention has been drawn to a class of unsaturated fatty acid converting enzymes called oleate hydratases. These enzymes turn unsaturated fatty acids into hydroxylated fatty acids. Most of them have a high prevalence for accepting oleic acid as a substrate,[3] which is a main fatty acid component of many sustainable plant-based and microbial triglycerides such as high-oleic sunflower oil or oil from yeast *Cutaneotrichosporon oleaginosus*.[4]

Hydroxylated fatty acids are predominantly found in a range of microorganisms, in which they are considered to be defense factors against toxic free fatty acids.[5] Free long chain unsaturated fatty acids are known for their antibacterial effects on gram positive bacteria. Originally, they were thought to disintegrate the microbial cell membrane. However, saturated fatty acids don't convey the same inhibiting effects as unsaturated ones. Instead, it has been reported that long chain unsaturated fatty acids inhibit the FabI enzyme, which is part of the bacterial type II fatty acid synthesis. Additionally, other fatty acid converting enzymes derived from other organisms are known to be inhibited by unsaturated fatty acids.[6] Gram negative bacteria show no inhibition from free long chain unsaturated fatty acids, which most likely is connected to their cell wall being less penetrable for fatty acids. Hydroxy-fatty acids functionally resemble more saturated fatty acids and thus most likely are less toxic than their unsaturated counterparts.[7] Even though it is reported that gram negative bacteria are resistant towards free long chain unsaturated fatty acids, many of them are known to carry fatty acid hydratases.[8,9] That is why most likely, there are other functionalities besides the detoxification. One of them might be their effect on the general cellular stability, most likely conveyed through alterations in the membrane. It was observed that bacteria harboring oleate hydratase activities are more resistant towards heat and solvent stress, which was attributed to a modulated membrane composition.[10] Additionally, it has been reported that marine microorganisms produce 2-OH-hydroxylated fatty acids and that their concentration increases upon exposure to a low pH, which presumably leads to a better flow control of ions into the cell.[11] Some organisms are known to carry more than one fatty acid hydratase further complementing their evolutionary advantages.[12–14] Additionally, microorganisms have been found to be more heat and solvent resistant by carrying an oleate hydratase gene.[10]

Oleate hydratases belong to the class of fatty acid hydratases (EC 4.2.1.53) and have great potential to become a useful industrial biocatalyst for the generation of valuable fatty acid derivates such as (*R*)-10-hydroxy stearic acid (10-HSA),

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which is considered as a replacement for (*R*)-12-hydroxy stearic acid (12-HSA), an industrial product applied as lubricant and emollient. 12-HSA is currently generated by chemical hydrogenation of castor oil under extreme pressure and temperature with the help of metal catalysts, thus calling for a more eco-friendly alternative process.[5] Furthermore, hydroxylated fatty acids are valuable precursors for flavor lactones, where some fatty acid based lactones convey fruity and milky odors.[15] For instance, γ-dodecalactone is a component responsible for the buttery flavor in milk fat and can be produced from oleic acid with 10-HSA as an intermediate.[16] Compared to chemical synthesis, these natural hydroxy fatty acids have higher value since they are stereospecific.

Oleate hydratases were applied in the form of whole-cell catalysis,[17] isolated as pure enzymes[18] and as cell-free extract.[19] Recently, an oleate hydratase and a linoleate hydratase were used as part of a microbial host process, where their genes were integrated into the genome of *Yarrowia lipolytica* to produce γ- and δ-dodecalactone.[15] Fatty acid hydratases act strictly on free fatty acids, whereas they can't convert fatty acids as part of triacyl glycerides.[20]

So far, a variety of oleate hydratases originating from different organisms were described and characterized. Economical industrial processes ask for distinct requirements, which can be sustained by a large portfolio of enzymes with varying characteristics. These comprise different pH- and temperature profiles, dimeric or monomeric structures, turnover rates and substrate specificity.[3] At present, the only reported monomeric oleate hydratase is OhyRe originating from *Rhodococcus erythropolis*, while all other reported enzyme varieties are functional dimers. Until now, crystal structures of OhyRe,[18] OhyEm (*Elizabethkingia meningoseptica*),[21] OySa (*Staphylococcus aureus*),[22] OhySt (*Stenotrophomonas sp.* KCTC 12332)[23] and OhyLa (linoleate hydratase from *Lactobacillus acidophilus*)[24] are available. The structures of OhyEm, OhySa and OhySt were determined with bound FAD and OhySa is the only structure, where the binding of 10-HSA and oleic acid was observed. FAD dependency is a characteristic of oleate hydratases and in Radka *et al.*,[22] it was suggested that upon binding of FAD a conformational shift allows the substrate oleic acid to enter deep into the inner active site cavity, which prevents water quenching of reactive intermediates, subsequently allowing for substrate conversion to the target product.

It is known that oleate hydratases have a substantially higher activity using $FADH_2$ as a cofactor.[21,25] On the other hand, upon cell lysis and purification, $FADH_2$ gets oxidized immediately to FAD, which makes an industrial application using $FADH_2$ impractical. Depleting oxygen and using additives increases the costs to produce a normally cheap molecule such as 10-HSA and additives also contaminate the final product. But also for small scale measurements and reactions, this causes several issues. The affinity measurement of $FADH_2$ towards the enzyme is elaborate and additives might influence the interaction between enzyme and ligand. In general, oleate hydratases have low affinity towards FAD and many lose it upon purification, hampering current industrial applications.[5]

In this study, a close relative of OhyRe has been identified and characterized. It is an oleate hydratase originating from *Pediococcus parvulus*, (OhyPp) (former name *Pediococcus damnosus*), a lactic acid bacterium, first extracted from ropy Basque Country ciders, which today is used as a starter culture for the fermentation of vegetables, meat and dairy products.[26] We could demonstrate that OhyPp is monomeric and has slightly higher affinity towards FAD than OhyRe. Additionally, this variant offers unexpected insights into the structural features of binding of FAD and expands the portfolio of monomeric oleate hydrases by a new member.

Results and Discussion

OhyPp – a close relative of OhyRe

OhyPp is predicted to be 558 amino acids long with a calculated molecular mass of 66.4 kDa. For protein expression in *Escherichia coli*, a codon-optimized DNA-sequence of OhyPp was cloned into the vector pET28a(+) and expressed with an N-terminal His-tag (Figure S1), (DNA and amino acid sequences see supporting information). After purification via IMAC, the enzyme solution was nearly colorless, which is a similar effect as for OhyRe and can be attributed to a loss of FAD. This subsequently leads to a loss of activity as well. Such an observation has been made for many fatty acid hydratases.[18,20,21,24]

Quaternary structure analysis

We employed size exclusion chromatography coupled to multi-angle light scattering to analyze the oligomerization state of OhyPp in solution. OhyPp can be characterized as a monomeric enzyme as OhyRe (Figure S2).[18] Monomeric oleate hydratases lack N- and C-terminal extensions in their amino acid sequence, which are thought to be responsible for dimerization.

Optimal reaction conditions

The optimal growth temperature of *P. parvulus* is at 30 °C[26] diverting from the optimal temperature of OhyPp at 18 °C (Figure 1(A)). The optimal pH is at 6 with a rather low tolerance for a wide range compared to OhyRe (Figure 1(B)). Furthermore, OhyPp requires a buffer containing 200 mM NaCl (Figure 2), which yielded in the highest activity. Interestingly, OhyPp was not stable at a pH of 6 and 6.5 using 50 mM MES, 200 mM of NaCl and phosphate buffer, observable by instant aggregation during desalting and loss of activity. Therefore, OhyPp was stored in 20 mM Tris/Base and 200 mM NaCl at pH 7.2, where no immediate aggregation was observed, while enzyme activity remained. For the reactions, MES buffer (50 mM MES, 200 mM NaCl, pH 6) was added to the reaction medium so that a final pH of 6 was reached.

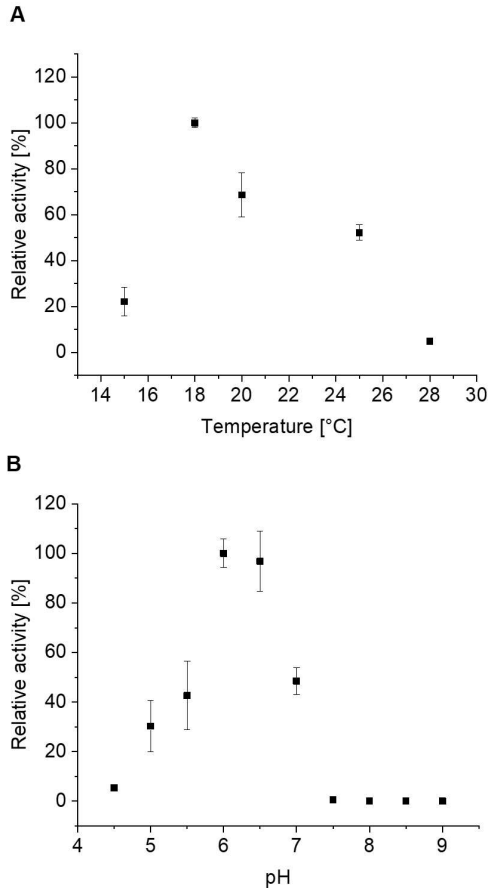

Figure 1. (A) Determination of the optimal reaction temperature. Values are normalized to maximal activity. Enzyme was stored in 20 mM Tris/Base, pH 7.2, 200 mM NaCl and 50 mM MES, 200 mM NaCl buffer was added to reach a pH of 6 in the reaction. Error bars represent the standard deviation of triplicate experiments. **(B)** Determination of the optimal pH. Values are normalized to maximal activity (pH 6). The different buffer types used can be found in the experimental section. Error bars represent the standard deviation of triplicate experiments.

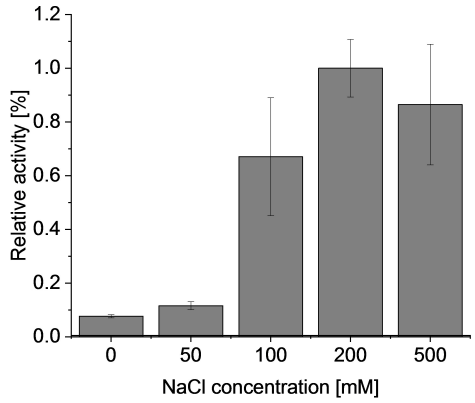

Figure 2. Determination of the optimal NaCl-concentration for activity. Values are normalized to the maximal activity at 200 mM NaCl. Enzyme was stored in 20 mM Tris/Base, pH 7.2, 200 mM NaCl and buffer was adjusted to a pH of 6 with MES buffer for the reaction. Error bars represent the standard deviation of triplicate experiments.

OhyPp having a theoretical pI of 5.33[27] is more active in acidic environments than in basic ones reflecting the strong acidity of both substrate and product. Oleic acid itself has a pK_a of 5.02 (https://pubchem.ncbi.nlm.nih.gov) and thus a strong acidifying effect on buffer systems, which leads to anticipated pHs not being reached. For instance, at experimental conditions with 100 mM Tris/Base, pH 7 and 0.15% oleic acid, the pH dropped down to 6.7. The pH was therefore measured and set to a specific value prior to the experiment. At a pH above 7, oleic acid turns immediately opaque upon mixing with water-based buffers and extended foaming occurs.

At pH-values between 7 to 9, at temperatures below the melting temperature, free fatty acids are known to form crystalline solids consisting of a mixture of acid and soap.[28] These are extremely insoluble in water and consequently only low concentrations of the monomeric fatty acids remains in solution. This monomer stays in equilibrium with the formed crystal. Therefore, this leads to less substrate availability for fatty acid hydratases. Crystals were macroscopically observable. However, the effect was more profound for Tris/Base buffer and thus at a pH above 7.5, N-Tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid (TAPS) buffer was used.

Fatty acid specificity

OhyPp was tested for conversion of a range of different unsaturated fatty acids, which are listed in Table 1. Palmitoleic, linoleic and α-linolenic acid get converted (Figure S3). In general, most fatty acid hydratases have the highest specificity for either linoleic or oleic acid and a rather low tolerance for other substrates.[12,3] A broad substrate spectrum is unique and there is currently just one hydratase known to convert substrates ranging from C16 to C22 unsaturated fatty acids.[12] However, most oleic acid hydratases convert linoleic and linolenic acid as a structural relative.[3]

Table 1. Fatty acids tested for conversion by OhyPp.

Fatty acid		Products	Conversion [%]
palmitoleic acid	C16:1 cis-9	10-hydroxy-hexadecanoic acid	2.3 ± 0.2
petroselinic acid	C18:1 cis-6	n.d.[a]	–
oleic acid	C18:1 cis-9	10-hydroxy octadecanoic acid	34.3 ± 7.7
elaidic acid	C18:1 trans-9	n.d.[a]	–
linoleic acid	C18:2 cis-9,12	10-hydroxy-12(Z)-octadece-noic acid	24.6 ± 7.1
α-linolenic acid	C18:3 cis-9,12,15	10-hydroxy-12(Z), 15(Z)- octa-decadienoic acid	8.9 ± 2.3
gadoleic acid	C20:1 cis-9	n.d.[a]	–
arachidonic acid	C20:4 cis-5,8,11,14	n.d.[a]	–
nervonic acid	C24:1 cis-9	n.d.[a]	–

[a] Not detected.

OhyPp was observed to convert palmitoleic, linoleic and α-linolenic acid besides oleic acid. As most OHs only accept cis-fatty acids for conversion, also OhyPp is not able to convert elaidic acid, the trans-isomer of oleic acid. Furthermore, petroselinic acid is not converted and in linoleic and linolenic acid, only the 9-double bound gets converted leading to the conclusion that OhyPp prefers the 10-position for hydroxylation. Longer fatty acids such as gadoleic and nervonic acid are most likely too large for the inner cavity of OhyPp. In Busch *et al.*,[14] an extensive substrate screening with OhyRe and another *Rhodococcus sp.* OH has been performed showing OhyRe to be the first OH to hydroxylate fatty acids at the 12-position. It will be interesting to investigate whether this is specific for HFam3 family members and compare them to other families leading to more insights into the hydration dynamics.

Sequence comparison

OhyPp is the current closest relative of OhyRe within the NCBI-data base holding a 74% identity and 91% similarity of the sequences. Hydratases were grouped into eleven different classes (HFam1-11) depending on their levels of sequence similarity (HyED, https://hyed.biocatnet.de).[9] OhyEm belongs to HFam11,[21] whereas OhyRe and OhyPp can be categorized into HFam3.[18] Within a class, there is an average global sequence identity of 62%, however, between classes, the identity can be significantly lower. Between OhyEm and OhyRe/OhyPp, there is only a 28/27% identity with a similarity of 56/58% (Table S1).

To gain structural insights in OhyPp, we intended to solve the crystal structure.

Extensive crystallization studies with and without His_6 tag did not lead to crystals diffracting to high resolution, therefore a model for OhyPp was build utilizing Robetta[29] (Figure 3). Compared to the overall structure of OhyRe, the Robetta model of OhyPp is very similar to OhyRe with a root mean square deviation of 1.3 Å for 518 pair of Cα-atoms (Figure S4). OhyPp is organized in four domains, with the FAD binding site composed of domain I and II. Characteristic of members of the HFam3 family, is an extended FAD binding loop, which is extended by about 25 amino acids compared to members of other subfamilies.

FAD binding

The binding of FAD in comparison to OhyEm and OhyRe was analyzed. To investigate how much FAD is bound to each protein, total FAD concentration was measured by fluorescence after the proteins were denatured with heat and the aggregated protein debris were separated by centrifugation since only unbound FAD returns a fluorescence signal. The occupancy defined as the molar ratio of total FAD per protein is very low in all three proteins. For OhyPp and OhyEm, there is a 15% occupancy after Ni-NTA purification for both and a 6 and 13% occupancy, respectively, after desalting. In contrast, in OhyRe, there is only a 1% occupancy after Ni^{2+}-NTA and no FAD could

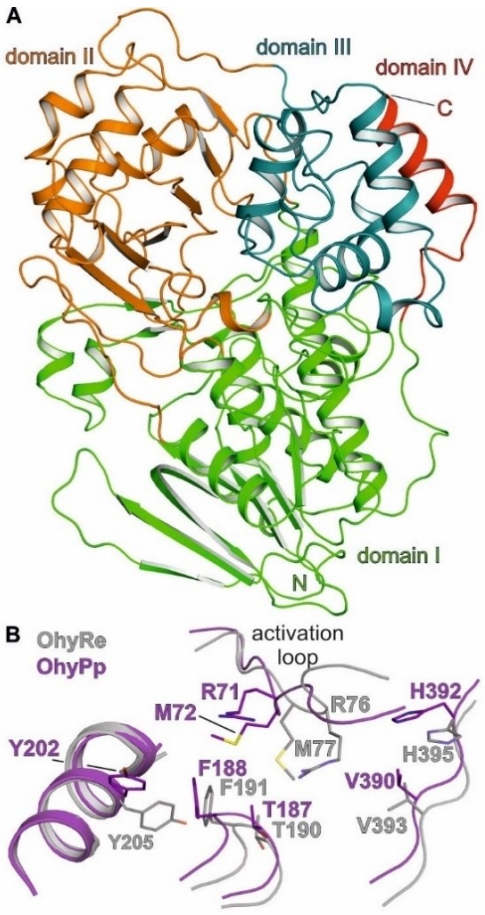

Figure 3. Domain organization of OhyPp with domain I in green, domain II in orange, domain III in deep teal and domain IV in red **(A)**. Superposition of the modelled active site of OhyPp (deep purple) and OhyRe (PDB-ID: 5odo;[18]) coloured in grey **(B)**. Important residues lining the active site are shown in stick representation.

be detected after desalting. Most FAD was unbound and free in solution of native OhyRe (99%) and OhyPp (78%), whereas in OhyEm most FAD was released only after denaturation. In OhyEm, only 9% of FAD was mobilized after purification. This can either be because parts of the proteins are already denatured or – more likely – that the affinity in OhyRe and OhyPp is so low that most FAD gets released immediately during the purification process. Since there seems to be a diverging behavior on FAD binding, the respective affinities were measured in form of dissociation constants (K_d) by using the quenching of tryptophan fluorescence. Indeed, the K_d of OhyPp was slightly lower than OhyRe's: 83.57 ± 4.11 μM and 204.47 ± 36.75 μM, respectively. Since OhyEm is a dimeric protein, it holds two K_d-values which can be obtained by regression analysis. The first one was measured to be 0.39 ± 0.06 μM, whereas the second one 120.11 ± 2.66 μM (Figure S5). Consequently, OhyEm holds a 500-fold higher affinity than OhyRe and a 200-fold higher one than OhyPp towards FAD. This is also reflected in the activity. After purification, OhyRe and OhyPp lost all activity whereas OhyEm was still active.

Hence, the conversion reaction with OhyPp needs an addition of external FAD.

FAD-binding motive

Characterized oleate hydratases have a commonly conserved FAD binding motive. Specifically, they all harbor a GXG fingerprint (starting at position 8 in OhyPp in Figure S6), which serves as a connecting loop between a β-strand and an α-helix as parts of the Rossman-fold. The first glycine leads to a sharp turn of the structure, whereas the second one allows the FAD's pyrophosphate to bind close to the following α-helix.[30]

The overall FAD binding patterns differ slightly since HFam3-members are having a GXGXXN and HFam11-members comprise the more common $GXGXXGX_{21}E/D$ motif. The latter acid is responsible for binding the 2'-OH of the ADP-ribose. In HFam3-members, the acidic amino acid is replaced by a hydrophobic one being mostly tyrosine, methionine or in the case of OhyPp a phenylalanine.

GGR – a conserved pattern

Oleate hydratases display a strongly conserved RGGREM pattern and has been firstly described by Radka *et al.*[31] It is located in a conserved loop and in proximity of the isoalloxazine ring of FAD and of the suggested substrate binding pocket. Whereas the first arginine is not strongly conserved amongst oleate hydratases, the following sequence "GGR" is. The second arginine and glutamic acid are suggested to bind the isoalloxazine ring in OhySa and additionally, the glutamic acid is described as crucial for activity since it stabilizes a hydronium ion enabling it to attack the oleate π-bond. In OhyRe and in OhyPp, however, the glutamic acid is replaced by a methionine, which is not able to conduct the catalytic reaction in the same way as suggested for OhySa.[22] Interestingly, this is in alignment with an oleate hydroxylase from *Ricinus communis* and the fatty acid desaturase FAD2 of *Arabidopsis thaliana*, as shown in Figure 4. Both are also carrying a methionine at the identical position.

Oleate hydroxylases are different compared to oleate hydratases in a way that they are membrane bound, convert fatty acids that are esterified to phosphatidylcholine and require NADH and cytochrome *b*5 as cofactors.[34] Furthermore, there is a charge-transfer reaction involved in the reaction, defining hydroxylases as oxidoreductases and these hydroxylases carry μ-oxo-bridged diiron cluster making the enzyme dependent on oxygen and iron.[35] Despite the differences between those fatty acid converting enzymes, GGR is a common motive, and it shows a certain degree of evolution between oleate hydratases and plant oleate desaturating enzymes. This sequence most likely is important for fatty acid or cofactor interaction or for activity.

A noticeable structural difference between HFam3 and HFam11 oleate hydratases is the so called "FAD-lid", which contains a conserved GXXXG pattern as opposed to a GGXXXG pattern in the latter. Furthermore, this region is prolonged in HFam3 by 13 amino acids compared to HFam11-type enzymes. This region together with the activation loop is undergoing large conformational changes after FAD interaction in OhySa.[22] These shield the FAD binding site from the solvent.

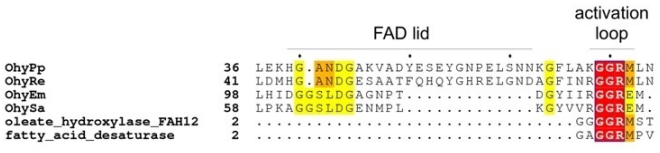

Figure 4. Alignment of the FAD lid and activation loop of two monomeric members of the HFam3 family (OhyRe and OhyPp), two dimeric members of the HFam11 family (OhyEm and OhySa), an oleate hydroxylase from *R. communis* (NP_001310650.1) and a fatty acid desaturase from *A. thaliana* (NP_001319529.1) using the COBALT constraint alignment tool[32] and ESPript from the Endscript-server for visualization (https://espript.ibcp.fr).[33]

Kinetic parameters

Kinetic parameters were measured for OhyPp using oleic acid as a substrate. They resulted in a K_m value of 21.06 ± 5.95 mM and a k_{cat} of 11.46 ± 0.05 min^{-1} (Figure S7). OhyPp is a rather slow enzyme with a half time of 3.7 h, where the maximal conversion could be observed after 24 h (Figure S8).

Kinetic parameters of enzymatic reactions are used to study enzymes' behavior in living organisms or to simulate conversion for industrial upscaling. However, oleate hydratases are a special case. For several oleate hydratases, kinetic parameters were measured. However, their scientific pertinence must be evaluated carefully. Oleic acid is not miscible in water but fatty acid hydratases need access towards their substrates, which can be achieved by creating a emulsion. However, the emulsion formation and its quality are dependent on the preparation protocol. Local and uneven dispersion effects are not avoidable. At a pH above 7, oleic acid forms a soap and is thus more miscible in water. So already the variation in pH has a strong effect on fatty acid dispersion in water and thus the measurement of kinetic parameters is not able to represent the enzyme's natural behavior. In previous experiments, where 10-HSA was enzymatically produced in larger scale and extracted as solid flakes, it could be observed that full conversion was not achieved noticeable by flakes, which still contained significant amounts of oleic acid. Consequently, 10-HSA precipitates together with oleic acid to a solid material even when the former is abundant only in low concentrations. Thus, the more 10-HSA is produced, the more oleic acid leaves the emulsion and is not available for the enzyme for conversion anymore. Also, the way the manual assay is carried out has a strong effect on the reaction's outcome. In summary, all the described challenges are reflected in strongly diverting kinetic parameters for the same enzymes as for example observed by Zhang *et al.*[3] Kinetic parameter assessment out of context meaning not under exactly equal and consistent reaction conditions as used

for upscaling or without a direct comparison are not useful and don't aid in understanding the *in vivo* behavior.

Long-term stability

Long-term stability of OhyPp at 4 °C was assessed and compared with the dimeric enzyme OhyEm. Dimeric enzymes are reported to lose activity due to subunit dissociation amongst other reasons and in an immobilization study, this has been named as a main reason for activity loss for OhyEm.[36] In that study, it has been reported that OhyEm loses 60% of its activity already after 7 days, however, our experiments were contradictory. OhyEm and OhyPp were both stable for a period over 9 days at an equal level and despite some fluctuations still retain 60 and 80% of their initial activity. However, after 27 days, OhyEm still carried 80% of its initial activity, whereas OhyPp only held around 20% (Figure 5).

OhyPp is thus less stable during extended storing than OhyEm, even though the latter is a dimeric enzyme. Consequently, other reasons besides the dissociation of subunits play a role. One might be the higher affinity of OhyEm towards FAD, since FAD binding will stabilize the overall fold of the protein. Whereas at 4 °C enzyme stability is high, during reaction conditions, oleate hydratases are reported to lose activity rather quickly.[9] This is also the case for OhyPp, which during the reaction started to denature forming a white debris. Resolubilization with ethyl acetate was not possible, arguing for protein aggregates, rather than precipitated 10-HSA. One of the reasons might be that free fatty acids can act as soaps and even though the enzymes are evolutionary adapted to these substrates, they still might denature upon exposure to a large excess of free fatty acids. As a result, much more stable enzymes are required for industrial processes.

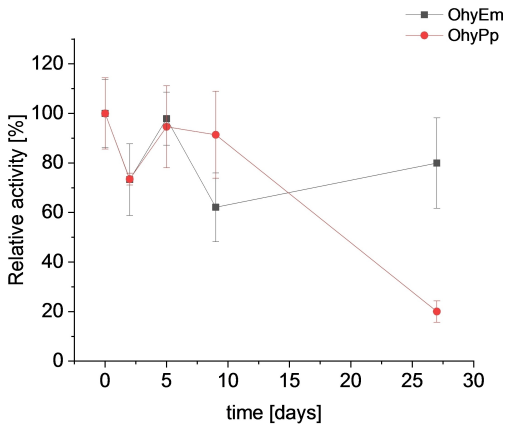

Figure 5. Stability test of monomeric OhyPp compared to dimeric OhyEm over the course of 27 days. Enzyme solutions were stored at 4 °C in each enzyme's respective optimal buffer. Error bars represent the standard deviation of triplicate experiments.

Melting temperatures

Additionally, the melting temperatures of OhyPp, OhyRe and OhyEm were analyzed and compared with each other. OhyPp has a lower long-term stability than OhyEm and this is also reflected in differences in the melting temperatures of these enzymes. OhyEm showed the highest melting temperature with 52.5 ± 0.2 °C. OhyRe's melting temperature was determined with 45.0 ± 0.0 °C and OhyPp has the lowest one with 41.1 ± 0.2 °C. The significant higher melting temperature of OhyEm could be explained by the dimeric occurrence of the protein. The protein-protein interface confers additional stabilization to the dimer. The lowest melting temperature of OhyPp could at least in parts explain the difficulties in the crystallization of the protein.

Conclusion

An oleate hydratase from HFam3 named OhyPp was identified, which is closely related to the already known OhyRe. OhyPp lacks N- and C-terminal extensions, which most likely are responsible for dimerization in agreement with our SEC-MALS experiments confirming OhyPp being monomeric in solution. Despite its high sequence similarity with OhyRe, OhyPp has different characteristics. Its optimal pH is at 6, however, the enzyme is much more stable at a pH of 7.2. Its temperature optimum is differentiating to OhyRe's but also to the organism's optimal growth temperature. OhyPp is able to convert palmitoleic acid and the structural relatives of oleic acid linoleic and linolenic acid. It prefers cis-configuration and doesn't accept substrates with carbons above 20.

Most FAD is lost upon purification, which appears to be typical for monomeric oleate hydratases belonging to the HFam3 subfamily. This low affinity towards FAD currently limits their application in industrial applications. Optimizing this characteristic by using means of protein engineering is thus desirable as OhyPp's sequence and structure might help due to its higher affinity towards FAD compared to OhyRe's.

OhyPp has a similar amino acid sequence structure as OhyRe with a methionine on the position where HFam11 subfamily members carry a glutamic acid, which is thought to be an active residue in conversion. As a result, the proposed reaction mechanism may not be transferable to OhyPp as well. Furthermore, a strongly conserved amino acid motif "GGR" could be observed, which not only exists in all characterized oleate hydratases but also in other fatty acid dehydrogenating enzymes originating from plants.

Finally, stability of OhyPp is given to a minimum of nine days at 4 °C but it is lower compared to dimeric OhyEm. During the reaction, however, strong aggregation was observed. Its low stability might in part explain why no crystals could be obtained for assessing the structure. Instead, a model was designed showing a similar structure compared to OhyRe.

Oleate hydratases belong to one of the few soluble and high expression rate exhibiting fatty acid converting enzymes. They create the valuable industrial product 10-HSA, however,

major challenges remain for a successful adaption of large-scale industrial process and they mainly comprise low FAD binding and process stability. This new enzyme expands the portfolio by a new member and helps to further understand their function.

Experimental Section

Chemicals

Chemicals were mostly purchased from Sigma-Aldrich and Carl Roth at the highest available grade.

Cloning

The sequence of the novel putative oleate hydratase OhyPp (WP_057784965.1) has been derived from the National Center for Biotechnology Information[37] as closest relative of OhyRe in the data base and was synthesized with codons optimized for *E. coli* by EurofinsGenomics. The gene was subsequently cloned into pET28a(+) using Gibson Assembly® Cloning Kit (NEB). For PCR and subsequent cloning, Phusion High-Fidelity Polymerase (Thermo Scientific) was used. After PCR, open vectors were treated with Fast digest *Dpn*I and circularized with T4 DNA Ligase (both from Thermo Scientific). Plasmids were transformed into DH5α and sequenced.

Protein expression

BL21DE3 chemical competent cells were transformed with pET28a(+) containing OhyPp and resulting colonies were used to inoculate an LB overnight-culture, where all liquid cultures contained 50 μg/ml Kanamycin. 500 mL of TB-medium was inoculated with the overnight-culture and grown to an optical density OD_{600} of 0.6–0.8 at 37 °C. Temperature was decreased to 16 °C and cells were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) with a final concentration of 0.1 mM. After 16 h, cells were harvested, resuspended in buffer (20 mM Tris/Base, 20 mM imidazole, 500 mM NaCl, pH 7.2) using a high pressure homogenizator (EmulsiFlex-B15, AVESTIN). Cell debris were removed by a centrifuge at 20.000xg for 40 min at 4 °C. Protein was purified by incubating cell-free lysate using Ni^{2+}-NTA beads (Thermo Fisher) at 4 °C overnight, washed with buffer and eluted with elution buffer (20 mM Tris/Base, 250 mM imidazol, 500 mM NaCl, pH 7.2). The protein solution was dialyzed into "storage buffer" (20 mM Tris/Base, 200 mM NaCl, pH 7.2). Protein concentration was assessed using ROTI®Quant (Roth) with Bovine Serum Albumine (Roth) as standard. OhyEm was prepared as described in Engleder *et al.*[21] and OhyRe according to Lorenzen *et al.*[18]

Size exclusion chromatography – multi-angle light scattering (SEC-MALS)

SEC-MALS experiments were performed at 18 °C. OhyPp was loaded onto a Superdex 200 increase 10/300 column (Cytiva) previously equilibrated with SEC buffer (20 mM Tris/Base pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$). The column was coupled to a miniDAWN TREOS three-angle light scattering detector (Wyatt Technology) in combination with a RefractoMax520 refractive index detector. For calculation of the molecular mass, protein concentrations were determined from the differential refractive index with a specific refractive index increment (dn/dc) of 0.185 ml g^{-1}. Data was analyzed with the ASTRA 6.1.4.25 software (Wyatt Technology).

Enzymatic assays

High-purity oleic acid (99%, Alfa Aesar) resulting in a final concentration of 0.4% was vortexed with 148 μl of storage buffer until emulsified. 100 μl of protein solution (15 μM) and 1 μl of FAD (3 mM) were added and solutions were incubated at their optimal temperatures with orbital shaking for 2 h. Reaction was stopped, and fatty acids were extracted using 1 mL of ethyl acetate. Reactions including ethyl acetate were vortexed, centrifuged at 15'000xg for 3 min and the upper ethyl acetate layer was transferred to a new vial. After drying off the ethyl acetate, fatty acids were methylated in a liquid handler (MultiPurposeSampler MPS Robotic, Gerstel) using a modified protocol originating from Griffiths *et al.*[38] with HCl methanol (Merck) instead of BF_3 methanol. In our modified protocol, 500 μl of toluene and 10 μl of the standard in toluene is added to the sample and mixed. Then 1 mL of sodium methoxide is added and incubated for 20 min at 80 °C. Afterwards, 1 mL of HCl methanol is added and the solution is incubated again for 20 min at 80 °C. After derivatization, 400 μl of water and 1 mL of hexane is added, the sample mixed, centrifuged and the upper toluene/hexane layer is taken for GC-FID (flame ion detector) analysis. As internal standard, lauric acid (Sigma–Aldrich) was used. Samples were analyzed on a SHIMADZU GC-2025 with an AOC-20i auto injector and ZBWAX 13 m/Ø 0.32 mm with a hydrogen flow rate of 14 ml/min, synthetic air flow of 400 ml/min and nitrogen with a flow rate of 30 ml/min and a temperature increase from 150 °C to 240 °C in 5 °C steps per minute.

Enzyme characterization

To measure the optimal temperature of OhyPp, reactions were conducted at 15, 20, 25 and 28 °C. The enzyme solution was preheated for 5 min to adjust to each respective temperature and the reaction was conducted for 20 min. For pH-tolerance, a pH-range of 4.5–9 was tested. 50 mM Citrate buffer was used for pH 4.5–5.5, 50 mM MES-buffer for pH 5.5–6.5 and 50 mM Tris/Base-buffer for pH 7–9. Reaction was performed for 2 h.

Fatty acid specificity and mass determination

1 mg of each fatty acid was mixed with 300 μl of storage buffer und 200 μl of OhyPp cell-free extract was added. Reactions were incubated for 24 h at 20 °C. For calculating the % conversion of oleic acid and linoleic acid, 1 mg of oleic acid and linoleic acid were mixed with 100 μl of storage buffer and 100 μl of 11.8 μM OhyPp for 24 h at 20 °C. Fatty acids were extracted with 1 mL of ethyl acetate and 1 mL of hexane. After the solvents were evaporated, the fatty acids were resuspended in 60 μl pyridine and diluted in a 1:20 ratio in 50 μl pyridine. To this, 20 μl of N-Methyl-N-(trimethylsilyl)trifluoracetamid (MSTFA) and 1% trimethylchlorosilane was added. Following incubation for 1 h at 50 °C, the samples were measured on a Trace GC Ultra with a mass spectrometer DSQ2, Triplus Autosampler and SSL Injektor. Temperature ramp started at 180 °C for 2.5 min, then 5 °C/min until 285 °C was reached. Used split ratio was 12 and the flow rate 0.8 ml/min. The ion source temperature was 250 °C, injection volume 1 μl and the mass range 50–650 m/z.

Measurement of FAD concentration

A calibration curve of FAD fluorescence in either 20 mM Tris/Base, 200 mM NaCl, pH 7.2 for OhyRe and OhyPp or 50 mM HEPES, 200 mM NaCl, pH 6 for OhyEm was measured using 450 nm as excitation and 525 nm as emission wavelength. For "free" FAD, the native protein solution was measured, for "total" FAD, proteins were denatured for 15 min at 70 °C and the clear supernatant was measured. "Bound" FAD was calculated by "total" FAD – "free" FAD.

K_d-measurement

OhyEm was depleted of FAD as described in Engleder *et al.*[21] FAD (Alfa Aesar) was titrated in 1 μl steps to a 300 μl protein solution with a concentration of 5 μM and the fluorescence signal of tryptophane was measured in a Quartz multi-well plate on a plate reader (Inspire) with an excitation wavelength of 295 nm and an emission wavelength of 335 nm. Curves were fitted using the formula Vmax*X/(Kd + X) for OhyPp and OhyRe and BmaxHi*X/(KdHi + X) + BmaxLo*X/(KdLo + X) for OhyEm.

Stability tests

For stability tests, the FAD concentration of OhyEm was measured using fluorescence after protein purification and to OhyPp the same molar amount of FAD was added to avoid any effect of a difference in FAD concentration. The protein solutions were stored without additional additives at 4 °C for a duration of 27 days in total and for each time step, the required amount of said solution was used. Assays were conducted as described under "enzymatic assays". For each enzyme, its optimal buffer and reaction temperature was used.

Melting temperature analysis

2.5 μl of 10 × SYPRO™ Orange (Thermo Scientific) was added to 5 μl of protein solution (5 μM) and 16.5 μl of storage buffer into qPCR tubes with optically clear lids. Samples were put on ice before transferring them to the RT-PCR cycler (CFX Opus 96, Bio-Rad). Ramp went from 10–95 °C in 0.5 °C steps holding for 10 s per cycle.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

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