Charakterisierung lebensmitteltechnologisch relevanter Ferulasäureesterasen aus Basidiomycota

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- Nieter, A.; Haase-Aschoff, P.; Kelle, S.; Linke, D.; Krings, U.; Popper, L.; Berger, R. G.; (2015). „A chlorogenic acid esterase with a unique substrate specificity from *Ustilago maydis*". Applied and Environmental Microbiology, *81*(5), 1679-1688.

- Nieter, A.; Kelle, S.; Takenberg, M.; Linke, D.; Bunzel, M.; Popper, L.; Berger, R. G.; (2016). „Heterologous production and characterization of a chlorogenic acid esterase from *Ustilago maydis* with a potential use in baking". Food Chemistry, *209*, 1-9.

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Nachfolgend gelistete Veröffentlichungen und Poster wurden im Rahmen dieser Doktorarbeit erarbeitet, sind jedoch nicht Gegenstand dieser Dissertationsschrift.

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Inhaltsverzeichnis

Abkürzungsverzeichnis

aa	*Amino acid* (Aminosäure)
ABTS	2,2'-Azino-di-(3-ethylbenzthiazolin-6-sulfonsäure)
AOX1	Alkoholoxidase 1-codierendes Gen
Ara_2F	2-O-*trans*-Feruloyl-*α*-L-Arabinofuranosyl-(1→5)-L-Arabinofuranose
Asp	Aspartat
AX	Arabinoxylan
BLAST	*Basic local alignment search tool*
bzw.	Beziehungsweise
CA-ME	Kaffeesäuremethylester
CGA	Chlorogensäure
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propansulfonat
CLEA	*Cross-linked enzyme aggregates* (quervernetzte Enzymaggregate)
CP	*Coffee pulp* (Kaffeesatz)
CTAB	Cetyltrimethylammoniumbromid
Da	Dalton
DiFA	Dehydrodiferulasäure
DMF	Dimethylformamid
DMSO	Dimethylsulfoxid
DSWB	*Destarched wheat bran* (stärkefreie Weizenkleie)
DTT	Dithiothreitol
EDTA	Ethylendiamintetraessigsäure
ESI-MS/MS	Elektronenspray-Ionisations-Tandem-Massenspektrometrie
FA	Ferulasäure
F-A	5-O-*trans*-Feruloyl-L-Arabinofuranose
FAE	*Ferulic acid esterase* (Ferulasäureesterase; Feruloylesterase)
F-AX	*β*-D-Xylopyranosyl-(1→2)-5-O-*trans*-Feruloyl-L-Arabinofuranose
F-AXG	*α*-L-Galactopyranosyl-(1→2)-*β*-D-Xylopyranosyl-(1→2)-5-O-*trans*-Feruloyl-L-Arabinofuranose
F-AXX	D-Xylopyranosyl-(1→4)-*β*-D-Xylopyranosyl-(1→3)-5-O-*trans*-Feruloyl-L-Arabinofuranose
FA-ME	Ferulasäuremethylester
Gal_2F	6-O-*trans*-Feruloyl-*β*-D-Galactopyranosyl-(1→4)-D-Galactopyranose

Glu	Glutamat
HPLC	*High performance liquid chromatography*
HIC	Hydrophobe Interaktionschromatographie
His	Histidin
HIV	Humanes Immundefizienz-Virus
IEF	Isoelektrische Fokussierung
k_{cat}	*Turnover number* (Wechselzahl)
K_m	Michaeliskonstante
Leu	Leucin
MM	*Molecular mass* (Molare Masse)
NaCl	Natriumchlorid
OD_{600}	Optische Dichte bei einer Wellenlänge von 600 nm
*p*CA-ME	*Para*-Cumarsäuremethylester
PCR	*Polymerase chain reaction* (Polymerasekettenreaktion)
PeFaeA	Ferulasäureesterase aus *Pleurotus eryngii*
PES	Polyethersulfon
pI	Isoelektrischer Punkt
PMSF	Phenylmethylsulfonylfluorid
PAGE	Polyacrylamid-Gelelektrophorese
RSV	Respiratorisches Synzytial-Virus
reUmChlE	Rekombinante Chlorogensäureesterase aus *Ustilago maydis*
SA-ME	Sinapinsäuremethylester
SAX	Solubilisiertes Arabinoxylan
SBP	*Sugar beet pectin* (Zuckerrübenpektin)
SDS	*Sodium dodecyl sulfate* (Natriumdodecylsulfat)
SEC	*Size exclusion chromatography*
SNL	Standardnährlösung
Ser	Serin
sp., spp.	Spezies
Thr	Threonin
U	Unit (μmol min^{-1})
u. a.	Unter anderen/m
UmChlE	Chlorogensäureesterase aus *Ustilago maydis*
UTR	*Untranslated region* (untranslatierter Bereich)

UV	Ultraviolett
vgl.	Vergleich
Vis	*Visible* (sichtbar)
v/v	*Volume by volume*
w/v	*Mass by volume*
WEAX	Wasserlösliches Arabinoxylan
WUAX	Wasserunlösliches Arabinoxylan
w/w	*Mass by mass*
z. B.	Zum Beispiel
3,4,5-MeO-ZA-ME	3,4,5-Trimethoxy-Zimtsäuremethylester

Zusammenfassung

In den letzten Jahren wurden zahlreiche Ferulasäureesterasen (FAE) aus Bakterien und Pilzen, insbesondere aus der Abteilung der Ascomycota, identifiziert, isoliert und charakterisiert. Durch ein umfangreiches Screening ist das Potential von Basidiomycota, den nächsten Verwandten der Ascomycota, als FAE-Produzenten aufgezeigt worden. Mit chromatographischen Reinigungsstrategien wurden FAE-aktive Enzyme aus den Kulturüberständen der beiden Basidiomycota *Pleurotus eryngii* und *Ustilago maydis* separiert. Anschließend erfolgte eine biochemische und molekularbiologische Charakterisierung der gereinigten Enzyme.

Anhand eines umfangreichen Substratscreenings mit artifiziellen und natürlichen FAE-Substraten wurde das isolierte Enzym aus *P. eryngii* (PeFaeA) als Typ A FAE klassifiziert. Ferner demonstrierte das Substratprofil, dass die PeFaeA eine Präferenz für voluminöse natürliche Substrate, wie feruloylierte Saccharide, besitzt. Die ermittelten kinetischen Konstanten zeigten, dass die PeFaeA eine dreifach höhere Affinität für Ferulasäuremethylester (FA-ME, K_m= 0,15 mM) als für β-D-Xylopyranosyl-(1→2)-5-O-trans-Feruloyl-L-Arabinofuranose (F-AX, K_m= 0,44 mM) aufweist. Demgegenüber verdeutlichten Vergleiche der k_{cat} und k_{cat}/K_m-Werte, dass die PeFaeA F-AX vierfach schneller und auch effizienter als FA-ME hydrolysiert. Weiterhin offenbarte das Enzym eine hohe Stabilität gegenüber diversen Metallionen (1 – 5 mM), EDTA und NaCl (0,1 – 3 M). In Gegenwart von 3 M NaCl erhöhte sich die Enzymaktivität um 28 %. Bei Behandlung von stärkefreier Weizenkleie, einem Nebenstrom der Getreidevermahlung, konnte die mittels PeFaeA freigesetzte Menge an Ferulasäure (FA) duch den Zusatz von Xylanasen synergistisch erhöht werden.

Das isolierte FAE-aktive Enzym aus dem Kulturüberstand von *U. maydis* (UmChlE) stellte sich angesichts seines Substratspektrums und seiner -affinität als Novum heraus. Neben einer breiten Aktivität gegenüber bekannten FAE-Substraten besaß das Enzym eine beträchtliche hydrolytische Aktivität gegenüber Chlorogensäure (CGA). Die fehlende hydrolytische Aktivität gegenüber Sinapinsäuremethylester resultierte in der Klassifizierung als Typ B FAE. Vergleiche der ermittelten kinetischen Konstanten für CGA, FA-ME, Kaffeesäure- und *p*-Cumarsäuremethylester offenbarten, dass das isolierte Enzym eine Chlorogensäureesterase mit Typ B FAE-Nebenaktivität darstellt. Damit ist in der vorliegenden Arbeit die erste Chlorogensäureesterase beschrieben, welche nicht nur Aktivität für CGA, sondern auch für gebräuchliche FAE-Substrate besitzt. Darüber hinaus wurde die Aktivität des Enzyms gegenüber komplexer pflanzlicher Biomasse angesichts der Freisetzung von FA, Kaffee- und *p*-Cumarsäure gezeigt.

Mithilfe der über ESI-MS/MS erhaltenen Peptidpartialsequenzen konnten für beide Esterasen degenerierte Primer abgeleitet werden, mit denen die vollständigen codierenden Sequenzen beider Enzyme amplifiziert wurden. Sequenzanalysen identifizierten für die PeFaeA und die UmChlE eine katalytische Triade aus Ser, His und Glu. Beide Esterasen wurden heterolog in *Pichia pastoris* produziert. Für die rekombinante UmChlE (reUmChlE) wurde die Produktion erfolgreich vom Schüttelkolben auf den 5 L-Bioreaktor adaptiert (45,9 U L^{-1}).

Das *Up-Scaling* der Produktion der reUmChlE ermöglichte es schließlich, ausreichend reine reUmChlE zu generieren, um den Effekt von FAE-aktiven Enzymen auf die Rheologie von Weizenmehlteigen zu untersuchen. Der Zusatz von reUmChlE führte zu einer deutlichen Teigerweichung, einer verminderten Klebrigkeit und einer verbesserten Handhabung.

Schlagwörter: Basidiomycota, Esterase, Ferulasäure, feruloylierte Saccharide, heterologe Expression, *Pichia pastoris*

Abstract

In recent years, numerous feruloyl esterases (FAEs) have been identified, isolated and characterized from bacteria and fungi; most of them from the phylum of ascomycota. The potential of basidiomycota, the closest relatives of ascomycota, as FAE-producers was shown in a comprehensive screening. After the development of chromatographic purification strategies, a FAE activity was separated from the culture supernatants of the basidiomycota *Pleurotus eryngii* and *Ustilago maydis*. Subsequently, the isolated enzymes were characterized biochemically and genetically.

Performing an extensive substrate screening with synthetic and natural FAE substrates the purified enzyme from *P. eryngii* (PeFaeA) was classified as type A FAE. Substrate specificity profiling demonstrated that the PeFaeA preferred bulky natural substrates, such as feruloylated saccharides. The kinetic constants revealed that the PeFaeA possessed a three-fold higher affinity to methyl ferulate (FA-ME, K_m= 0.15 mM) than to β-D-xylopyranosyl-(1 → 2)-5-O-trans-feruloyl-L-arabinofuranose (F-AX, K_m= 0.44 mM). In contrast, the comparison of k_{cat} and k_{cat}/K_m values showed that the PeFaeA hydrolyzed F-AX four times faster and more efficiently than FA-ME, respectively. Furthermore, the enzyme exhibited a high stability towards various metal ions (1 – 5 mM), EDTA, and NaCl (0.1 – 3 M). In the presence of 3 M NaCl, the enzyme activity increased by 28 %. When destarched wheat bran, a side stream of the milling process, was treated with PeFaeA, the release of ferulic acid (FA) was synergistically improved in the presence of a xylanase.

The isolated FAE-active enzyme from the culture supernatant of *U. maydis* (UmChlE) represented a novelty in view of its substrate spectrum and affinity. In addition to a broad specificity for common FAE substrates, the enzyme possessed a superior hydrolytic activity towards chlorogenic acid (CGA). The lack of a hydrolytic activity towards methyl sinapate resulted in the classification as type B FAE. However, the comparison of the determined kinetic constants for CGA, FA-ME, methyl caffeate and methyl *p*-coumarate showed that the isolated enzyme was a chlorogenic acid esterase with type B FAE side activity. Thus, the present work describes the first chlorogenic acid esterase, which was also active towards substrates other than CGA, such as typical FAE substrates. In addition, the activity of the enzyme on complex substrates was demonstrated by its ability to release FA, *p*-coumaric and caffeic acid from plant biomass.

Degenerated primers were deduced for both esterases from partial peptide sequences, which were obtained via ESI-MS/MS, to allow for the amplification of the full length coding sequences of both enzymes. Sequence analysis identified a catalytic triad consisting of serine, histidine and glutamic acid for PeFaeA and UmChlE. Both esterases were heterologously produced in *Pichia pastoris*. The production of the recombinant UmChlE (reUmChlE) was successfully scaled-up from shake flasks to a 5 L bioreactor (45.9 U L^{-1}).

The up-scaling of the reUmChlE production eventually allowed the generation of sufficient amounts of pure reUmChlE to investigate the effect of FAE-active enzymes on wheat doughs. Rheological studies showed that the addition of reUmChlE led to a significant dough softening, a reduced stickiness and improved handling characteristics.

Key words: basidiomycota, esterase, ferulic acid, feruloylated saccharides, heterologous expression, *Pichia pastoris*

1. Einleitung

1.1 Pflanzliche Zellwände als Ressource erneuerbarer Energien

Der weltweit steigende Energiebedarf, versiegende Erdölquellen sowie die Sorge über den globalen Klimawandel sind nur einige der Punkte, die die Notwendigkeit der Entwicklung alternativer Energien begründen. Um dieser Problematik zu begegnen, sind insbesondere pflanzliche Zellwände, die mit einer jährlichen Produktionsrate von 10^{11} Tonnen die häufigste erneuerbare Ressource auf der Erde darstellen, in den Fokus der Forschung gerückt (Pauly & Keegstra 2008; Topakas et al. 2007). Bisher werden pflanzliche Zellwände nur zu 2 % von der Menschheit genutzt, obwohl sie eine wichtige Quelle an natürlichen Biomolekülen wie Zuckern und Phenolsäuren darstellen. Derzeit finden pflanzliche Zellwandmaterialien hauptsächlich Anwendung in Form von Holz für die Wärmeproduktion, als Baumaterial sowie als Ausgangsmaterial in der Textil-, Papier- und Zellstoffindustrie (Benoit et al. 2006b; Pauly & Keegstra 2008). Die Hauptursache für die noch ineffiziente Nutzung pflanzlicher Zellwände ist die komplexe Struktur dieser Gefüge, welche die Freisetzung industriell relevanter Bestandteile erschwert. Die Gewinnung dieser Bausteine unterliegt allerdings einem wachsenden wirtschaftlichen Interesse, um sie z. B. als Aromastoffe, Antioxidantien oder in der Bioethanolproduktion einzusetzen (Benoit et al. 2006b). Schätzungen zufolge ist der Energiegehalt der Zucker, die jährlich beim Abbau pflanzlicher Zellwände freigesetzt werden, äquivalent zu 640 Billionen Barrel Öl (Topakas et al. 2007).

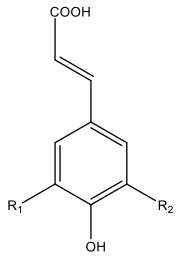
Um die weltweite Abhängigkeit von nicht erneuerbaren Ressourcen zu reduzieren, werden Alternativen zu Erdöl-basierten Kraftstoffen gesucht. Der häufigste erneuerbare Kraftstoff ist gegenwärtig Bioethanol, welches aus Getreidekörnern (Stärke) und Zuckerrohr (Saccharose) gewonnen wird. Allerdings wird die Versorgung mit diesen Rohstoffen in der nahen Zukunft wegen ihrer Lebensmitteleigenschaft ebenfalls begrenzt sein. Als Reaktion auf diese Entwicklung wurde in den letzten Jahren die Produktion von Bioethanol aus erneuerbaren Lignocellulosematerialien intensiv erforscht (Eriksson et al. 2002). Insbesondere Holz, Getreide von einjährigen Pflanzen, landwirtschaftliche Nebenströme und Altpapier werden als Quellen lignocellulolytischer Biomasse für die Ethanolproduktion genutzt. Mit einer Produktion von 170 Millionen Tonnen pro Jahr zählt Weizenstroh zu den am häufigsten vorkommenden pflanzlichen Nebenströmen in europäischen Ländern und stellt damit einen ökonomischen und nützlichen Rohstoff für die Bioethanolproduktion dar (Fang et al. 2002). Bisher ist die enzymatische Hydrolyse von Weizenstroh, welches hauptsächlich aus Cellulose

(~ 40 % der Trockenmasse), Hemicellulose (~ 25 %) und Lignin (~ 20 %) besteht, jedoch noch stark limitiert (Faulds 2010). Speziell die Kompaktheit und Komplexität von Lignocellulose gestaltet den enzymatischen Abbau in fermentierbare Zucker schwieriger als vergleichsweise bei Stärke (Gray et al. 2006). Daher sind momentan die Kosten für die Herstellung einer Gallone (entspricht ca. 3,78 L) Bioethanol aus Lignocellulose höher als für die Produktion aus Stärke (Wyman 2003). Um die Bioethanolproduktion aus lignocellulolytischer Biomasse ökonomisch zu gestalten, bedarf es zukünftig effizienter und kostengünstiger degradierender Enzyme.

1.1.1 Vorkommen von Phenolsäuren in pflanzlichen Zellwänden

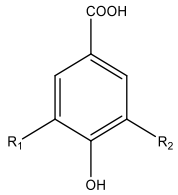
Pflanzliche Zellwände bestehen aus einem heterogenen Gemisch verschiedener Polymere. Die Hauptkomponenten pflanzlicher Zellwände bilden die drei Polysaccharide Cellulose, Hemicellulose und Pektin, welche einen Anteil von bis zu 70 % an der Gesamtbiomasse haben (Jørgensen et al. 2007). Neben Lignin sind Phenolsäuren (**Fig. 1-1**) wichtige Strukturkomponenten pflanzlicher Zellwände. Sie sind sowohl an der Ausbildung eines komplexen Netzwerkes zwischen den pflanzlichen Zellwandpolysacchariden untereinander als auch zwischen pflanzlichen Zellwandpolysacchariden und Lignin beteiligt. Phenolsäuren werden in zwei Untergruppen unterteilt: zum einen in die Hydroxyzimtsäuren und zum anderen in die Hydroxybenzoesäuren (**Fig. 1-1**).

Hydroxyzimtsäuren



	R_1	R_2
Ferulasäure	H	OCH_3
p-Cumarsäure	H	H
Sinapinsäure	OCH_3	OCH_3
Kaffeesäure	H	OH

Hydroxybenzoesäuren



	R_1	R_2
Syringasäure	OCH_3	OCH_3
Vanillinsäure	OCH_3	H
Gallussäure	OH	OH

Fig. 1-1 Strukturen von in pflanzlichen Zellwänden vorkommenden Phenolsäuren.

Die Biosynthese der Phenolsäuren erfolgt in Pflanzen über den Phenylpropanoidweg ausgehend von den aromatischen Aminosäuren L-Phenylalanin und L-Tyrosin (Kumar & Pruthi 2014). Die am häufigsten vorkommende Phenolsäure in pflanzlichen Zellwänden ist Ferulasäure (FA,

4-Hydroxy-3-Methoxy-Zimtsäure). Sie kommt hauptsächlich in der Kleie von Getreidekörnern, den Schalen von Früchten sowie in den Wurzeln und Schalen von Gemüse vor (Clifford 1999). In Getreidekörnern beträgt der Anteil von FA an der Gesamtmenge phenolischer Verbindungen 64 – 88 %. In geringeren Mengen sind in Getreidekörnern ebenfalls *p*-Cumar-, Sinapin-, Vanillin-, Kaffee-, Syringa- und Hydroxybenzoesäure nachgewiesen worden (vgl. **Fig. 1-1**) (Mattila et al. 2005). Demgegenüber liegt FA in Obst und Gemüse vorwiegend in Form von Konjugaten mit Chinasäure (Kaffee, Kohl, Sellerie und Karotten), mit Glucar- und Galactarsäure (Zitrusfrüchten), mit Weinsäure (Weintrauben), mit Äpfelsäure (Rettich), oder mit Mono-/Disacchariden wie Glucose (Äpfeln und Kohl), Digalactose (Spinat) und Gentiobiose (Brokkoli) vor (Clifford 1999; Herrmann & Nagel 1989).

Generell sind Hydroxyzimtsäuren wie Ferula- und *p*-Cumarsäure in pflanzlichen Zellwänden sowohl verestert mit Polysacchariden wie Arabinoxylan (Süßgräser) und Pektin (Dikotyledonen) als auch verethert mit Lignin (Ishii 1997) enthalten. In Folge von Oligomerisierungsreaktionen der Hydroxyzimtsäuren entsteht ein komplexes Netzwerk aus Polysacchariden, Proteinen und Lignin (Ishii 1997), welches zur Zellwandintegrität beiträgt und der Biomasse eine erhöhte Stabilität gegenüber enzymatischem Abbau verleiht (Wong 2006).

Speziell in der weit verbreiteten Pflanzenfamilie der Süßgräser (Gramineae), zu der u. a. alle Getreide-, aber auch Bambusarten zählen, ist ein hoher Gehalt an veresterter FA am Arabinoxylan (AX) vorhanden (Weizenkleie: 0,5 – 1 % (w/w) (Benoit et al. 2006b); Maiskleie: 3,1 % (w/w) (Saulnier et al. 1995)). Arabinoxylane stellen den Hauptbestandteil von Hemicellulose dar und bestehen aus einem linearen Rückgrat aus β-1,4-glykosidisch verknüpften D-Xylopyranose-Einheiten (**Fig. 1-2 (1)**), die in unregelmäßigen Abständen sowohl an der O-2 **(2)** als auch an der O-3 Position **(3)** substituierte α-L-Arabinofuranose-Reste tragen (Faulds et al. 2003). Einige der α-L-Arabinofuranose-Reste sind an der O-5 Position mit Phenolsäuren, insbesondere (Di-) Ferula- und *p*-Cumarsäure, verestert (**Fig. 1-2; A, B**).

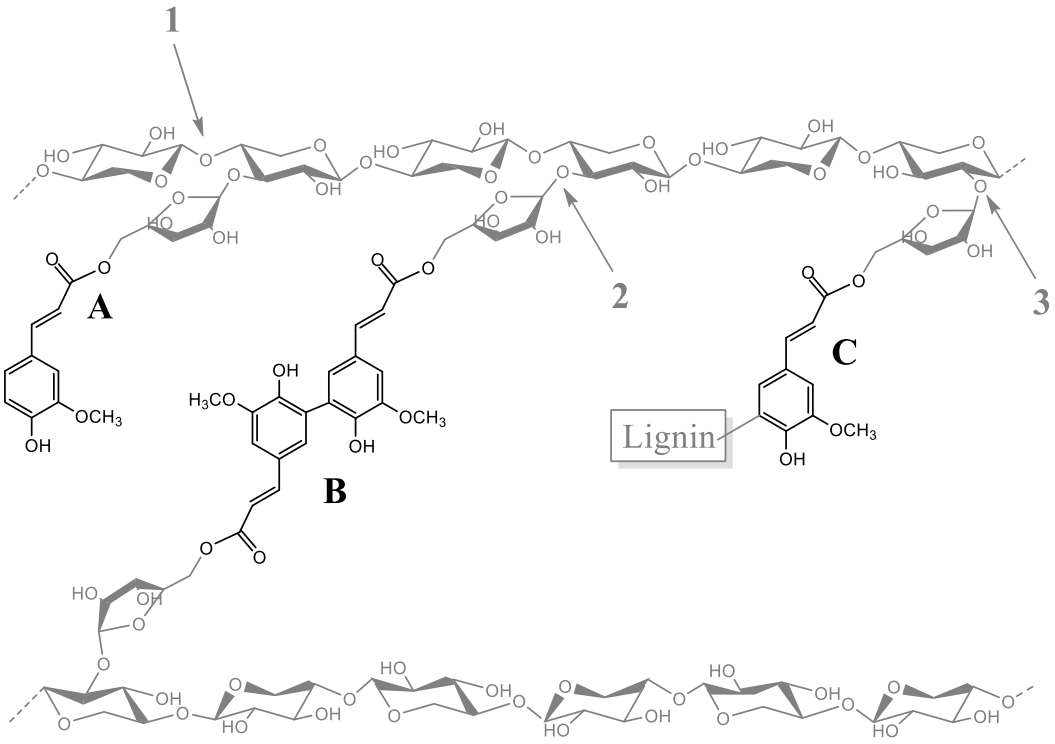


Fig. 1-2 Strukturausschnitt des Arabinoxylans (AX) von Monokotyledonen (Gramineae) mit veresterten FA-Resten (schwarz) (nach Wong 2006). AX, die Hauptkomponente von Hemicellulose, besteht aus einem Rückgrat von *β*-1,4-glykosidisch verknüpften D-Xylopyranose-Einheiten **(1)**, welche in unregelmäßigen Abständen sowohl an der O-2 **(2)** als auch an der O-3 Position **(3)** substituierte *α*-L-Arabinofuranose-Reste tragen. Teilweise ist die O-5 Position der Arabinoseseitenketten mit FA verestert **(A)**. Ferner sind die AX sowohl über Dehydrodiferulasäuren **(B, 5-5‘ Dimer)** untereinander als auch über feruloylierte Arabinoseseitenketten mit Lignin **(C)** verbunden.

Außerdem wurden veresterte Hydroxyzimtsäuren (bis zu 1 % (w/w) FA) in den Zellwänden einiger Dikotyledonen wie Zuckerrübe, Spinat, wilde Möhre, Amarant und Quinoa nachgewiesen (Bunzel et al. 2005; Parr et al. 1997; Wefers et al. 2015). Im Gegensatz zu Monokotyledonen (Veresterung der FA-Reste an den O-5 Position der Arabinoseseitenketten des AX), liegt FA in Dikotyledonen verestert an den O-2 Positionen der Arabinane (50 – 55 %) und O-6 Positionen der Galactane (40 – 45 %) des Pektins vor (Ralet et al. 1994b). Pektine sind komplexe Moleküle, deren Rückgrat aus alternierenden L-Rhamnose- und D-Galacturonsäure-Einheiten (Rhamnogalactorunan) besteht. An den Rhamnose-Resten des Rückgrates befinden sich *β*-1,4-glykosidisch gebundene D-Galactane und *α*-1,3-glykosidisch gebundene L-Arabinane (Wong 2008). Ferner liegt FA in den Zellwänden von Bambus verestert an der O-4 Position der *α*-D-Xylopyranose-Reste des Xyloglucans vor (Ishii et al. 1990).

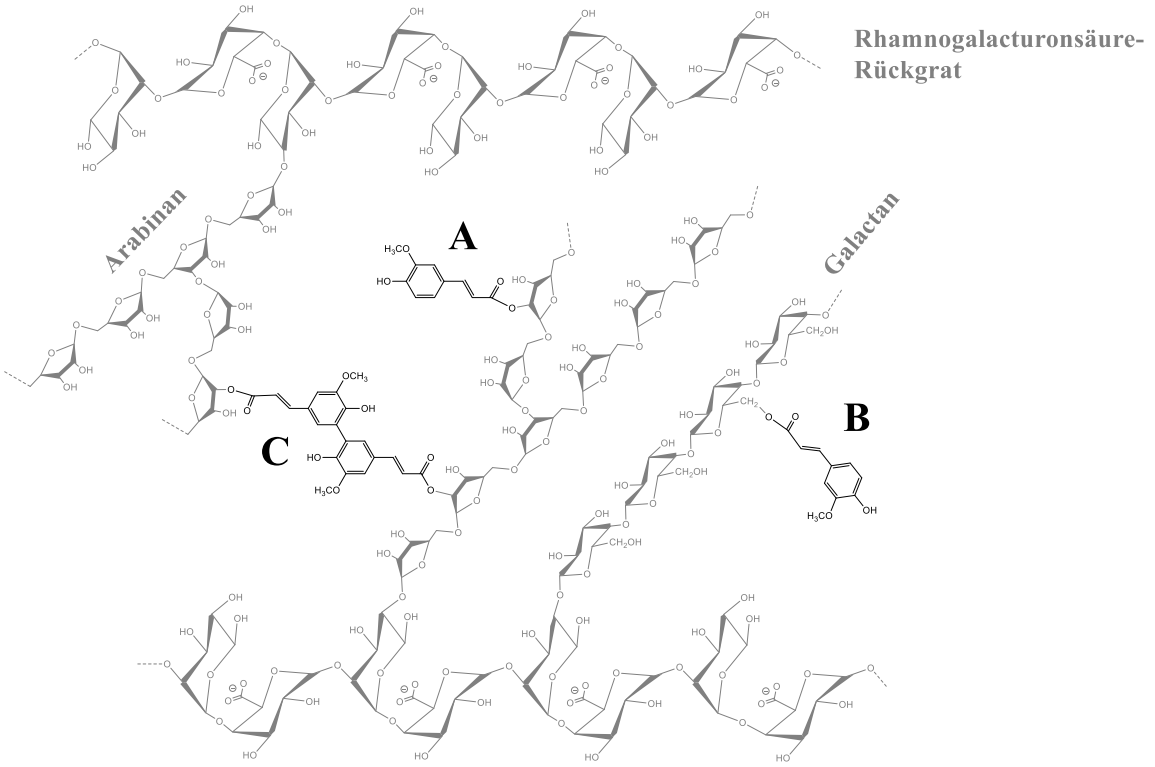


Fig. 1-3 Strukturausschnitt des Pektins (Rhamnogalacturonan) von Dikotyledonen mit veresterten FA-Resten (schwarz) (nach Wong 2006). Das Rückgrat des Pektins besteht aus alternierenden L-Rhamnose- und D-Galacturonsäure-Einheiten (Rhamnogalacturonan). An den Rhamnose-Resten des Rückgrates befinden sich glykosidisch verknüpfte D-Galactane und L-Arabinane. Ferner befinden sich FA-Reste sowohl verestert an der O-2 Position der Arabinane **(A)** als auch an der O-6 Position der Galactane **(B)**. Des Weiteren werden die verschiedenen Seitenketten des Rhamnogalacturonans über Dehydrodiferulasäuren **(C, 5-5‘ Dimer)** miteinander verbunden.

Wie in **Fig. 1-2** und **1-3** dargestellt, existiert FA in pflanzlichen Zellwänden nicht nur als Monomer (Ralph et al. 1994; Wong 2006). Geissmann und Neukom (1971) demonstrierten in ihren Versuchen mit Weizenmehl, dass wässrige Lösungen von AX die ungewöhnliche Eigenschaft haben, bei Zugabe von Wasserstoffperoxid und einer Peroxidase zu gelieren. Sie identifizierten die FA-Reste als Auslöser für dieses Phänomen. Im Verlauf einer oxidativen phenolischen Kupplungsreaktion zwischen den an AX veresterten FA-Resten entstehen Dehydrodiferulasäuren (DiFA). Saulnier und Thibault (1999) kalkulierten in ihrer Studie, dass jedes AX-Makromolekül etwa 75 veresterte FA-Reste trägt, von denen ca. 30 Reste Diferulasäure-Brücken ausbilden. Eine Auswahl identifizierter Dehydrodiferulasäuren pflanzlicher Zellwände ist in **Fig. 1-4** gezeigt. Darüber hinaus wurden in den letzten Jahren Dehydrotriferulasäuren und Dehydrotetraferulasäuren isoliert (Bunzel 2010). In der Literatur werden als Erklärung für die Entstehung von Ferulasäure-Oligomeren zwei verschiedene Mechanismen angeführt: eine UV-Licht-katalysierte Photodimerisierung (Morrison et al. 1992)

oder eine Radikal-vermittelte oxidative Kupplungsreaktion enzymatischen Ursprungs (Thibault et al. 1991).

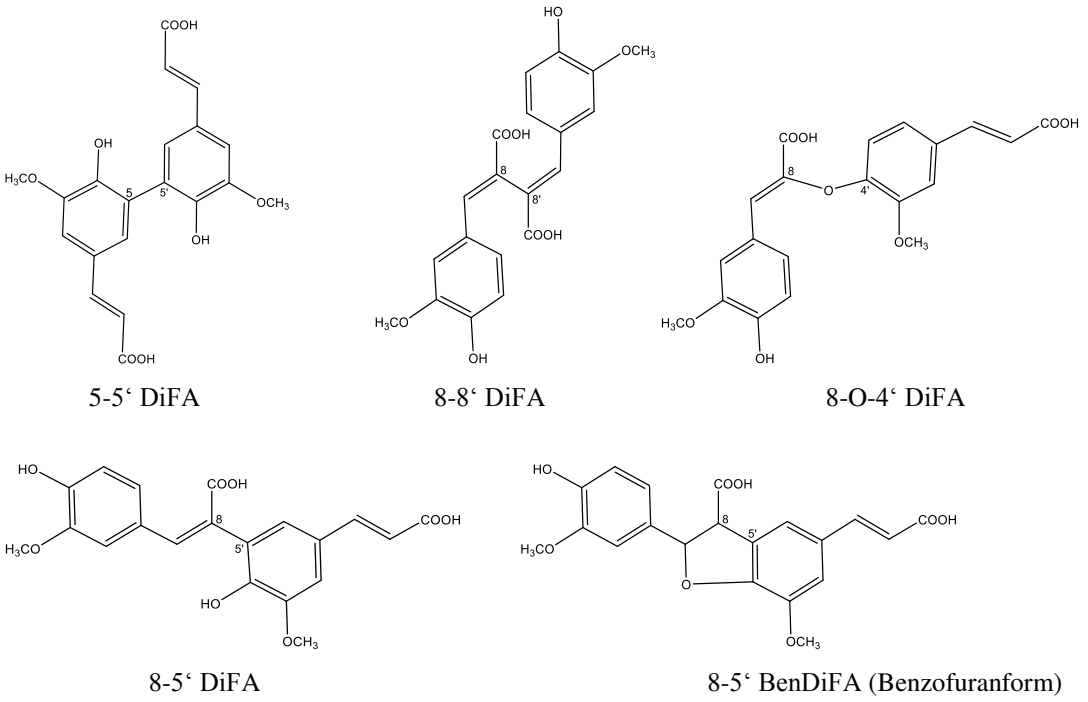


Fig. 1-4 Strukturen von in pflanzlichen Zellwänden vorkommender Dedydrodiferulasäuren (DiFA) (nach Wong 2006).

1.1.2 Bedeutung pflanzlicher Hydroxyzimtsäuren für die Industrie

In den letzten Jahren ist das Bewusstsein für die Bioaktivität und die potentiellen nutritiven Wirkungen von Hydroxyzimtsäuren und deren Konjugaten gestiegen. Im Speziellen für FA wurden zahlreiche Anwendungen in der Pharma-, Kosmetik- und Lebensmittelindustrie vorgeschlagen.

Pharmazeutische Industrie

Der Angriff von freien Radikalen und reaktiven Sauerstoffspezies auf den menschlichen Körper führt zu einer Reihe von Krankheiten wie Arteriosklerose, Krebs oder grauer Star (Young & Woodside 2001). Obwohl endogene antioxidative Systeme im menschlichen Körper existieren, ist die Aufnahme exogener Antioxidantien hilfreich, um das Abwehrsystem zu stärken. Die Bildung von freien Radikalen im menschlichen Körper wird sowohl von endogenen als auch exogenen Faktoren beeinflusst. Insbesondere die bei verschiedenen endogenen Prozessen gebildeten Superoxid- und Hydroxyl-Radikale sind assoziiert mit zahlreichen Krankheiten.

Als exogene Antioxidantien eignen sich Hydroxyzimtsäuren, da sie ein Resonanz-stabilisiertes Phenoxy-Radikal ausbilden können. Dabei ist FA aufgrund ihres geringen Redoxpotentials ein

ausgezeichneter Fänger von Sauerstoffradikalen wie beispielsweise Superoxid- und Hydroxyl-Radikalen (Wang et al. 1993).

Zahlreiche Studien belegen eine inhibitorische Wirkung von FA auf die Vermehrung von Viren wie Grippeviren, Respiratorische Synzytial-Viren (RSV) und Humane Immundefizienz-Viren (HIV) (Edeas et al. 1995; Hirabayashi et al. 1995; Sakai et al. 1999). Sakai et al. (1999) zeigten, dass RSV-infizierte Zellen in Gegenwart von FA eine verringerte Menge an Chemokinen produzieren. Die Minderung der Produktion von Chemokinen impliziert eine verringerte Immunantwort und deutet damit auf eine FA-abhängige Inhibierung des RSV-Zyklus hin. Ferner verfügt FA über eine antimikrobielle Aktivität gegen eine Reihe von Gram-positiven und Gram-negativen Bakterien sowie Hefen (Jeong et al. 2000). Überdies konnte gezeigt werden, dass FA, Chlorogensäure und Kaffeesäure eine starke Inhibierung des Wachstums von Mikroorganismen der humanen Magen-Darm-Mikroflora wie *Escherichia coli, Klebsiella pneumoniae, Enterobacter aerogenes, Citrobacter koseri, Pseudomonas aeruginosa* und *Shigella sonnei* herbeiführen. Der antimikrobielle Effekt der aufgeführten Hydroxyzimtsäuren wurde zurückgeführt auf die Inhibierung der Arylamin *N*-Acetyltransferase von Bakterien (Lo & Chung 1999; Tsou et al. 2000).

Lebensmittelindustrie

Aufgrund ihrer antioxidativen und antimikrobiellen Aktivität finden Hydroxyzimtsäuren ebenfalls Beachtung in der Lebensmittelindustrie. Sie können u. a. die Oxidation von Proteinen und Lipiden verhindern. Graf (1992) zeigte, dass Gemische aus FA und Aminosäuren/Dipeptiden die Peroxidation von Linolsäure synergistisch hemmen. Ferner wird FA verbreitet als ergogene Substanz in Sportlernahrung diskutiert, da es neben der antioxidativen Aktivität auch die Hormonsekretion im menschlichen Körper stimulieren soll (Berning & Steen 2005; Headley & Massad 1999). O‘ Connell und Fox (1999) demonstrierten, dass Kaffeesäure (5,5 mM) die Hitzestabilität von Milch bei 140 °C erhöhen kann. Der Zusatz von Kaffeesäure bewirkte eine Reduktion der reaktiven Lysin- und Cysteingruppen und inhibierte die Freisetzung der κ-Caseine aus den Caseinmycellen bei Erwärmung.

Wie in Kapitel 1.1.1 beschrieben, bilden pflanzliche Zellwände über die Verknüpfung von veresterten Hydroxyzimtsäuren ein komplexes Netzwerk aus Polymeren. Die Eigenschaft, als Vernetzungsmittel zu agieren, ermöglicht es, neuartige Gele für die Lebensmittelverarbeitung bereitzustellen. Hydroxyzimtsäuren können mit Aminosäureresten von Proteinen wie Tyrosin, Lysin und Cystein reagieren und Proteinmoleküle miteinander verknüpfen (Hurrell & Finot 1984). Ou et al. (2005) nutzten FA, um die Eigenschaften Protein-basierter Filme zu verbessern.

Die Zugabe von FA erhöhte die Reißfestigkeit, die Dehnbarkeit und die antioxidativen Eigenschaften von Filmen für die Konservierung von frischem Schweinefett.
Des Weiteren ist FA ein wirksames Molekül, um die Verfärbung von Nahrungsmitteln zu inhibieren. Bei Zusatz von FA behielten grüne Erbsen ihre Farbe, und der Farbverlust von grünem Tee wurde unterbunden. Meerbrassen, die mit FA und γ-Oryzanol gefüttert wurden, behielten ihre leuchtende Farbe infolge der verhinderten Photooxidation von Lutein und Astaxanthin (Maoka et al. 2008).

Kosmetik- und Textilindustrie

In der Regel stellen antioxidative Wirkstoffe eine erfolgreiche Strategie dar, um die Haut vor UV-vermittelten oxidativen Schäden zu schützen (Mathew & Abraham 2004). Dementsprechend finden Hydroxyzimtsäurederivate als UV-Lichtschutzmittel in vielen Sonnen- und Hautcremes sowie Haarpflegeprodukten Verwendung. Ferner patentierten Danoux et al. (2004) Sinapinsäure und deren Derivate als *Anti-aging*-Mittel in Hautkosmetika und Sonnencremes sowie als Mittel zur Verbesserung der DNA-Reparatur. Einige japanische Textilhersteller verarbeiten FA bei der Produktion von Golfbekleidung, um den biologischen Materialien einen Schutz gegen UV-Licht zu verleihen (Graf 1992).

Aromaindustrie

Der Trend und die Nachfrage nach natürlichen und „gesunden“ Lebensmitteln der letzten Jahre wirkten sich auch auf die Aroma-produzierende Industrie aus. Heutzutage fordern die Mehrheit der Verbraucher natürliche Aromen. Alle Aromastoffe, die unter den derzeitigen amerikanischen und europäischen Rechtsvorschriften chemisch synthetisiert wurden, können nicht als „natürliche Aromen“ gekennzeichnet werden (Verordnung (EG) Nr. 1334/2008). Um natürliche Aromen zu gewinnen, werden häufig physikalische Prozesse wie Extraktion oder Destillation genutzt. Jedoch gelingt es auf diese Weise selten, reine Aromastoffe zu ökonomischen Preisen zu erzeugen. Eine Alternative, um natürliche Aromastoffe zu generieren, stellen Fermentationen und enzymatische Reaktionen dar (Krings & Berger 1998; Muheim & Lerch 1999). Insbesondere FA ist in den vergangenen Jahren in den Fokus der Aromaindustrie gerückt, da es sich als gutes Substrat für die Produktion von natürlichem Vanillin erwiesen hat. Zahlreiche Forschungsgruppen haben über die Entstehung von Vanillin als Intermediat beim enzymatischen Abbau von FA berichtet (Muheim & Lerch 1999; Rosazza et al. 1995). Speziell für Vanillin besteht ein hoher Bedarf auf dem Aromamarkt, da es vielfältig als Aromastoff in der Lebensmittel-, Getränke, Parfum- oder auch Pharmaindustrie eingesetzt wird (Hagedorn &

Kaphammer 1994). Marktpreise von 1200 Dollar pro kg natürlichem Vanillin aus Vanilleschoten veranschaulichen die Bedeutung von FA als Aroma-Präkursor. Ferner ist es mit Decarboxylasen möglich, natürliches 4-Vinylguajacol, einen Aromastoff mit intensiv rauchiger Note, aus FA als Ausgangssubstrat zu generieren (Donaghy et al. 1999; Rosazza et al. 1995). Letztendlich haben Hydroxyzimtsäuren als Aroma-Präkursoren aufgrund ihres ubiquitären Vorkommens (vgl. Kapitel 1.1.1) ein großes Potential zur Herstellung natürlicher Aromastoffe.

1.1.3 Industrielle Nebenströme als Quelle für Hydroxyzimtsäuren

Insbesondere Nebenströme der Lebensmittelindustrie, von denen in Europa jährlich mehrere Millionen Tonnen u. a. in der Müllerei, Brauerei- und Zuckerindustrie erzeugt werden, verfügen über ein hohes energetisches und chemisches Potential. Anfallende Nebenströme dieser Industriezweige sind beispielsweise Stroh, Schalen, Stängel, Kleie, Zuckerrohrbagasse, diverse Trester und Presskuchen (Mandalari et al. 2008; Pandey et al. 2000; Saha 2003). Jährlich fallen in der Europäischen Gemeinschaft bei der Raffination von Zucker etwa 14 Millionen Tonnen an Zuckerrübenschnitzel und in der Getreideindustrie etwa 5 Millionen Tonnen an Kleie (Weizen, Mais) an (Bonnina et al. 2001). Obwohl diese Produkte eine reichhaltige erneuerbare Quelle für organischen Kohlenstoff darstellen, werden sie derzeit noch unzureichend genutzt. Bis dato finden diese industriellen Nebenströme vorwiegend Anwendung als Futter- und Düngemittel oder sie werden thermisch entsorgt (Mandalari et al. 2008). Um die anfallenden Kosten für die Behandlung und Entsorgung der Nebenströme auszugleichen, kommt der Entwicklung effektiver Methoden für die Gewinnung hochwertiger Verbindungen aus diesen „Abfallprodukten“ immer mehr Bedeutung zu. Neben einem hohen Gehalt an Polysacchariden ist für zahlreiche industrielle Nebenströme ein hohes Vorkommen an Hydroxyzimtsäuren beschrieben (**Tab. 1-1**), welche nach enzymatischer Freisetzung für die Weiterverarbeitung in verschiedenen Industriezweigen eingesetzt werden könnten. Ferner liegen Hydroxyzimtsäuren in pflanzlichen Materialien oft auch als lösliche Konjugate von Chinasäure vor, z. B. Chlorogensäure. Die häufigste Chlorogensäure stellt dabei 5-*O*-Kaffeoylchinasäure dar (Benoit et al. 2006b; Clifford 1999).

Tab. 1-1 Überblick über den Gehalt an Hydroxyzimtsäuren in industriellen Nebenströmen.

Industrieller Nebenstrom	Hydroxyzimtsäurederivat	Gehalt	Quelle
Mandelschalen	5-*O*-Kaffeoylchinasäure 4-*O*-Kaffeoylchinasäure 3-*O*-Kaffeoylchinasäure	42,5 mg/100 g[a] 7,9 mg/100 g[a] 3,0 mg/100 g[a]	Takeoka und Dao (2003)
Artischocken-Blanchierwasser	5-*O*-Kaffeoylchinasäure 4-*O*-Kaffeoylchinasäure 3-*O*-Kaffeoylchinasäure Kaffesäurederivate	11,3 g Phenol-säuren/100 mL	Llorach et al. (2002)
Kartoffelschalen	Chlorogensäure Kaffeesäure	4,84 mg/g[b] 0,02 mg/g[b]	De Sotillo et al. (1994)
Apfeltrester	Chlorogensäure Cumaroylchinasäure *p*-Cumarsäure Ferulasäure	14,3 mg/g[b] 1,8 mg/g[b] 0,5 mg/g[b] 0,4 mg/g[b]	Schieber et al. (2003)
Kokosnussschalen	Ferulasäure	0,24 mg/g[b]	Dey et al. (2003)
Kaffeesatz	Kaffeesäure *p*-Cumarsäure Ferulasäure	2,66 mg/g[b] 0,08 mg/g[b] 0,24 mg/g[b]	Benoit et al. (2006b)
Weizenstroh (nach Wasserdampfbehandlung)	*p*-Cumarsäure Ferulasäure	2,13 mg/g[b] 1,35 mg/g[b]	Benoit et al. (2006b)
Zuckerrübenschnitzel	Ferulasäure	6,4 mg/g[b]	Benoit et al. (2006b)
Maiskleie (autoklaviert)	*p*-Cumarsäure Ferulasäure	3,12 mg/g[b] 31,22 mg/g[b]	Benoit et al. (2006b)
Biertreber	*p*-Cumarsäure Ferulasäure Hydroxyzimtsäurederivate	0,05 mg/g[b] 0,21 mg/g[b] 0,54 mg/g[b]	McCarthy et al. (2013)

Die Gehalte an Hydroxyzimtsäuren sind bezogen auf die Feuchtmasse (a) oder die Trockenmasse (b).

Trotz des reichhaltigen Vorkommens von Hydroxyzimtsäuren in pflanzlichen Zellwänden (vgl. Kapitel 1.1.1) ist die kommerzielle Anwendung natürlicher Hydroxyzimtsäuren aufgrund ihrer eingeschränkten Zugänglichkeit limitiert (Faulds et al. 1997). Um eine effiziente Degradation pflanzlicher Zellwände zur Freisetzung von Hydroxyzimtsäuren aus Nebenströmen der Lebensmittelindustrie zu realisieren, bedarf es des Einsatzes von Enzymen mit differenzierten Aktivitäten (Mandalari et al. 2008). Für eine komplette Hydrolyse der Hemicellulosefraktion sind zwei verschiedene Enzymgruppen notwendig: (1) Endoxylanasen (EC 3.2.1.8) oder β-Xylosidasen (EC 3.2.1.37), um die AX-Hauptkette zu spalten, sowie (2) α-Arabinofuranosidasen (EC 3.2.1.55) und Esterasen (EC 3.1.-.-), um die Seitenketten abzuspalten und die Vernetzung zwischen AX und anderen pflanzlichen Polymeren zu hydrolysieren (Wong 2006). Eine Schlüsselrolle zur Verbesserung der Hydrolyse pflanzlichen Materials besitzen Ferulasäureesterasen (FAE, EC 3.1.1.73). Im Folgenden wird näher auf die Eigenschaften und Applikationen dieser besonderen Enzymklasse, welche Gegenstand der vorliegenden Arbeit ist, eingegangen.

1.2 Ferulasäureesterasen (FAE)

Ferulasäureesterasen, ebenfalls bekannt als Feruloyl- oder Zimtsäureesterasen, stellen eine Untergruppe der Carboxylesterhydrolasen (EC 3.1.1.-) dar. Sie katalysieren die Hydrolyse von Esterbindungen zwischen FA bzw. verwandter Hydroxyzimtsäuren und komplexen pflanzlichen Zellwandpolysacchariden (Williamson et al. 1998).

Erstmalig wurde eine FAE-Aktivität in den Arbeiten von MacKenzie et al. (1987), welche das cellulo- und xylanolytische Enzymsystem von *Streptomyces* spp. untersuchten, beschrieben. An diese Entdeckung schloss sich im darauffolgenden Jahr die erste Publikation über die partielle Reinigung einer FAE aus dem Basidiomyceten *Schizophyllum commune* an (MacKenzie & Bilous 1988). In den nachfolgenden Jahren folgten zahlreiche Veröffentlichungen über die Isolierung und Charakterisierung von FAE aus verschiedenen mikrobiellen Quellen. Mit Beginn der 1990er Jahre ist das Forschungsinteresse an FAE beachtlich gestiegen (**Fig. 1-5**). Während bei SciFinder unter dem Suchbegriff „*Ferulic acid esterase*“ für das Jahr 1990 nur eine Publikation vorhanden ist, sind für das Jahr 1999 bereits 16 Veröffentlichungen aufgelistet. Weitere Analysen hinsichtlich der Anzahl der Veröffentlichungen zu FAE im Zeitraum von 1990 – 1999 (76 Publikationen) und 2000 – 2009 (209 Publikationen) verdeutlichen das wachsende wissenschaftliche Interesse an dieser Enzymklasse.

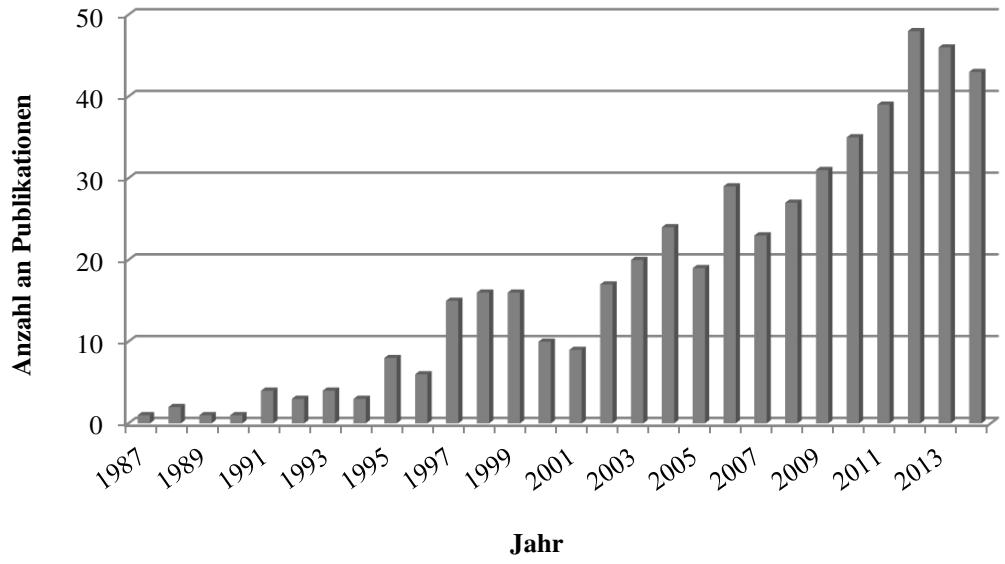


Fig. 1-5 Entwicklung der Anzahl an Publikationen zu FAE (SciFinder Recherche vom 22.09.2015 unter dem Suchbegriff „*Ferulic acid esterase*“).

Bisher wurden FAE hauptsächlich aus Pilzen isoliert; insbesondere die den Ascomycota zugehörige Gattung *Aspergillus* gehört zu den effektivsten FAE-Produzenten (Topakas et al. 2007). Demgegenüber sind aus der Abteilung der Basidiomycota bis dato nur vier FAE

gereinigt und charakterisiert worden (Hashimoto et al. 2010; Linke et al. 2013; Wang et al. 2014a; Wang et al. 2014b), exklusive der zwei in dieser Arbeit isolierten Enzyme. Ferner sind diverse FAE in Bakterien wie *Streptomyceten* (Faulds & Williamson 1991; Ferreira et al. 1999), *Clostridien* (Blum et al. 2000; Donaghy et al. 2000) und *Bacillus* (Donaghy et al. 1998) identifiziert worden. Neben einigen Vertretern in Gerste (Sancho et al. 1999) und Fingerhirse (Latha et al. 2007) ist das Vorkommen von FAE auch in Ratten (Andreasen et al. 2001) und im Darm von Termiten (Rashamuse et al. 2014) beschrieben.

1.2.1 FAE aus Basidiomycota

Basidiomycota, welche über 32.000 Arten aller bisher beschriebenen Pilze (30 % der Pilze) umfassen, stellen eine sehr vielseitige und ökologisch bedeutsame Abteilung der Pilze dar. Sie gehören gemeinsam mit den Ascomycota (Schlauchpilze, 60 % der Pilze) dem Unterreich der Dikarya an (Kirk et al. 2008; Lüttge et al. 2010; Schwantes 1996). Die Taxonomie der Basidiomycota ist am Beispiel des Pflanzenpathogens *Ustilago maydis* (Maisbeulenbrand) und des Holzfäulepilzes *Pleurotus eryngii* in **Fig. 1-6** illustriert; mit diesen beiden Pilzen wurden umfangreiche Arbeiten im Rahmen dieser Dissertation durchgeführt.

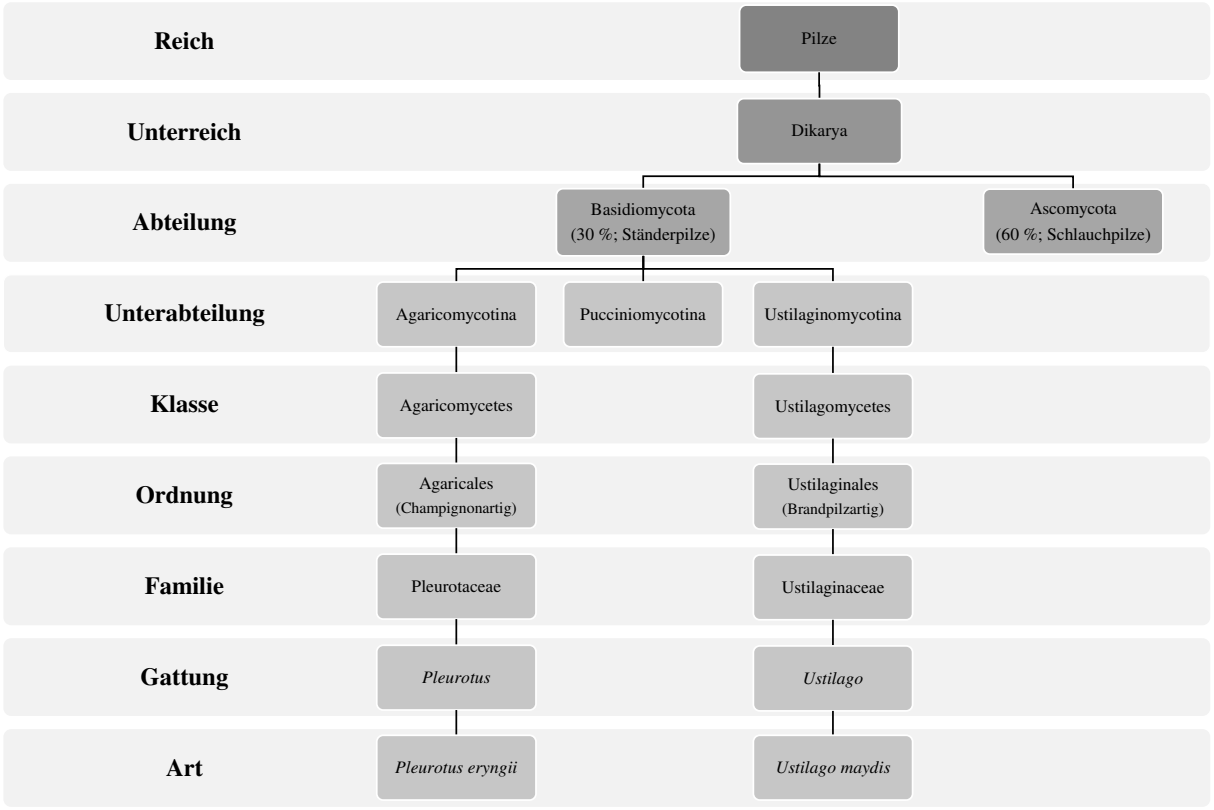


Fig. 1-6 Taxonomie der Pilze am Beispiel von *Pleurotus eryngii* (Brauner Kräuter-Seitling) und *Ustilago maydis* (Maisbeulenbrand) (nach NCBI Taxonomy Browser).

Als Adaption an ihre Lebensweise auf Laub- und Nadelhölzern sekretieren Basidiomycota eine Vielzahl von Enzymen wie Laccasen (EC 1.10.3.2), Peroxidasen (EC 1.11.1.-) und Hydrolasen (EC 3.-.-.-) (Bouws et al. 2008). Obwohl Basidiomycota attraktive Enzymproduzenten sind, ist die Anzahl an identifizierten FAE aus Basidiomycota im Vergleich zu dem Portfolio bekannter FAE aus Ascomycota sehr gering (vgl. Kapitel 1.2). Zu dieser Wissenslücke scheinen insbesondere die ubiquitär in Braunfäule- und Weißfäulepilzen vorkommenden Laccasen einen erheblichen Beitrag geleistet zu haben (Haase-Aschoff et al. 2013a). Laccasen gehören zur Klasse der Oxidoreduktasen (EC 1.-.-.-) und katalysieren die Polymerisation von phenolischen Verbindungen wie beispielsweise FA und deren Ester (Dwivedi et al. 2011; Eastwood et al. 2011; Knezevic et al. 2013; Martinez et al. 2009). Demzufolge ist in Gegenwart von Laccasen weder der phenolische Ester als Substrat für den FAE-Assay verfügbar noch ist das phenolische Produkt der FAE-katalysierten Reaktion nachweisbar. Um dieser Problematik zu begegnen, wurde von Haase-Aschoff et al. (2013a) ein neuartiger FAE-Assay entwickelt. Im Vergleich zu allen zuvor in der Literatur beschriebenen Assays erfolgt die Detektion von FAE-Aktivitäten nicht nur über ein phenolisches Substrat, sondern zusätzlich über zwei Zimtsäurederivate (Zimtsäuremethylester (ZA-ME) und 3,4,5-Trimethoxy-Zimtsäuremethylester (3,4,5-MeO-ZA-ME)), die zugleich keine Laccase-Substrate darstellen.

Mithilfe des neu entwickelten Assays führten Haase-Aschoff et al. (2013a) das erste gezielte *Screening* von Basidiomycota auf FAE-Aktivität durch. Von den 41 getesteten Basidiomycota waren 25 Stämme (61 %) in der Lage, zumindest einen der vorgelegten Ester zu hydrolysieren (**Fig. 1-7**). Die gleichzeitige Verwendung von drei FAE Substraten (FA-ME, ZA-ME und 3,4,5-MeO-ZA-ME), in equimolaren Konzentrationen im Inkubationsansatz, ermöglichte es, anhand der Hydrolysegrade eine Substratpräferenz der jeweiligen FAE abzuleiten.

Innerhalb der FAE-aktiven Basidiomycota hydrolysierten *Auricularia auricula-judae* (100 mol-%), *Marasmius scorodonius* (94 mol-%) und *Phellinus robustus* (93 mol-%) ZA-ME am besten. Die beiden letzteren waren zudem mit 68 und 56 mol-% die besten FA-ME hydrolysierenden Organismen. *Micromphale foetidum* war der drittbeste FA-ME-spaltende (42 mol-%) und der beste 3,4,5-MeO-ZA-ME-spaltende (42 mol-%) Organismus. Durchschnittlich war ZA-ME das am besten umgesetzte Substrat, gefolgt von FA-ME und 3,4,5-MeO-ZA-ME (Haase-Aschoff et al. 2013a). Insgesamt belegt dieses umfassende *Screening* das große Potential von Basidiomycota zur Bildung diverser FAE. Es wurden verschiedene Substratspezifitäten aufgezeigt, die auf unterschiedliche FAE-Typen hindeuten und somit die Isolierung und Charakterisierung verschiedener basidiomycetischer FAE erstrebenswert machen, um den Kenntnisstand zu dieser Enzymklasse zu erweitern.

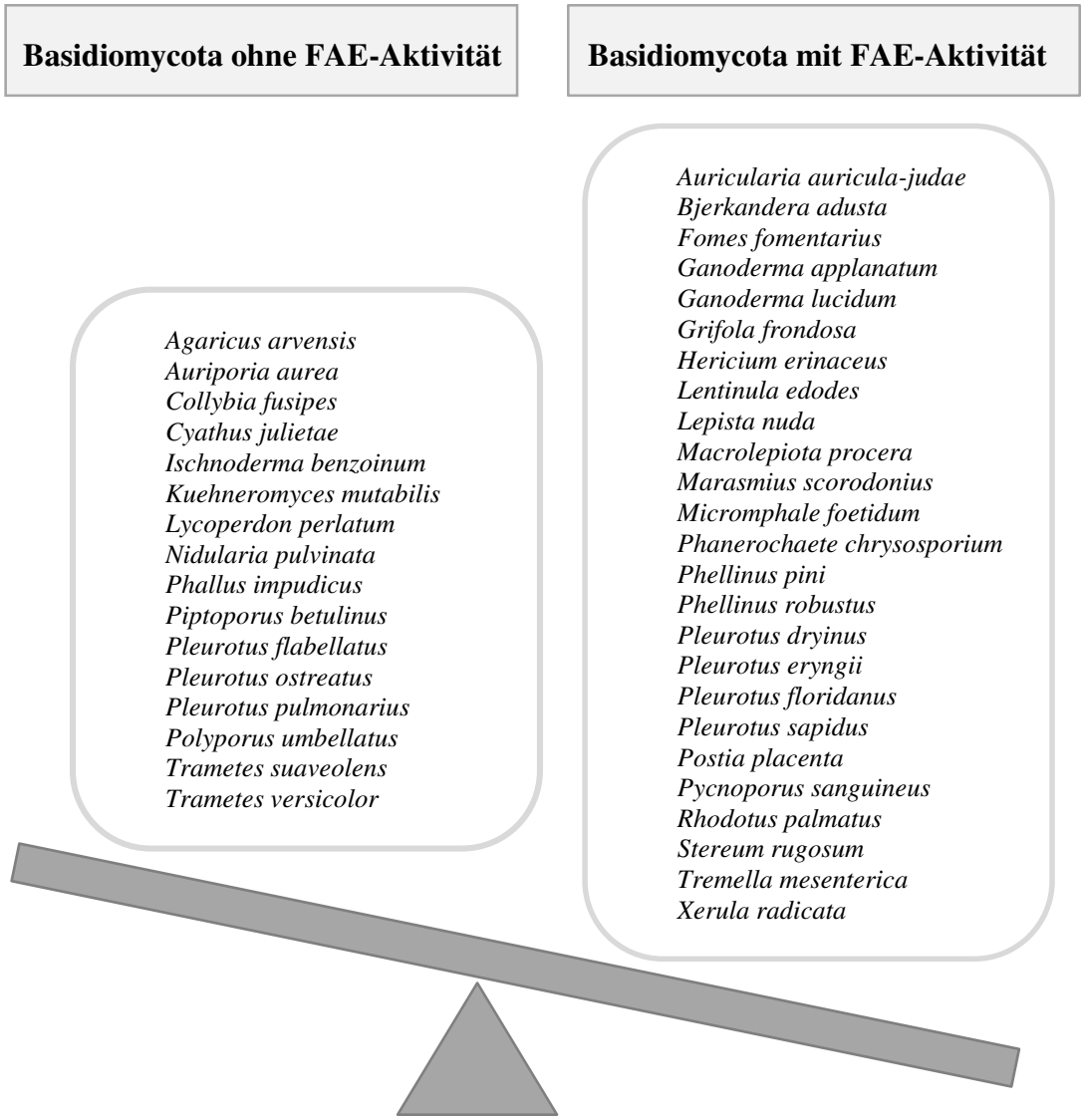


Fig. 1-7 *Screening* von Basidiomycota bezüglich FAE-Aktivität (Haase-Aschoff et al. 2013a).

1.2.2 Klassifizierung von FAE

Die erste Differenzierung von FAE erfolgte im Jahr 1993 durch Faulds und Williamson. Sie untersuchten die Substratspezifitäten von zwei *Aspergillus niger* FAE und identifizierten Unterschiede, u. a. bezüglich der Hydrolyse von FA-ME, Kaffeesäuremethylester (CA-ME), *p*-Cumarsäuremethylester (*p*CA-ME) und Sinapinsäuremethylester (SA-ME). Im nachfolgenden Jahr ergänzten Ralet et al. (1994a) das betrachtete Substratspektrum um feruloylierte Saccharide, welche aus Zuckerrübenpektin und Weizenkleie isoliert wurden (vgl. Kapitel 1.1.1). Kroon et al. (1996) führten schließlich die ursprüngliche Unterteilung von FAE anhand ihrer Induktion und Substratspezifität in die Typen A und B ein. Im Jahr 1999 veröffentlichten Kroon et al. eine Erweiterung dieser Arbeiten und zeigten, dass lediglich eine der beiden FAE in der Lage war, DiFA aus Xylanase-vorbehandelten Pflanzenmaterialien

freizusetzen. Infolge des steigenden Forschungsinteresses an FAE seit den 1990er Jahren (vgl. Kapitel 1.2) und der damit einhergehenden Identifizierung, Reinigung und Charakterisierung zahlreicher FAE in Verbindung mit der Zugänglichkeit erster Sequenzinformationen von FAE veröffentlichten Crepin et al. (2004b) ein Klassifizierungsmodell. Dieses System gliederte FAE in vier Typen A – D auf Basis von Sequenzhomologien und der Substratpräferenz für die Hydrolyse der vier artifiziellen Substrate FA-ME, CA-ME, *p*CA-ME und SA-ME (**Tab. 1-2**). Ferner wurde für die Klassifizierung das Vermögen zur Freisetzung von DiFA aus komplexen Substraten berücksichtigt. Allerdings ließen die phylogenetischen Analysen das Vorhandensein weiterer noch ungenannter Enzymunterklassen vermuten. Auf Basis von primären Sequenzinformationen vermuteten Crepin et al. (2004b) die Existenz vermeintlicher Typ „E" FAE mit unbekannten biochemischen Charakteristika.

Tab. 1-2 Klassifizierung von FAE anhand von Substratspezifitäten und Sequenzhomologien (modifiziert nach Crepin et al. 2004b).

	Typ A FAE	Typ B FAE	Typ C FAE	Typ D FAE
Aktivität gegenüber Hydroxyzimtsäureester:				
Ferulasäuremethylester	✓	✓	✓	✓
p-Cumarsäuremethylester	✓	✓	✓	✓
Sinapinsäuremethylester	✓	---	✓	✓
Kaffeesäuremethylester	---	✓	✓	✓
Aktivität gegenüber feruloylierten Saccharidenᵃ:				
Substrat mit FA-Rest an der O-5 Position	✓	✓	✓	✓
Substrat mit FA-Rest an der O-2 Position	---	✓	✓	✓
Freisetzung von DiFA	✓ (5-5')	---	---	✓ (5-5')
Sequenzhomologie	Lipasen	Kohlenhydrat-esterase Klasse 1; Acetylxylan-esterase	Chlorogensäure-esterase; Tannase	Xylanase
Bevorzugter Induktorᵇ	Weizenkleie; Haferspelzenxylan	Zuckerrübenpektin	Zuckerrübenpektin; Weizenkleie	Weizenkleie
Enzym-Beispiel	AnFaeA bzw. FAEIII aus *Aspergillus niger*	FoFaeB aus *Fusarium oxysporum*	TsFaeC aus *Talaromyces stipitatus*	NcFaeD aus *Neurospora crassa*
Referenz	Faulds et al. 1994	Topakas et al. 2003	Garcia-Conesa et al. 2004	Crepin et al. 2004a

* Angaben wurden aus der Literatur zum bestehenden Klassifizierungssystem von Crepin et al. (2004b) ergänzt.
*ᵃ Angaben nach Topakas et al. (2007); *ᵇ Angaben nach Fazary et al. (2007) und Kroon et al. (1996).

Im Jahr 2008 wurde die Typisierung der FAE unter Berücksichtigung von phylogenetischen Analysen auf sieben Klassen erweitert (Benoit et al. 2008). Diese sieben Klassen enthielten ausschließlich charakterisierte FAE der Typen A – C; Vertreter der Typ D FAE konnten in diesem System nicht zugeordnet werden. Maßgeblich für die Differenzierung zwischen den Unterfamilien des Klassifizierungssystems von Benoit et al. (2008) waren phylogenetische, und

nicht funktionelle Parameter. Ein alternatives Klassifizierungssystem für FAE lieferten Udatha et al. (2011) basierend auf Deskriptor-gestützten Sequenzanalysen verbunden mit pharmakophoren Modellen. Ausgehend von 365 FAE-Aminosäuresequenzen, welche vorwiegend hypothetisch annotierte Sequenzen aus Genomprojekten umfassten, wurden 12 FAE-Familien gebildet. In Abhängigkeit von der Distanz der Aminosäurereste der katalytischen Triade (bestehend aus Serin, Aspartat und Histidin) wurden die 12 FAE-Familien zusätzlich in 32 FAE-Untergruppen unterteilt.

Die Einordnung neuer FAE in der Literatur erfolgt bis dato ausschließlich anhand des Klassifizierungssystems von Crepin et al. (2004b), welches die FAE Typen A – D umfasst. Neben der ubiquitären kommerziellen Verfügbarkeit der artifiziellen Substrate (FA-ME, SA-ME, CA-ME und *p*CA-ME) ist häufig ein Mangel an Sequenzinformationen sowie die Problematik einer fehlenden Plattform zur FAE-Sequenzanalyse nach dem Deskriptor-basierten Modell ursächlich. Vergleiche von erweiterten Substratspektren demonstrieren jedoch die Unzulänglichkeit des Klassifizierungssystems von Crepin et al. (2004b). Die klassifizierten Typ B FAE aus *Sporotrichum thermophile* (StFaeB) und *Fusarium oxysporum* (FoFaeB) zeigten unterschiedliche Substratspezifitäten für 26 Methylphenylalkanoate (Topakas et al. 2005a). Die Typ C FAE aus *Talaromyces stipitatus* (TsFaeC) und *S. thermophile* (StFaeC) wiesen ebenfalls Unterschiede für die Hydrolyse der 34 untersuchten Substrate auf (Vafiadi et al. 2006).

Außerdem zeigten Haase-Aschoff et al. (2013b), dass die Hydrolyse artifizieller FAE-Modellsubstrate nicht zwangsläufig auf eine genuine FAE-Aktivität schließen lässt. Während die Carboxylesterase aus *Auricularia auricula-judae* (EstBC) zunächst wegen der Hydrolyse der vier Modellsubstrate (FA-ME, SA-ME, CA-ME und *p*CA-ME) als FAE charakterisiert wurde, zeigte das Enzym keine Aktivität gegenüber feruloylierten Sacchariden oder komplexeren natürlichen Substraten wie Zuckerrübenpektin oder Weizenkleie. Diese Ergebnisse demonstrieren die Notwendigkeit, im Assay neben Modellsubstraten auch feruloylierte Saccharide einzusetzen. Zur Identifizierung neuer FAE sollte die Hydrolyse von natürlichen Substraten wie feruloylierten Sacchariden (vgl. **Fig. 7-4**) ein essentielles Kriterium sein.

Auf Basis diverser Studien wurde das in **Tab. 1-2** dargestellte Klassifizierungssystem von Crepin et al. (2004b) um die Aktivität für O-5- und O-2-feruloylierte Sacchariden ergänzt (Puchart et al. 2007). Typ A FAE wie *A. niger* AnFaeA (Williamson et al. 1998), *T. stipitatus* TsFaeA (Garcia-Conesa et al. 2004) und *F. oxysporum* FoFaeA (Topakas et al. 2003a) sind nur aktiv gegenüber Substraten, die FA verestert an der O-5 Position von L-Arabinofuranose tragen.

Demgegenüber hydrolysierten alle untersuchten Typ B FAE wie AnFaeB aus *A. niger* (Williamson et al. 1998), PfFaeB aus *Penicillium funiculosum* (Kroon et al. 2000), TsFaeB aus *T. stipitatus* (Garcia-Conesa et al. 2004), FoFaeB aus *F. oxysporum* (Topakas et al. 2003b) und StFaeB aus *S. thermophile* (Topakas et al. 2004) sowohl die O-5- als auch O-2-feruloylierten Substrate. Jedoch unterschieden sich die Typ B FAE hinsichtlich der Präferenz für die unterschiedlich verknüpften feruloylierten Substrate, was wiederum eine Möglichkeit zur Differenzierung in Unterklassen aufzeigt, um die Klassifizierung von FAE spezifischer zu gestalten. Ferner setzten sowohl die Typ C FAE aus *S. thermophile* (StFaeC, Topakas et al. 2005b) und aus *T. stipitatus* (TsFaeC, Garcia-Conesa et al. 2004) als auch die Typ D FAE aus *Pseudomonas fluorescens* subsp. *cellulosa* (XYLD, Faulds et al. 1995b) FA aus beiden Substrattypen frei.

1.2.3 Struktur und katalytischer Mechanismus von FAE

Röntgenkristallstrukturen bisher untersuchter FAE zeigen eine kanonische Faltung der *α/β*-Hydrolasen und weisen im aktiven Zentrum eine katalytische Triade aus Ser-Asp-His auf, wobei das Nucleophil Serin im Zentrum des hochkonservierten Pentapeptids G-X-S-X-G (X: jede beliebige Aminosäure) lokalisiert ist (Faulds et al. 2005; Goldstone et al. 2010; Hermoso et al. 2004; Schubot et al. 2001; Suzuki et al. 2014). Demgegenüber zeigen neuere Studien, dass FAE-Vertreter der Basidiomycota (Est1 aus *P. sapidus* (Linke et al. 2013) und die zwei in dieser Arbeit bearbeiteten FAE aus *P. eryngii* und *U. maydis*) als Säurerest in der katalytischen Triade Glutamat anstatt Aspartat tragen.

Generell verläuft die FAE-katalysierte Reaktion (**Fig. 1-8**) analog zu den katalytischen Mechanismen von Serinpeptidasen, Lipasen und anderen Esterasen (Wong 2013). Im ersten Schritt (**1**) erfolgt ein nucleophiler Angriff des katalytischen Serins an den Carbonylkohlenstoff des Substrates unter Ausbildung eines tetraedrischen Übergangszustandes. Die Aktivierung des katalytischen Serins verläuft über ein Ladungs-Relay-System, in welchem der katalytische Histidin- und Aspartat-Rest involviert sind. Der kurzlebige tetraedrische Übergangszustand wird durch Wasserstoffbrückenbindungen zwischen dem negativ geladenen Carbonylsauerstoffatom („Oxyanion“) und zwei NH-Gruppen des Hauptstranges über das sogenannte „Oxyanion-Loch“ stabilisiert (**2**). Beim anschließenden Zerfall des tetraedrischen Übergangszustandes bildet das nucleophile Serin mit der Säurekomponente des Substrates ein stabiles kovalentes Intermediat (Acyl-Enzym-Intermediat) (**4**) aus, während die Alkoholkomponente des Substrates entlassen wird (**3**). Im nachfolgenden Deacylierungsschritt erfolgt der Angriff eines Nucleophils (Wasser im Falle der Hydrolyse bzw. ein Alkohol oder

Ester bei der reversen Hydrolyse) an den Carbonylkohlenstoff des Acyl-Enzym-Intermediats (**4**) unter Ausbildung eines zweiten tetraedrischen Übergangszustandes (**5**). Das Nucleophil wird analog zum initialen Katalyseschritt über das Ladungs-Relay-System aktiviert. Der Zerfall des kurzlebigen Übergangszustandes führt zur Freisetzung des Produktes und des regenerierten Enzyms (**6**) (Blow et al. 1969; Prates et al. 2001; Schubot et al. 2001; Wong 2006).

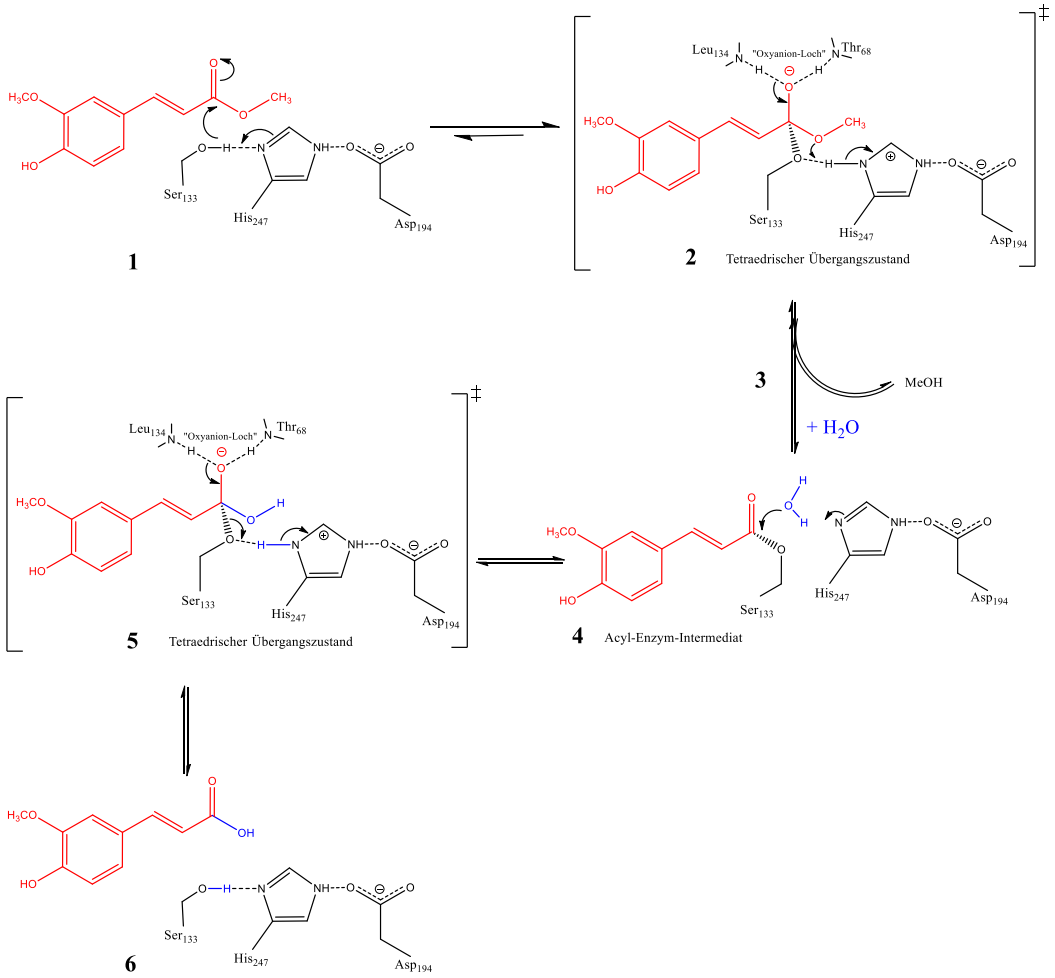


Fig. 1-8 Katalytischer Mechanismus von FAE (modifiziert nach Wong 2006). Die Katalyse erfolgt über die Bildung und der anschließenden Aufspaltung des kovalenten Acyl-Enzym-Intermediates unter Ausbildung von tetraedrischen Übergangszuständen. Die Nummerierung der katalytischen Triade (Asp_{194}, His_{247} und Ser_{133}) und der „Oxyanion-Loch"-Reste (Leu_{134} und Thr_{68}) basiert auf der 3D-Struktur der AnFaeA aus *A. niger*. In diesem Beispiel stellt FA-ME (rot) das Substrat der Reaktion dar.

1.2.4 Industrielle Applikationen von FAE

Ferulasäureesterasen gehören im Vergleich zu Peroxidasen, Laccasen oder Polyphenoloxidasen (EC 1.10.3.1) zu den weniger untersuchten Mitgliedern der an Phenolen agierenden Enzyme. Dennoch besitzen sie großes Potential in vielen Bereichen der Pflanzenverarbeitung, wie beispielsweise zur Verbesserung der Bioverfügbarkeit von pflanzlichen Inhaltsstoffen aus

Lebensmitteln, oder als Hilfsenzyme für die Gewinnung nachhaltiger Energien (Faulds 2010). FAE haben nicht nur die Fähigkeit, pflanzliche Biomasse zu dekonstruieren, sondern auch das Potential neue Verbindungen für die Gesundheits- und Pharmaindustrie zu synthetisieren. Die Aussicht auf diverse industrielle Applikationen von FAE führte seit Beginn der 1990er Jahre zu einer Erhöhung des Forschungsinteresses an dieser Enzymklasse und verdeutlicht sich anhand der zunehmenden Anzahl an neu entdeckten FAE in den letzten Jahren (vgl. Kapitel 1.2).

1.2.4.1 Papier- und Zellstoffindustrie

Die Hauptproblematik bei der Herstellung von hochqualitativen Fasern für die Industrie stellt die Eliminierung von Lignin dar. Lignin ist strukturell in das Cellulose- und Hemicellulose-Netzwerk eingebettet und für die dunkle Farbe der Pulpe verantwortlich. Zum Bleichen und Entfernen von Lignin wird industriell u. a. eine chemische Behandlung mit Chlorverbindungen durchgeführt, aber diese Verfahren erzeugen hohe Gehalte an Dioxin und Chlorlignin, welche als umweltbelastend eingestuft sind. Die hohe Spezifität enzymatischer Reaktionen repräsentiert eine interessante Alternative zum chemischen Bleichen. Hemicellulasen und Laccasen werden seit Jahren zur Bleichung von Zellstoffen eingesetzt, um den Chlorverbrauch zu verringern und die Helligkeit der Pulpe zu erhöhen (Bajpai 1999; Paice et al. 1995). Ausgehend von ihrer Funktion Esterbindungen zu hydrolysieren, welche u. a. pflanzliche Zellwandpolysaccharide und Lignin verbinden, wird FAE ein großes Potential zur Delignifizierung von Biomasse zugeschrieben (Topakas et al. 2007). Record et al. (2003) zeigten die Anwendbarkeit der rekombinanten *A. niger* FAE (AnFaeA) zur Delignifizierung und Bleichung von Weizenstrohpulpe. Sie untersuchten nicht nur die individuelle Eignung der FAE, sondern betrachteten auch Kombinationen mit etablierten Enzymen dieses Industriesektors wie Laccasen und/oder Xylanasen. Eine effektive Delignifizierung der Pulpe (38 %) wurde bereits bei alleinigem Einsatz der FAE erzielt, obgleich die individuelle Behandlung mit einem Laccase-Mediatorsystem (44 %) oder Xylanase (40 %) effizienter war. Die höchste Delignifizierungsrate (74 %) lieferte ein bisequentieller Prozess, bestehend aus einer kombinierten FAE-Xylanase-Vorbehandlung gefolgt von einer Laccase-Behandlung. Im Vergleich dazu erzielten Herpoël et al. (2002) bei alleiniger Xylanase-Laccase-Behandlung nur Delignifizierungsraten von 60 %. Diese ersten Ergebnisse über den synergistischen Einsatz einer FAE zur enzymatischen Behandlung von Pulpe sind sehr erfolgsversprechend, da vergleichbare Delignifizierungsraten (75,5 %) nur mittels intensiver chemischer Behandlung (Hypochlorit und Schwefelsäure) erzielt werden (Herpoël et al. 2002).

1.2.4.2 Produktion von Bioethanol

Konventionelle Methoden zur Produktion von Bioethanol aus Cellulose und Hemicellulose umfassen eine saure oder enzymatische Hydrolyse der Biopolymere in lösliche Oligosaccharide gefolgt von einer Fermentation zu Ethanol. Wie in Kapitel 1.1.3 dargestellt, benötigt die effiziente Degradation pflanzlicher Biomasse ein Zusammenspiel von Enzymen mit unterschiedlichen Aktivitäten. Beim Abbau von Weizenstroh zur Bioethanolproduktion beschrieben Tabka et al. (2006) einen synergistischen Effekt von Cellulasen (10 U g^{-1}), Xylanasen (3 U g^{-1}) und FAE (10 U g^{-1}). Bei gleichzeitigem Einsatz der aufgeführten kritischen Enzymkonzentrationen betrug der prozentuale Anteil an freigesetzter Glucose 51,4 % (bei 50 °C und Zusatz von Tween 20). Ferner wurde bei Haferspelzen eine Steigerung der freigesetzten Menge an reduzierenden Zuckern (38,6 % auf 73,4 %) nach 24 h erreicht, wenn bei der Inkubation neben einer Cellulase (1024 U) zusätzlich eine *A. niger* FAE (13 mU) und eine *Trichoderma* Xylanase (256 U) eingesetzt wurden (Yu et al. 2003). Diese Studien verdeutlichen, dass durch das Zusammenspiel von cellulolytischen, xylanolytischen und FAE die Gesamtausbeute an freigesetzten reduzierenden Zuckern beim Abbau von lignocellulolytischer Biomasse erhöht wird. Bei der Produktion von Bioethanol wäre ein Vorbehandlungsschritt empfehlenswert, um die Polysaccharide von den aromatischen Verbindungen der pflanzlichen Zellwand zu trennen. Eine FAE-katalysierte Vorhydrolyse der Biomasse und anschließende Abtrennung der Hydroxyzimtsäuren aus dem Hydrolysat wäre aufgrund des großen industriellen Potentials dieser Substanzen ökonomisch wertvoll (vgl. Kapitel 1.1.3).

1.2.4.3 Hydrolyse und Synthese von bioaktiven phenolischen Verbindungen

Hydroxyzimtsäuren besitzen industrielles Potential aufgrund ihrer antimikrobiellen, UV-schützenden und antioxidativen Eigenschaften sowie ihrer Eignung als Aromapräkursoren (vgl. Kapitel 1.1.2). Beim Abbau pflanzlicher Biomasse spielen FAE als Hilfsenzyme von Glycosidasen zur Freisetzung von Polysacchariden für die Bioethanolproduktion sowie zur Generierung von Phenolsäuren als wertvolle Nebenprodukte eine wichtige Rolle. Generell setzen FAE allein weder große Mengen an Hydroxyzimtsäuren frei, noch bewirken sie signifikante Veränderungen in der Struktur oder Löslichkeit der Biomasse (Faulds 2010). Zahlreiche Studien veranschaulichen, dass FAE nur 3 – 13 % der Gesamtmenge an extrahierbarer FA aus Getreidereststoffen freizusetzen vermögen (Shin et al. 2006). Eine Erhöhung der Ausbeute an Hydroxyzimtsäuren kann durch synergistische Verwendung mit Hemicellulasen wie Xylanasen oder Arabinofuranosidasen erzielt werden. Faulds und

Williamson (1995) zeigten, dass eine 24-fache Steigerung der freigesetzten Menge an FA erreicht wurde, wenn die Weizenkleie zusätzlich zu AnFaeA aus *A. niger* mit einer Xylanase aus *T. viride* inkubiert wurde. Diese synergistische Wechselwirkung der zwei Enzymklassen bewirkte letztendlich eine FA-Ausbeute von 95 %.

Aufgrund der relativ geringen Löslichkeit von Hydroxyzimtsäuren in aprotischen Medien sind ihre Anwendungen in Öl-basierten Nahrungsmitteln und Kosmetika beschränkt. Eine Verstärkung der Hydrophobität kann durch Veresterung der Carbonsäurefunktion mit geeigneten Gruppen wie Glycerolen, Alkoholen oder Lipiden erreicht werden (Faulds 2010). Der Einsatz von Lipasen zur direkten Veresterung von Hydroxyzimtsäuren mit aliphatischen Alkoholen wurde in verschiedenen Studien beschrieben; die erzielten Reaktionsraten und Ausbeuten waren jedoch gering (Compton et al. 2000; Stamatis et al. 1999, 2001). Die Lipase von *Candida antarctica* katalysierte die Veresterung von aliphatischen Alkoholen zu Phenolsäureestern in protischen Lösungsmitteln mit sehr variablen Ausbeuten (3 – 98 %). Insbesondere für FA wurden nur sehr geringe Reaktionsausbeuten erzielt (2 % nach 3 Tagen (Guyot et al. 1997)). Demgegenüber gelang es Giuliani et al. (2001) mit einer FAE aus *A. niger*, Pentylferulat mit Ausbeuten von 50 – 60 % zu synthetisieren. Vafiadi et al. (2006) nutzen die Typ C FAE aus *T. stipitatus* (TsFaeC) erfolgreich zur Umesterung von Ferulasäuremethylester und L-Arabinose zu Feruloylarabinose. Außerdem wurde gezeigt, dass die StFaeC aus *S. thermophile* in der Lage war, L-Arabinose in einem ternären Wasser/*n*-Hexan/*t*-Butanol-System zu etwa 40 % in feruloylierte Derivate zu verestern (Topakas et al. 2005b). Die Synthese von feruloylierten Sacchariden birgt ein großes industrielles Potential, da diese Substanzen bis dato nicht kommerziell verfügbar sind. Ferner könnte die Verfügbarkeit eines Portfolios an feruloylierten Sacchariden zu einer Verfeinerung des Klassifizierungssystems von FAE führen, wie in Kapitel 1.2.2 aufgezeigt.

1.2.4.4 Landwirtschaft

Die Quervernetzung pflanzlicher Zellwände über Hydroxyzimtsäuren wird als Hauptfaktor für die schlechte Verdaulichkeit von Pflanzennahrung im Magen von Wiederkäuern angeführt (Yu et al. 2005a). Um die Nährstoffassimilation von Getreide und Gräsern zu erleichtern, wäre der Einsatz von FAE als Tierfutterzusatz denkbar. Buanafina et al. zeigten, dass durch die heterologe Produktion der FAE aus *A. niger* (AnFaeA) in den Futtergräsern *Lolium multiforum* (2006) und *Festuca arudinacea* (2008) das Feruloylierungsmuster und die Verzweigungen der pflanzlichen Zellwände verändert wurden, was eine verbesserte Verdaulichkeit des Pflanzenmaterials für Wiederkäuer zur Folge hatte. Ferner erzielte eine Inkubation von

Haferschalen mit einem Multi-Enzym-Cocktail, bestehend aus einer gereinigten *A. niger* FAE sowie Xylanase, Cellulase, β-Glucanase, Endoglucanase I und II, eine Verbesserung der Zellwandverdaulichkeit (86 %) und des Futterabbaus im Pansen *in vitro* (Yu et al. 2005b).
Bei der Silierung von Tierfutter, einem anaeroben Prozess zur Konservierung und Verbesserung der Verwertbarkeit von Futtermitteln für Wiederkäuer, spielen FAE ebenfalls eine Rolle. Viele silierende Milchsäurebakterien produzieren FAE, und diese Stämme erhöhen *in situ* wesentlich den Pansenfaserabbau und führen dementsprechend zu einer verbesserten Verdaulichkeit der Futtermittel für die Nutztiere (Nsereko et al. 2008).

1.2.4.5 Backwarenindustrie

Ein zentraler Angriffspunkt zur Veränderung von rheologischen Eigenschaften der Teige sind die teilweise mit FA veresterten AX. Die FA-Reste sind involviert in die oxidative Vernetzung von AX miteinander als auch von AX mit Glutenproteinen (vgl. Kapitel 6); dies erfolgt sowohl über oxidative Verknüpfung von FA-Resten miteinander als auch von FA-Resten mit Tyrosin- oder Cysteinresten der Glutenproteine (Hilhorst et al. 2002). Im Verlauf dieser Reaktion bildet sich ein elastisches Gel, welches das Glutennetzwerk stabilisiert, das Wasserbindevermögen der AX signifikant erhöht und letztendlich in einer reduzierten Teigklebrigkeit resultiert (Hilhorst et al. 2002; Mutsaers 1997). Der Vernetzungsgrad der AX korreliert dabei mit den Gehalt an veresterter FA und ist ausschlaggebend für die Dehnbarkeit der Teige. Im Verlauf der FAE-katalysierten Hydrolyse der veresterten FA-Reste wird die Teigviskosität reduziert, da die verbleibenden veresterten FA-Reste nicht nur mit anderen veresterten FA-Resten Verknüpfungen eingehen können, sondern auch mit freien FA-Resten. Dementsprechend können FAE eingesetzt werden, um den Vernetzungsgrad der Makromoleküle des Mehls zu reduzieren (Wang et al. 2002). Dabei kann der Einsatz von FAE, abhängig von dem Ausmaß und der Position der hydrolysierten Verknüpfungen, positive oder negative Effekte auf die Teigrheologie haben. Bis dato existiert nur eine Veröffentlichung über den Einsatz von FAE in der Backwarenindustrie (Crepin 2003). Crepin (2003) berichtete, dass der Zusatz der Typ C FAE aus *T. stipitatus* (TsFaeC; 6,5 mU – 6,5 U) zum Teig in einem erhöhten Brotvolumen, einer verbesserten Krumenweichheit und Krustenfarbe resultierte. Die Auswahl der Typ C FAE für die Backversuche basierte auf Vorversuchen, in denen diese FAE im Vergleich zu der Typ A FAE aus *A. niger* (AnFaeA) und der Typ B FAE aus *Neurospora crassa* (NcFae-1) den signifikantesten Effekt verursachte.

1.3 Zielsetzung der Arbeit

Aufgrund der Vielzahl von potentiellen Anwendungen ist das Interesse an FAE in der Lebensmittel- und Pharmaindustrie in den letzten Jahren bemerkenswert gestiegen (vgl. Kapitel 1.2.3). Zahlreiche Mitglieder dieser Enzymfamilie sind patentiert worden; im Jahr 2008 lagen in Patentdatenbanken und Patentanmeldungen 165 Treffer für FAE vor (Fazary & Ju 2008). Dennoch war zu Beginn der eigenen Arbeiten keine FAE kommerziell verfügbar. Seit der Beschreibung der ersten FAE-Aktivität im Jahr 1987 (MacKenzie et al. 1987), wurden viele FAE in Pilzen und Bakterien identifiziert, welche sich jedoch auf wenige Gattungen von Mikroorganismen wie *Streptomyces, Bacillus, Penicillium, Aspergillus* und *Fusarium* konzentrieren.

Nachdem in Vorarbeiten (Haase-Aschoff et al. 2013a) das Potential von Basidiomycota zur Produktion von diversen FAE aufgezeigt wurde, lag der Fokus dieser Arbeit auf der Identifizierung und Isolierung neuartiger FAE aus Basidiomycota. Nach der Entwicklung einer Proteinreinigungsstrategie sollten die elektrophoretisch reinen FAE sowohl biochemisch als auch molekularbiologisch charakterisiert werden. Ferner galt es, die Eignung der basidiomycetischen FAE für industrielle Applikationen, wie der Freisetzung von Hydroxyzimtsäurederivaten aus Nebenströmen der Lebensmittelindustrie als auch der Verbesserung von Weizenmehlteigen, zu analysieren. Um ausreichende Mengen an reinen FAE für Applikationsstudien bereitzustellen, stellte die heterologe Produktion der neuen FAE einen unabdingbaren weiteren Schwerpunkt der vorliegenden Arbeit dar.

2. Vorwort zur Publikation „ A halotolerant type A feruloyl esterase from *Pleurotus eryngii*"

Zu Beginn der Arbeiten war eine Vielzahl von identifizierten FAE aus Bakterien, Pflanzen und Ascomycota in der Literatur beschrieben (Fazary & Ju 2007), während aus der großen Gruppe der Basidiomycota lediglich zwei Enzyme biochemisch charakterisiert vorlagen (Hashimoto et al. 2010; Linke et al. 2013). Ferner existierte eine Veröffentlichung von MacKenzie und Bilous (1988) über die partielle Aufreinigung einer FAE aus dem Kulturüberstand von *Schizophyllum commune*.

Diese Kenntnislücke über basidiomycetische FAE war insofern erstaunlich, als das typische Habitat der Basidiomycota ferulasäurereiche Materialien wie Holz und Laub bilden. Zu diesem Defizit beigetragen haben mag die Tatsache, dass Laccasen, Enzyme der Klasse der Oxidoreduktasen, die insbesondere von Braunfäule- und Weißfäulepilzen produziert werden, FA und deren Ester polymerisieren (Dwivedi et al. 2011; Eastwood et al. 2011; Knezevic et al. 2013; Martinez et al. 2009). Dementsprechend versagen die üblichen FAE-Assays, da die Produkte der enzymatischen Hydrolyse bereits vor der Detektion nicht mehr vorliegen. Um die Problematik der Beeinträchtigung der Detektion von FAE-Aktivität in Gegenwart von Laccasen zu begegnen, wurde ein neuartiger FAE-Assay entwickelt (Haase-Aschoff et al. 2013a). Mithilfe dieser Methode wurden Kulturüberstände von 41 Basidiomycota hinsichtlich ihrer FAE- und Laccase-Aktivität untersucht. Dabei wurde für mehr als die Hälfte der Organismen (61 %) eine FAE-Aktivität detektiert, womit ferner das Potential der Basidiomycota als Produzent FA-Ester-spaltender Enzyme bestätigt wurde. Aus den Vorarbeiten von Haase-Aschoff et al. (2013a) ging *Pleurotus eryngii* aufgrund der hohen hydrolytischen Aktivität gegenüber FA-ME als vielversprechender FAE-Produzent hervor.

Die nachfolgende Publikation beschreibt die Reinigung und biochemische Charakterisierung einer FAE (PeFaeA) aus dem Kulturüberstand von *P. eryngii*. Mit dem isolierten Enzym wurde ein umfassendes Substratscreening durchgeführt. Hierbei wurden nicht nur herkömmliche artifizielle Zimtsäure- und Benzoesäure-Derivate, sondern auch natürliche Substrate wie Chlorogensäure und feruloylierte Saccharide eingesetzt. Ferner wurde die Aktivität der PeFaeA gegenüber Weizenkleie als komplexes natürliches Substrat überprüft, um die Anwendbarkeit des Enzyms für biotechnologische Prozesse, wie beispielsweise der Freisetzung von Ferulasäure aus industriellen Nebenströmen, zu bewerten.

3. A halotolerant type A feruloyl esterase from *Pleurotus eryngii*

Highlights

- A new feruloyl esterase from the basidiomycete *P. eryngii* was characterized
- A catalytic triad of Ser-Glu-His makes the enzyme unique
- The enzyme possessed a broad tolerance towards metal ions and organic solvents
- Increased activity in the presence of 3 M NaCl was observed
- Ferulic acid was released from destarched wheat bran and feruloylated saccharides

Abstract

An extracellular feruloyl esterase (PeFaeA) from the culture supernatant of *Pleurotus eryngii* was purified to homogeneity using cation exchange, hydrophobic interaction, and size exclusion chromatography. The length of the complete coding sequence of PeFaeA was determined to 1668 bp corresponding to a protein of 555 amino acids. The catalytic triad of Ser-Glu-His demonstrated the uniqueness of the enzyme compared to previously published FAEs. The purified PeFaeA was a monomer with an estimated molecular mass of 67 kDa. Maximum feruloyl esterase (FAE) activity was observed at pH 5.0 and 50 °C, respectively. Metal ions (5 mM), except Hg^{2+}, had no significant influence on the enzyme activity. Substrate specificity profiling characterized the enzyme as a type A FAE preferring bulky natural substrates, such as feruloylated saccharides, rather than small synthetic ones. K_m and k_{cat} of the purified enzyme for methyl ferulate were 0.15 mM and 0.85 s^{-1}. In the presence of 3 M NaCl activity of the enzyme increased by 28 %. PeFaeA alone released only little ferulic acid from destarched wheat bran (DSWB), whereas after addition of *Trichoderma viride* xylanase the concentration increased more than 20-fold.

Keywords

Fungi; basidiomycete; feruloyl esterase; halotolerance; feruloyl saccharides

3.1 Introduction

The carbohydrate moieties of plant cell walls constitute the largest source of renewable chemical energy on earth. Many basidiomycetes thrive on such substrates, because they produce a complex set of degrading enzymes (Fazary & Ju 2007; Mandalari et al. 2008). Thus, a strong motivation for the interest into the enzymes of xylotrophic fungi is the increasing importance of biofuels. Complete hydrolysis of the hemicellulose fraction to fermentable saccharides requires (1) endoxylanases and β-xylosidases, which cleave the xylan main chain, and (2) accessory enzymes, such as α-arabinofuranosidases and esterases. Feruloyl esterases (FAE; EC 3.1.1.73), a subclass of the carboxylic ester hydrolases, play a key role among the accessory enzymes. Also known as ferulic acid, cinnamic acid, or cinnamoyl esterases, they are characterized by an α/β-hydrolase structure and, usually, a Ser-Asp-His catalytic triad (Nardini & Dijkstra 1999; Udatha et al. 2011). Their substrates are the ester linkages between ferulic acid or related cinnamic acids and plant cell wall polysaccharides (Williamson et al. 1998). Hydrolysis may result in either the liberation of short phenylpropanoid side chains, or in the removal of oligoferuloyl-crosslinks between adjacent hemicelluloses chains (Wong 2006). The first reaction yields phenolic compounds with application potential as antioxidants and preservatives in the food, health, cosmetic, and pharmaceutical industries (Kroon et al. 1999). The second facilitates the accessibility of the polysaccharides for other hydrolases and is thus of interest for a more efficient utilization of biomass. The industrial attractiveness of FAEs is amplified by a broad substrate profile, good stereospecificity, and high operational stability (Bornscheuer 2001). To date, more than 50 FAEs with different molecular mass, substrate preference, isoelectric point, and optimum reaction conditions were characterized from fungi and bacteria (Topakas et al. 2007; Topakas et al. 2012). Moukouli et al. (2008) concluded that the missing correlation between the physicochemical characteristics of the FAEs and their optimal reaction conditions required the biochemical characterization of more FAEs in relation to their amino acid sequences. In contrast to cellulolytic ascomycetes, however, there is a noticeable lack of knowledge on FAEs from basidiomycetes. Looking at their ecological niche this is remarkable. Haase-Aschoff et al. (2013a) revealed that the frequent presence of interfering laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) explained the analytical problems. High laccase activities are often found in white-rot fungi (Dwivedi et al. 2011), and this also applies to *Pleurotus eryngii* (Knezevic et al. 2013), the object of the present study. Laccases disturb the common FAE assay by polymerization of the product ferulic acid as well as the substrate(s) (ferulic acid ester derivatives): as a result, FAE activity remains hidden. To

reliably detect an FAE activity, laccases would have to be perfectly removed, an unrealizable demand in practical laboratory work. By developing a novel assay for FAEs the detection problem was eventually overcome (Haase-Aschoff et al. 2013a). This paper describes the purification and biochemical characterization of an FAE from *P. eryngii* (PeFaeA). The inhibitory effect of solvents and other agents was tested. The ability of the enzyme to release ferulic acid from the natural complex substrate destarched wheat bran (DSWB), both in absence or presence of a *Trichoderma viride* xylanase was investigated. Finally, the full sequence of PeFaeA was amplified and the amino acid residues responsible for the catalytic activity of the protein were identified.

3.2 Material and methods

3.2.1 Chemicals and substrates

All chemicals used had highest purity grade and were from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), and Sigma-Aldrich (Taufkirchen, Germany). Ferulic acid methyl and ethyl esters were obtained from Alfa Aesar (99 %, Karlsruhe, Germany). Methyl caffeate, coumarate, and sinapate were synthesized according to Borneman et al. (1990). Feruloylated saccharides (5-O-transferuloyl-L-arabinofuranose (F-A); *β*-D-xylopyranosyl-(1→2)-5-O-trans-feruloyl-L-arabinofuranose (F-AX); *α*-L-galactopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-5-O-trans-feruloyl-L-arabinofuranose (F-AXG)) prepared from destarched corn bran were kindly supplied by M. Bunzel (Institut für Angewandte Biowissenschaften, KIT, Karlsruhe, Germany). The esterase substrates were purified and identified as described (Allerdings et al. 2006; Linke et al. 2013). PCR primers were ordered from Eurofins MWG Operon (Ebersberg, Germany).

3.2.2 Cultivation of *Pleurotus eryngii*

The basidiomycete *P. eryngii* used in this study was from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany; No. 613.91). The strain was grown on standard nutrition liquid (SNL)-agar and maintained at 4 °C. SNL medium was 30.0 g L^{-1} D-(+)-glucose monohydrate, 4.5 g L^{-1} L-asparagine monohydrate, 3.0 g L^{-1} yeast extract, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$; 1.0 mL L^{-1} trace element solution (0.08 g L^{-1} $FeCl_3$ x 6 H_2O, 0.09 g L^{-1} $ZnSO_4$ x 7 H_2O, 0.03 g L^{-1} $MnSO_4$ x H_2O, 0.005 g L^{-1} $CuSO_4$ x 5 H_2O, 0.4 g L^{-1} EDTA); the pH was adjusted to 6.0 with 1 M NaOH prior to autoclaving. For SNL-agar plates, 15 g L^{-1} agar was added to SNL.

Main cultures of *P. eryngii* were cultivated in SNL with a reduced level of glucose (10 g L^{-1}) supplemented with 0.4 % (v/v) Tween 80 as effector for FAE production (Linke et al. 2005). After 13 d of cultivation at 24 °C and 150 rpm on a rotary shaker (Infors, Bottmingen, Switzerland) cultures were harvested and stored until further processing at -20 °C.

3.2.3 Purification of PeFaeA

Culture supernatant of *P. eryngii* was separated from the frozen cells by centrifugation at 5000 x g followed by filtration (filter paper, Schleicher und Schuell, Dassel, Germany). One litre supernatant was diluted one time with buffer A (36.5 mM acetate buffer pH 4.0) and applied on a 25 mL self-casted SP Sepharose FF column (XK 16/20, GE Healthcare) preequilibrated with buffer A. Fractions of 6.0 mL were collected at a flow rate of 4 mL min^{-1}. FAE was eluted with 20 % buffer B (buffer A with 1 M NaCl). FAE active fractions were pooled, concentrated, desalted using an ultrafiltration module (10 kDa cut off, Polyethersulfone (PES), Sartorius, Goettingen, Germany) and 5 mL of the concentrate (one time diluted with buffer A) was loaded on a HiTrap SP XL column (1 mL, GE Healthcare) preequilibrated with buffer A. Elution of FAE occurred in the range of 10 – 20 % buffer B at a flow rate of 1 mL min^{-1}. The collected 18 mL of FAE active fractions were pooled and concentrated, as described above. Following 500 µL of concentrate was diluted one time with buffer (50 mM Bis-Tris pH 6.5 with 2 M $(NH_4)_2SO_4$) and applied on a HiTrap Butyl FF preequilibrated with buffer C (50 mM Bis-Tris pH 6.5 with 1 M $(NH_4)_2SO_4$). FAE eluted by a linear gradient of 60 – 90 % of 50 mM Bis-Tris buffer pH 6.5 at a flow rate of 1 mL min^{-1}. Fractions containing FAE activity (10 mL) were pooled, concentrated, desalted using an ultrafiltration module (10 kDa cut off, PES, Sartorius), and 500 µL sample was applied on a Superdex 75 column (10/300 GL, GE Healthcare) preequilibrated with buffer containing 50 mM Bis-Tris pH 6.5 and 100 mM NaCl. Proteins were separated with a flow rate of 0.4 mL min^{-1}.

3.2.4 SDS-PAGE

Enzyme purity and molecular mass were determined after each purification step using SDS-PAGE (12 % resolving gel, 4 % stacking gel) according to Laemmli (1970). Samples were diluted in denaturing SDS sample buffer (0.1 M Tris-HCl pH 6.8, 0.2 M DTT, 4 % SDS, 20 % glycerol, 0.2 % bromophenol blue) and boiled for 10 min. After electrophoresis at 15 mA per gel, gels were stained with either silver or InstantBlue (Expedeon, Cambridgeshire, Great Britain). Laccase activity staining was performed after seminative SDS-PAGE directly on the gel using an overlay of 2,2′-Azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS). For

the seminative SDS-PAGE, samples were diluted with SDS sample buffer (as described above, but without DTT) without boiling. After the run, the gel was washed with Triton X-100 (2.5 %) for 1 min and subsequently incubated with a solution of 5 mM ABTS in 200 mM sodium tartrate buffer pH 4.5.

3.2.5 Peptide mass fingerprint

Partial sequences of the PeFaeA were obtained by ESI-MS/MS. An Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) with electrospray ionization was used as described by Linke et al. (2013). The amino acid sequences received were investigated for homology in the protein database using a Blast search.

3.2.6 cDNA synthesis

Isolation of total RNA from mycelium of *Pleurotus eryngii* grown for 13 d in SNL supplemented with 0.4 % (v/v) Tween 80 and ss cDNA synthesis were performed as described by Linke et al. (2013).

3.2.7 Sequence fishing

Degenerated primers Per1_fw (5´-AGYTTRCCCTTTGCSGARCCWCCA-3´) and Per8_rev (5´-GAAGGTCGTNCCCTCATCAAGAAC-3´) were deduced from partial amino acid sequences obtained from ESI-MS/MS; wobbled bases were encoded as follows: Y= C/T, R= A/G, W= A/T, S= G/C and N= A/G/C/T. PCRs were performed by mixing 10 µL 5x Phusion High-Fidelity DNA Polymerase buffer, 0.4 µL dNTP mix (10 mM each), 25 pmol forward primer, 25 pmol reverse primer, 0.02 U Phusion High-Fidelity DNA Polymerase (Fermentas, St. Leon-Roth, Germany), 1 µL ss cDNA, and ddH_2O to 50 µL. Gradient PCR was performed in a MasterCycler gradient (Eppendorf, Hamburg, Germany) by incubating the reaction mixture at 98 °C for 2 min, then for 35 cycles at 98 °C for 30 s, (55 – 65 °C) for 30 s and 72 °C for 60 s. Final elongation was performed at 72 °C for 10 min. PCR products were analyzed using agarose gel electrophoresis [1.5 % agarose (Serva, Heidelberg, Germany), boiled in TAE-buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8)]. DNA was extracted from agarose gels with the NucleoSpin Extract II Kit (Macherey & Nagel, Düren, Germany). DNA fragments were ligated into the *Eco*RV digested pUC57-vector (Thermo Scientific, St. Leon-Roth, Germany) by mixing 1 µL vector, 1 µL 10× T4 DNA Ligase buffer, 5 U T4 DNA Ligase and 17 µL Insert-DNA. The reaction mixture was incubated at 4 °C overnight. For transformation, the whole ligation reaction was added to 50 µL chemically competent *Escherichia coli* TOP10 (Invitrogen, Darmstadt, Germany), incubated on ice for 20 min, heat shocked at 42 °C for 90 s,

and transferred back on ice. In the next step, one milliliter of LB medium (Carl Roth) was added immediately. After incubating (200 rpm, 37 °C, 60 min) cells were plated out on LB agar containing 50 µg mL^{-1} ampicillin and 20 µg mL^{-1} X-Gal (Roth). Inoculated plates were incubated at 37 °C overnight. Positive clones were selected by colony PCR as described by Linke et al. (2013). Plasmid DNA was isolated with the NucleoSpin Plasmid DNA Kit (Macherey & Nagel). Sequencing was performed by Seqlab (Goettingen, Germany). In order to complete the sequence, 3´-RACE and PCR with untranslated region (UTR) primers, which were derived from the genome of *P. ostreatus* PC15 v2.0, was performed using a proof reading Pfu DNA Polymerase (Fermentas).

3.2.8 Activity assays

The FAE activity was determined by HPLC using methyl ferulate as standard substrate. Besides methyl ferulate, the hydrolysis of other benzoic and cinnamic acid ester derivatives as well as feruloylated saccharides was investigated by mixing 1 mM substrate and 50 mM acetate buffer pH 5.0. For laccase inhibition 1 mM of sodium azide was added additionally to the reaction mixture. After incubation for 1 h at 37 °C the reaction was terminated by doubling the reacting volume with methanol. Samples were centrifuged before injection to the HPLC. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of free acid per minute under the specified conditions. All measurements were performed at least twice.

Laccase activity was quantified at 420 nm in a microtiter plate (ε_{420}= 36 000 M^{-1} cm^{-1}) using ABTS as substrate. The reaction mixture contained 0.5 mM ABTS in 200 mM sodium tartrate buffer pH 4.5 and 15 µL sample in a total volume of 300 µL. The increase of absorbance was monitored over 20 min in a Synergy 2 microplate reader (BioTek, Bad Friedrichshall, Germany). One unit of enzyme activity was defined as 1 µmol of substrate oxidized per minute under the experimental conditions.

3.2.9 HPLC

HPLC was performed using a Chromolith Performance RP-18e reverse-phase column (Merck, Darmstadt, Germany) with the dimension of 100 x 4.6 mm. Substrates and products were separated using a step-wise gradient at a flow velocity of 1.5 mL min^{-1}: 10 µL sample loaded in 90 % buffer A (0.1 % formic acid), 10 % buffer B (acetonitrile); 10 to 36 % buffer B in 3.5 min; 36 to 66 % buffer B in 1.5 min; 66 to 100 % buffer B in 0.5 min; 100 % buffer B for 1.5 min; 100 to 10 % buffer B in 0.5 min; re-equilibrate with 10 % buffer B for 2 min; total run time 9.5 min. Substance elution was detected at 275 nm (cinnamic and gallic acid), 323 nm

(ferulic, caffeic, chlorogenic and sinapic acid), 232 nm (benzoic and 3-hydroxybenzoic acid), 254 nm (4-hydroxybenzoic and vanillic acid) and 306 nm (coumaric acid) using a Shimadzu UV-Vis detector (SPD-10A VP, Shimadzu Deutschland GmbH, Duisburg, Germany).

3.2.10 Preparative isoelectric focussing (IEF)

Preparative IEF was carried out to determine the pI of the purified enzyme. The sample was mixed with 0.5 % ampholytes (pH 3.0 – 10.0, SERVA, Heidelberg, Germany) and loaded into the system (Rotofor Cell, Biorad, Hercules, USA). IEF was performed at 12 W constant power for 4 – 5 hours at 4 °C. After the run 20 fractions were analyzed for FAE activity and pH values.

3.2.11 Effects of pH, temperature, solvents, and inhibitors on enzyme activity

For the determination of FAE activity methyl ferulate was used as substrate. A stock solution of the substrate (3 mM) was prepared in 10 % (v/v) DMSO. Twenty-five microliters of the purified enzyme sample were mixed with 65 µL of substrate and 10 µL of 500 mM buffer. Afterwards the mix was incubated for 60 min at 37 °C. The reaction was terminated by addition of 50 % methanol (v/v). Subsequently, the hydrolysis of the substrate methyl ferulate was analyzed using HPLC-UV. For all following measurements controls were carried out under identical conditions but without organic solvents, potential inhibitors or enzyme, respectively. The activity of PeFaeA in the absence of organic solvents, metal ions and inhibitors was set to 100 %.

The pH optimum of the enzyme was determined at 37 °C by varying the pH of the reaction mixture in the range from 3.0 to 9.0. The following buffer systems were used: 50 mM citrate (3.0 – 3.5), acetate (4.0 – 5.5), Bis-Tris (5.5 – 7.0) or Tris buffer (7.0 – 9.0). All further experiments were performed in 50 mM acetate buffer pH 5.0.

For the determination of the temperature optimum the activity assay was performed for 60 min from 20 °C to 60 °C.

The temperature stability of the enzyme was analyzed as follows: 25 µL of enzyme sample was incubated for 60 min at temperatures ranging from 20 °C to 60 °C. Afterwards the substrate methyl ferulate and 50 mM acetate buffer pH 5.0 were added, the reaction mixture was incubated for 60 min at 37 °C.

Enzyme tolerance to different solvents was investigated in the presence of 50 % (v/v) acetonitrile, ethanol, methanol, *n*-hexane, *n*-heptane, toluene, acetone, diethyl ether, DMSO, DMF, isobutyl alcohol, or *tert*-butyl methyl ether, respectively, in 50 mM acetate buffer pH 5.0 containing 0.7 mM methyl ferulate. After 60 min of incubation at 37 °C and 900 rpm, the mixture was applied for HPLC analysis as described above.

To study inhibition, the enzyme solution was incubated with different inhibitors, such as phenylmethylsulfonylfluoride (PMSF), SDS, EDTA and various metal ions, such as K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, Fe^{2+}, Fe^+, Cu^{2+}, Li^+ and Ni^{2+}. Apart from PMSF (1 mM) all other potential inhibitors were added at a concentration of 5 mM. The effect of high salt concentrations (0.1 – 3 M NaCl) on FAE activity was investigated too.

3.2.12 Substrate specificity and kinetic parameters

Substrate specificity for the PeFaeA against different cinnamic- and benzoic acid esters as well as natural substrates, such as F-A, F-AX, and F-AXG was performed by replacing methyl ferulate by the different substrates. Three mM stock solutions of all substrates were prepared in water. Kinetic parameters were determined from hydrolysis rates of 1 µM – 1 mM methyl ferulate and F-AX (at 37 °C, pH 5.0, 1 h). K_m and v_{max} values were calculated with nonlinear regression using SigmaPlot 10.0 (Systat Software Inc., Chicago, USA). Blanks were carried out for all assays using water instead of enzyme. For calculation of k_{cat} the molecular mass was determined using denaturing SDS-PAGE.

3.2.13 Release of ferulic acid from DSWB

DSWB was prepared as described by Johnson et al. (1988). The ability of the purified PeFaeA to liberate ferulic acid from DSWB as natural substrate was investigated with both the esterase and in combination with *Trichoderma viride* xylanase (2.3 U mg^{-1}, Fluka, Buchs, Switzerland). For the experiment 200 µL of a DSWB-water mixture (3 g of DSWB in 50 mL ddH_2O) was incubated with 100 µL of PeFaeA (10 mg mL^{-1}), xylanase (100 mg mL^{-1}) or both in 50 mM acetate buffer pH 5.0 (37 °C, 650 rpm, 16 h). Additionally, 1 mM sodium azide was added to the reaction mixture. Afterwards the amount of released ferulic acid was quantified using HPLC.

3.3 Results and discussion

3.3.1 Purification of PeFaeA

The FAE activity of the culture supernatant of *Pleurotus eryngii* increased with cultivation time and reached a maximum activity on the 13th day. The culture was harvested and biomass separated by filtration. PeFaeA was purified from the culture supernatant using cation exchange, hydrophobic interaction (HIC), and size exclusion chromatography (SEC). Cation exchange chromatography fractions showing FAE activity exhibited also high laccase activity in the ABTS assay. Laccase activity staining on a seminative SDS-PAGE gel showed that the

pooled fractions of the first purification step contained at least three laccases. Neither preparative IEF nor size exclusion chromatography separated this enzyme from PeFaeA. By using a HiTrap Butyl FF column for hydrophobic interaction chromatography the laccases passed through the column without binding, while the esterase eluted in the range from 60 to 90 % of elution buffer. Ninety-nine percentage of the total laccase activity was eventually removed. After the final purification step, the enzyme showed electrophoretical purity and a recovery of 1.5 % with a specific FAE activity of 0.6 U mg^{-1}. The purified FAE gave a single band on a 12 % SDS-PAGE gel with a molecular mass of 67 kDa (**Fig. 3-1**).

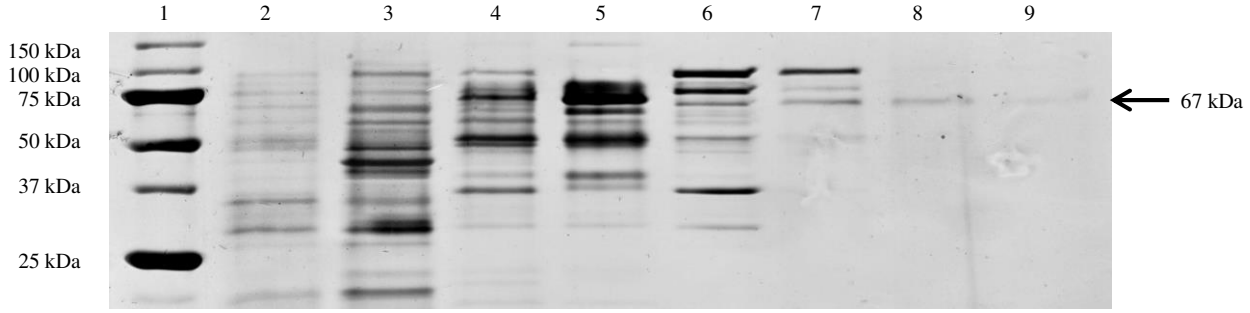


Fig. 3-1 Purification of the FAE (PeFaeA) from *P. eryngii* (silver stained SDS-PAGE gel). Precision plus Protein™ Standard (1), culture supernatant (2), flowthrough SP Sepharose FF (3), FAE active SP Sepharose FF fractions that were applied to SP XL (4), flowthrough HIC (5), HIC fraction with highest FAE activity (6), FAE active SEC fractions (7 – 9).

The molecular mass of the purified protein estimated by size exclusion chromatography suggested that the PeFaeA was a monomer. The molecular masses of FAEs known to date range from 11 to 210 kDa (Koseki et al. 2009a). Most of them possess a molecular mass of 30 – 60 kDa. However, the molecular mass of all published type A FAEs is much lower than the 67 kDa of the PeFaeA, for example *Aspergillus awamori* (35 kDa) (Koseki et al. 1998), *Aspergillus niger* (36 kDa) (de Vries et al. 1997; 2002), *Fusarium oxysporum* (27 kDa) (Topakas et al. 2003a), *Talaromyces stipitatus* (35 kDa) (Garcia-Conesa et al. 2004), and *Pleurotus sapidus* (55 kDa) (Linke et al. 2013). The pI of the enzyme was estimated by IEF to be 5.2.

3.3.2 Sequence analysis of the PeFaeA

By means of ESI-MS/MS 12 tryptic peptides were identified (**Tab. 3-1**). Homology search with Blastp (NCBI Blast) identified the enzyme as member of the Esterase_Lipase superfamily and showed a maximum identity of 78 – 100 % with the Est1 of *Pleurotus sapidus* (GenBank accession no. CBE71381.1) for all 12 peptides.

Tab. 3-1 Sequence of 12 peptide fragments deduced from MS spectra of PeFaeA**;** in total 199 amino acids (aa).

Peptide fragment		Amino acid sequence [a]
1	(12 aa)	GTPVVFVSLNYR
2	(18 aa)	LPFLAGTVLDEGTTFTPK
3	(19 aa)	DVPQCASTAGSKDTFSCLR
4	(24 aa)	SIPEFGQELFGGLPFAEPPVGQLR
5	(15 aa)	QAPQVQVGNTALLGR
6	(32 aa)	AGSLADDQSGELFAWDPTLDGPGGLLPDLPSK
7	(15 aa)	RAAALFGDVSFQSQR
8	(20 aa)	AALFESGFTATSLNFPASHR
9	(15 aa)	LGPLGFPQGVEAQKR
10	(11 aa)	SDSIDEATLLK
11	(9 aa)	FLTTEDQLR
12	(9 aa)	EQSWANFVK

[a] Leucine and isoleucine were not distinguishable via ESI-MS/MS technique.

A Blast search using the Est1 DNA sequence was performed within the fully sequenced genome of *Pleurotus ostreatus* PC15 v2.0. The matching sequence (protein ID 1033001) was then used to design the UTR primers. The determination of the codon usage of *Pleurotus* sp. and the deduction of degenerated primers to fish the sequence of PeFaeA were also facilitated. Using the degenerated primers Per1_fw and Per8_rev, which were derived from the identified tryptic peptides P4 and P2, resulted in the amplification of an 864 bp product. The deduced amino acid (aa) sequence of the amplified PCR product contained the sequence of the identified tryptic peptides P1, P3, P5, P6, P8, P9, P10, and P12. 3'-RACE and PCR with UTR primers resulted in the amplification of the full sequence of PeFaeA. The length of the complete coding sequence of PeFaeA was 1668 bp corresponding to a protein of 555 aa (GenBank accession no.CDI44666.1). The full aa sequence of PeFaeA possessed highest similarity (93 %) and identity (87 %) to Est1 sequence. In **Fig. 3-2** PeFaeA was aligned with Est1.

```
PeFaeA    MAILRGVATA LALLPFIAAQ APQVQVGNTA VIGRSIPEFG QELFGGIPFA EPPVGQLRLS NPVLKTRLGT PTFDASNFGP ACLQS-ASVP LMSEDCLRIN
Est1 Psa  .VL..A..A. .......... .......... I........S K.F....... .........N ........D. .....G.Y.. ....PDP... VI........
Identity  *.:**.**:* ********** ********** :********. :*:******* *********. ********.* *****.*:** ****. .*** ::********

PeFaeA    VLRPAGIPTG VVLPVMAWVY GGGFDFGDSS IYNASAIVAQ SVIRGTPVVF VSLNYRLGPL GFPQGVEAQK RGALNLGLKD QLAALEWVQA NIGLFGGDKS
Est1 Psa  ...T..V.A. .L........ .......... .......... .AL....... .......... ...K.Q..HD .......... .......... ...F......
Identity  ***.**:*:* *:******** ********** ********** *.:******* ********** ***:* **:. ********** ********** ***:******

PeFaeA    KVTIFGQSAG SISLSILFLN SNIKRLARAA IFESGFTATS LNFPASHREQ SWANFVKDVP QCASTAGSKD TFSCLRSDSI DEATLLKAGS LADDQSGELF
Est1 Psa  ...V...▲.. ..C...H... .P.QS..... ......P... Q....A...K ..T.....I. .......... ........T. .Q....Q..A ..........
Identity  ***:****** **.*** *** * *: ***** ******.*** ****:***: **:*****:* ********** ********:* *:****:**: **********

PeFaeA    AWDPTIDGPG GILPDIPSKL LARGQFARLP FIAGTVLDEG TTFTPKFITT EDQIRQSIIA NFTPSPFGPA VLAKSAETIL QLYPDVPALG SPFGTGNETF
Est1 Psa  .......... .......... ..K.K.V... .........▲ ......TV.. .......L.. .........T ....DV.... K....I.... ..........
Identity  ********** ********** **:*:*.*** *********. ****** :** *******:** *********: ****..**** :****:**** **********

PeFaeA    GLSSQYKRAA AIFGDVSFQS QRRFWIQTLS KAGLKTFGYL FADPQSSDPV NGVPHASEIP YVYGALGILG GTVTPQALAL SRIMVDYWVS FATSLDPNDG
Est1 Psa  ........T. .....L.... ...S...... ...V...... .T.....N.A ...S▲..... .....P..F. .....E.I.. .......... ..........
Identity  ********:* *****:**** *** ****** ***:****** *:*****:*. ***.****** ***** **:* *****:*:** ********** **********

PeFaeA    KGLPRPLWTQ YTPSNQAIML LNSTGTTMIP DDYRKKQIDF INSNPAVWHH RRSFST
Est1 Psa  ..F...V... .......... ......S... .......... .......... ....--
Identity  **:***:*** ********** ******:*** ********** ********** ****
```

Fig. 3-2 Sequence alignment of *P. eryngii* PeFaeA (present work, GenBank accession no. CDI44666.1) and *P. sapidus* Est1 (GenBank accession no. CBE71381.1). Peptide fragments identified by ESI-MS/MS analysis were underlined. The catalytic triad of serine, glutamic acid, and histidine was marked by triangles.

Based upon prediction using the web-based program SignalP 4.01, a cleavage site IAA-QA between aa 19 and 20 was identified indicating a 19 aa signal peptide at the N-terminus of the deduced amino acid sequence of PeFaeA. The predicted molecular mass of the mature protein was calculated to be 59.2 kDa (http://web.expasy.org/compute_pi/). The difference to the molecular mass of 67 kDa deduced from SDS-PAGE analysis can be explained by a high degree of glycosylation. Four potential *N*-glycosylation sites were identified at positions 132, 360, 396, and 521 (http://www.cbs.dtu.dk/services/NetNGlyc/). ESI-MS/MS definitely confirmed the presence of at least one glycopeptide with mainly high mannose type glycans. Furthermore, the enzyme had a G-X-S-X-G motive characteristic of the serine esterase family (Brenner 1988; Dodson & Wlodawer 1998). While the motive G-Q-S-A-G was the same for Est1, other known type A FAEs possess G-H-S-L-G, such as those from *Aspergillus awamori* and *Aspergillus niger*. The active sites were predicted as Ser207, Glu338, and His454 (http://pfam.sanger.ac.uk/). Here, the Ser207 was located at the centre of the conserved pentapeptide G-X-S-X-G, as was also shown for other FAE sequences and crystal structures (Udatha et al. 2011). PeFaeA and Est1 contain an uncommon catalytic triad of Ser-Glu-His, while Udatha et al. (2011) reported a catalytic triad of Ser-Asp-His for 324 analyzed FAE sequences.The change in the catalytic triad from Asp to Glu makes PeFaeA and also Est1 special among all previously published FAEs. This catalytic triad is uncommon for FAEs, but not for other proteins of the α/β-hydrolase-fold superfamily (Carr & Ollis 2009; Ollis et al. 1992). These enzymes are characterized by a catalytic triad composed of a nucleophilic serine, an absolutely conserved histidine and an acidic residue (aspartic or glutamic acid). Both aspartate and glutamate can act as proton acceptors in the catalytic triad of hydrolysis; thus, only the direct comparison of enzyme mutants will reveal the impact on the catalytic process.

3.3.3 Influence of pH, temperature, and organic solvents on FAE activity

Maximum FAE activity for the hydrolysis of methyl ferulate was observed at pH 5.0 (**Fig. 3-3A**). Additionally the enzyme **s**howed more than 50 % residual activity over a broad pH range from 3.5 to 7.0. However, the hydrolytic activity of PeFaeA towards methyl ferulate decreased rapidly at pH 8.0 11 % and at pH 8.5 2 %. The pH values described in the literature for FAEs ranged from 4.5 to 7.6, but most were located around pH 6.0 (Koseki et al. 2009a). Therefore the pH optimum (pH 5.0) of the PeFaeA was similar to known *Aspergillus* sp. type A FAEs.

The optimum temperature for maximum enzyme activity was recorded at 50 °C. The PeFaeA showed good activity in the temperature range from 40 to 50 °C whereas temperatures above 55 °C decreased the enzyme activity strongly (**Fig. 3-3B**). The enzyme lost about 50 % of its

activity after 1 h at 50 °C (**Fig. 3-3C**). In contrast, 90 % of enzyme activity was retained at 40 °C. The determined temperature optimum of the PeFaeA was comparable with the optimum of fungal FAEs in the literature. The major part of them had a temperature optimum in the range from 45 to 60 °C (Koseki et al. 2009a).

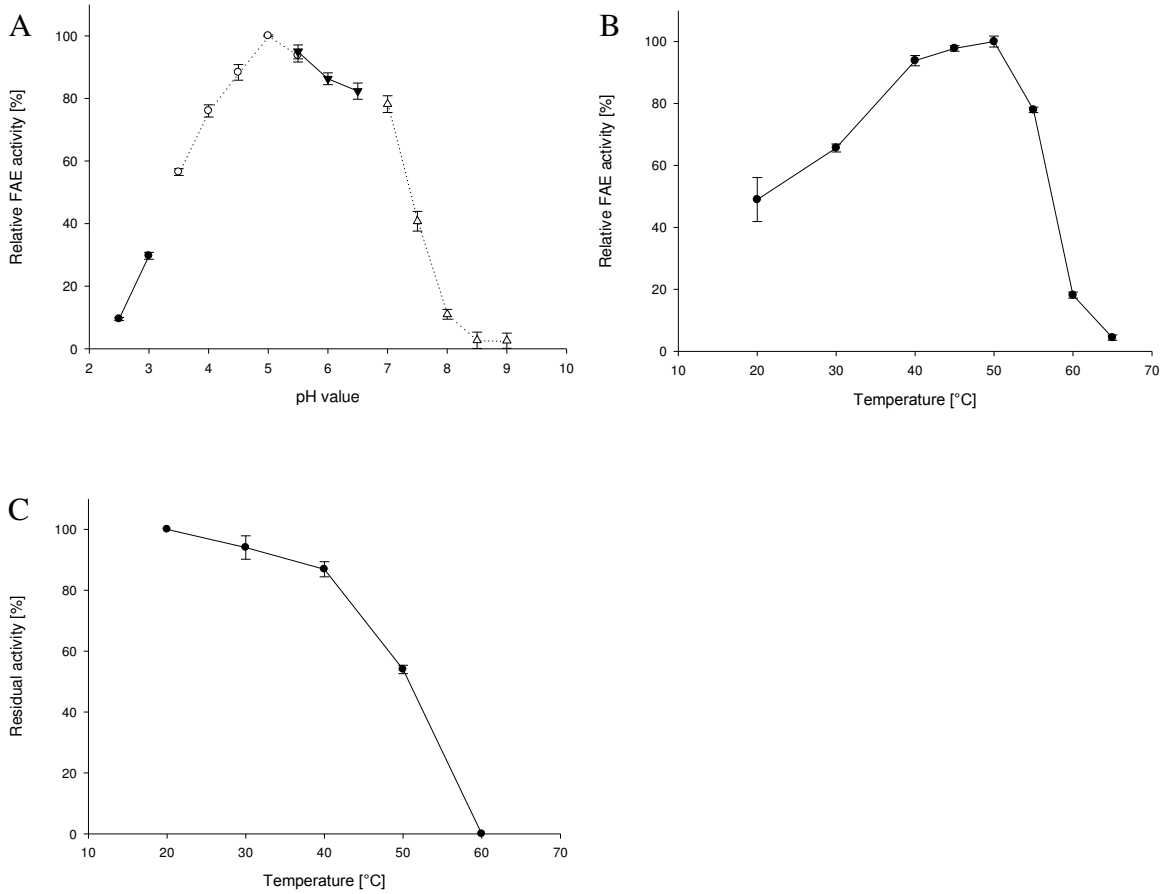


Fig. 3-3 Effect of pH and temperature on the activity of PeFaeA. **(A)** pH optimum was determined using citrate buffer (filled circle), acetate buffer (open circle), Bis-Tris buffer (filled inverted triangle), and Tris buffer (open triangle), **(B)** temperature optimum, **(C)** thermostability profile. Residual activities were determined after preincubating of the enzyme for 1 h at temperatures in the range of 20 – 60 °C. Remaining enzyme activity was determined after 1 h incubation with 1 mM methyl ferulate. Relative enzyme activity [%] was defined as the percentage of activity detected with respect to the maximum observed FAE activity in each experiment. Values are the average of triplicate experiments with standard deviation shown as error bars.

To investigate its usefulness in ester synthesis, the activity was measured in 1:1 (v/v) buffer emulsions with acetonitrile, ethanol, methanol, *n*-hexane, *n*-heptane, toluene, acetone, diethyl ether, DMSO, DMF, isobutyl alcohol, and *tert*-butyl methyl ether. A sample without solvent, incubated under the same experimental conditions (37 °C, 1 h, pH 5.0, 900 rpm), was set to 100 % and used for the calculation of the relative enzyme activities. In case of water-immiscible solvents, the real concentration of the organic solvent in the aqueous phase is not 50 %. Nevertheless, a large interfacial area between organic and polar phases was ensured by vigorous

shaking of the reaction mixture. Among the tested organic solvents the FAE showed good stability in *n*-hexane and *n*-heptane, where 74 % and 76 % of enzyme activity was retained (**Tab. 3-2**). In all other systems the residual enzyme activity was less than 15 %. Especially incubation with 50 % methanol inhibited PeFaeA completely.

Tab. 3-2 Effect of organic solvents on PeFaeA activity.

Solvent	Relative FAE activity [%]
n-heptane	76 (4.9)
n-hexane	74 (4.71)
diethyl ether	14 (0.9)
ethanol	12 (0.77)
tert-butyl methyl ether	12 (0.77)
toluene	11 (0.68)
DMF	8 (0.49)
isobutyl alcohol	7 (0.44)
acetonitrile	6 (0.38)
DMSO	2 (0.12)
acetone	1 (0.09)
methanol	ND

Residual activities of PeFaeA for the hydrolysis of 0.7 mM methyl ferulate (1 h, 37 °C, 900 rpm) in a binary system composed of acetate buffer pH 5.0 and organic solvents in the ratio 1:1 (v/v). FAE activity determined without organic solvent was set to 100 %. Numbers in parentheses are the estimates of the standard error. ND: activity not detectable.

A similar inhibitory effect of alcohols, such as ethanol and methanol, was reported by Faulds et al. (2011). In contrast, the Est1 of *Pleurotus sapidus* lost 95 % of its enzyme activity in the presence of 50 % hexane in the reaction mixture, while the addition of 50 % toluene or *tert*-butyl methyl ether resulted in moderate to good rates of hydrolysis of methyl ferulate (Linke et al. 2013). When comparing different studies concerning the effect of solvents on FAE activity it can be seen that the influence of the solvent varied for the different FAEs. Therefore, the effect seems not to be based on a change in solubility of the substrate in the solvent, but on changes in the active site of each esterase (Faulds et al. 2011).

3.3.4 Inhibition studies

Inhibition of PeFaeA by metal ions (Ca^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Li^{+}, K^{+}) as well as by EDTA, SDS, and PMSF was examined by addition of the tested compounds into the reaction mixture at a final concentration of 5 mM (except PMSF, 1 mM). Furthermore, the tolerance of PeFaeA towards high salt concentrations (NaCl 0.1 – 3 M) was investigated. FAE activities were measured using methyl ferulate as substrate and expressed as a percentage of the activity obtained in the absence of the added compounds.

In general, most of the investigated metal ions had either no or a slight stimulatory effect (**Tab. 3-3**). Among the metal ions tested only Hg^{2+} decreased the enzyme activity significantly to 30 %. In fact, it is known that Hg^{2+} react with thiol groups and can also reduce disulphide bonds (Vallee & Ulmer 1972). A complete inhibition of FAE activity by Hg^{2+} was reported by Kanauchi et al. (2008) and Kumar et al. (2013).

Tab. 3-3 Effect of metal ions and inhibitors on the activity of PeFaeA.

Concentration [mM]	Substance	Relative FAE activity [%]
5	$FeCl_3$	102 (0.06)
5	$FeSO_4$	100 (0.12)
5	KCl	96 (0.06)
5	$CaCl_2$	95 (0.03)
5	$Cu(NO_3)_2$	95 (0.03)
5	$NiSO_4$	91 (0.03)
5	$CoCl_2$	90 (0.05)
5	$ZnSO_4$	89 (0.07)
5	$MnSO_4$	88 (0.05)
5	LiCl	84 (0.07)
5	$MgCl_2$	82 (0.05)
5	$HgAc_2$	32 (0.01)
50	$MgCl_2$	123 (0.04)
50	$CaCl_2$	121 (0.08)
50	KCl	116 (0.03)
50	LiCl	109 (0.09)
1	PMSF	46[a] (0.01)
5	EDTA	95 (0.08)
5	SDS	5 (0.03)
100	NaCl	116 (0.06)
500	NaCl	124 (0.01)
1000	NaCl	130 (0.02)
2000	NaCl	133 (0.1)
3000	NaCl	128 (0.16)

Remaining enzyme activity was measured after 1 h incubation with 1 mM methyl ferulate and different metal ions as well as inhibitors in concentrations listed above (at 37 °C, 50 mM acetate buffer, pH 5.0). Numbers in parentheses are the estimates of the standard error. A sample without additive was used for the calculation of relative enzyme activities.

[a] After incubation with 1 mM PMSF at pH 7.0 no enzyme activity was detectable.

Both Fe^{2+} and Fe^{3+} had no effect on PeFaeA activity. Koseki et al. (1998) reported that the FAE from *A. awamori* which contains a catalytic triad of serine, aspartic acid and histidine was inhibited by Hg^{2+} and Fe^{2+}.

A positive effect on PeFaeA activity was detected in the presence of higher concentrations of both alkali and earth alkali metal ions. The addition of 50 mM Ca^{2+}, K^+, Mg^{2+} and Li^+, respectively, increased the initial activity by 10 – 20 %. In contrast, 5 mM SDS resulted in

about 95 % loss of enzyme activity, whereas 5 mM EDTA showed no significant inhibition of the FAE activity. Against expectation, 1 mM PMSF lowered the PeFaeA activity by only 50 % instead of a complete inhibition. PMSF is a known FAE inhibitor, because it binds to the serine residues in the active site (Gold & Fahrney 1964). In the presence of another buffer at pH 7.0 1 mM PMSF inhibited the PeFaeA completely (Gold & Fahrney 1964).

The addition of NaCl to the activity assays increased the initial FAE activity in all cases up to 15 – 30 %. Especially NaCl concentrations of 1 M and more had a stimulatory effect on enzyme activity (**Tab. 3-3**). It is possible that higher concentrations of Na^+ enhance the thermal stability of the enzyme and are consequently involved in maintaining the active conformation of the enzyme, as was reported for Ca^{2+}, K^+ and Mg^{2+} (Aurilia et al. 2007). PeFaeA is a halotolerant enzyme and still active at 3 M NaCl.

In addition, PeFaeA activity was investigated towards typical lipase substrates such as Tween 80 and olive oil according to the methods of Freire et al. (1997) and Linke et al. (2005). No activity was observed by incubation (1 h, 37 °C and 120 rpm) of PeFaeA with olive oil. A slow release of free fatty acids was determined in the presence of the substrate Tween 80 (0.4 mU mg^{-1}). The low specific activity of PeFaeA for Tween 80 suggested a weak lipase side activity.

3.3.5 Substrate specificity and kinetic constants

Substrate specificity of the PeFaeA towards different cinnamic and benzoic acid esters as well as natural substrates, such as F-A, F-AX, and F-AXG was investigated (**Tab. 3-4**). The PeFaeA hydrolyzed methyl ferulate (16.6 %), methyl coumarate (1.9 %), and methyl sinapate (4.6 %), but no methyl caffeate. These four typical synthetic feruloyl ester substrates were used for the FAE classification according to Crepin et al. (2004b), who divided FAEs into four types (A – D) based on their substrate specificity, sequence homology, and biochemical analysis. In the meantime the classification system was extended by phylogenetic analysis of fungal FAEs by Benoit et al. (2008), while a descriptor-based computational analysis with pharmacophore modelling (Udatha et al. 2011) offered an alternative attempt for classification. The lack of hydrolysis of methyl caffeate would result in type A according to Crepin et al. (2004b). Sequence homology to Est1 of *Pleurotus sapidus* (Linke et al. 2013) also encouraged the classification of PeFaeA as type A FAE. Likewise, chlorogenic acid, benzoic acid esters, and vanillic acid esters were no substrates. In contrast, by addition of a hydroxyl group such as 3-hydroxy benzoic acid esters and 4-hydroxy benzoic acid esters slow hydrolysis was observed. Moreover, the position of the substituted hydroxyl groups had an impact on the specificity of the FAE; for example the relative activities determined for the 4-hydroxy benzoic acid esters

(1.4 %) were nearly threefold higher than those calculated for the 3-hydroxy benzoic acid esters (0.5 %). However, the additional insertion of a methoxy group did not improve hydrolysis, as in the case of methyl vanillate. A similar lack of activity against benzoic acid esters, methyl vanillate as well as methyl syringate was previously reported for an FAE of *Aspergillus niger* (Faulds & Williamson 1994) and *Penicillium expansum* (Donaghy & McKay 1997). Furthermore, the addition of a second methoxy group in meta position decreased enzyme activity (methyl ferulate 16.6 % → methyl sinapate 4.6 %). As shown in **Tab. 3-4** the specific enzyme activities for the hydrolysis of methyl and ethyl esters of cinnamic acid, ferulic acid, and in the case of 4-hydroxy benzoic acid also for the propyl ester were nearly the same.

Tab. 3-4 Substrate specificity of purified PeFaeA.

Substance	Relative FAE activity [%]	Specific FAE activity [U mg^{-1}]
F-AX	100 (0.01)	3.89
F-A	38.3 (1.10)	1.49
F-AXG	27.2 (1.81)	1.06
methyl ferulate	16.6 (0.08)	0.65
ethyl ferulate	15.2 (0.56)	0.59
methyl sinapate	4.6 (0.13)	0.18
methyl gallate	2.6 (0.06)	0.10
methyl coumarate	1.9 (0.47)	0.07
ethyl 4-hydroxy benzoate	1.4 (0.07)	0.05
methyl 4-hydroxy benzoate	1.4 (0.07)	0.05
propyl 4-hydroxy benzoate	1.3 (0.73)	0.05
methyl 3-hydroxy benzoate	0.5 (0.02)	0.02
ethyl cinnamate	0.4 (0.02)	0.02
methyl cinnamate	0.3 (0.04)	0.01
butyl 4-hydroxy benzoate	ND	ND
chlorogenic acid	ND	ND
ethyl benzoate	ND	ND
ethyl vanillate	ND	ND
methyl benzoate	ND	ND
methyl caffeate	ND	ND
methyl vanillate	ND	ND

Purified PeFaeA was incubated with 1 mM of different substrates for 1 h (at 37 °C, 50 mM acetate buffer, pH 5.0). Relative enzyme activities were calculated based on the most preferred substance of PeFaeA: F-AX which was set to 100 %. Numbers in parentheses are the estimates of the standard error.

Abbreviations: F-A: 5-O-trans-feruloyl-L-arabinofuranose, F-AX: *β*-D-xylopyranosyl-(1→2)-5-O-trans-feruloyl-L-arabinofuranose, F-AXG: *α*-L-galactopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-5-O-trans-feruloyl-L-arabinofuranose; ND: activity not detectable.

On the other hand, the butyl ester of 4-hydroxy benzoic acid was not hydrolyzed at all. Consequently the lengthening of the ester-linked alkyl chain up to propyl had only little influence on PeFaeA activity as in the case of cinnamic acid, ferulic acid, and 4-hydroxy

benzoic acid. These results are in agreement with the kinetic results for TsFaeC from *Talaromyces stipitatus* of Vafiadi et al. (2006), who reported the same K_m values for methyl, ethyl, and propyl ferulate (0.04 mM), while the *n*-butyl group (0.106 mM) resulted in the highest K_m values. Consequently, TsFaeC showed the same affinity for alkyl isomers C1 – C3. The relative enzyme activities calculated for the different substrates showed that the PeFaeA preferred natural substrates, such as feruloylated mono-, di-, and trisaccharides (F-A, F-AX, F-AXG) over synthetic substrates. F-AX was hydrolyzed most effectively (3.9 U mg^{-1}) followed by F-A (1.5 U mg^{-1}) and F-AXG (1.1 U mg^{-1}). Likewise, Est1 of *P. sapidus* (Linke et al. 2013) preferred the natural FAE substrates in the same order, whereas *A. niger* FAEA preferred the natural substrate F-AX after F-A (Benoit et al. 2006b). A preference of type A FAEs for hydrophobic substrates with more bulky substituents was also reported by Kroon et al. (1999; 1997) and Williamson et al. (1998). In contrast, the FAE of *P. expansum* had higher specific activities for methyl ferulate (14.4 U mg^{-1}) than for F-AX (5.3 U mg^{-1}) (Donaghy & McKay 1997).

Kinetic constants (k_{cat}, K_m) were determined from initial hydrolysis rates for the most preferred synthetic substrate methyl ferulate and the most preferred natural one, F-AX. K_m for methyl ferulate was 0.145 mM and k_{cat}= 0.85 s^{-1} which resulted in a k_{cat}/K_m value of 5.85 mM^{-1} s^{-1}. K_m was determined for F-AX to be 0.44 mM and k_{cat} to be 3.41 s^{-1} which resulted in a k_{cat}/K_m value of 7.75 mM^{-1} s^{-1}. The K_m values demonstrated that the enzyme had a threefold higher affinity for methyl ferulate, and the comparison of k_{cat} and k_{cat}/K_m values showed that the PeFaeA hydrolyzed F-AX four times faster and more efficiently, respectively. The K_m value determined for methyl ferulate was fivefold or around 13-fold lower, respectively, than those recorded for other type A FAEs, such as the AnFaeA of *A. niger* (K_m 0.76 mM) (de Vries et al. 2002) and the Est1 of *P. sapidus* (K_m 1.95 mM) (Linke et al. 2013). As a result, the PeFaeA had a much higher affinity to methyl ferulate than the other two published type A FAEs. Other FAE types showed comparable K_m values, for example FAE of *P. expansum* (2.6 mM) (Donaghy & McKay 1997), FoFAE-I of *Fusarium oxysporum* (0.6 mM) (Topakas et al. 2003a), and StFAE-A of *Sporotrichum thermophile* (0.71 mM) (Topakas et al. 2004). There are also FAEs with lower K_m values for methyl ferulate, such as Fae1A of *Anaeromyces mucronatus* (0.007 mM) (Qi et al. 2011) and TsFAE of *T. stipitatus* (0.04 mM) (Vafiadi et al. 2006). In contrast k_{cat} values listed in the literature for *F. oxysporum*, *S. thermophile*, *T. stipitatus*, and *A. niger* ranged from 0.16 to 6.85 s^{-1} (Crepin et al. 2003a; Kroon et al. 1997; Topakas et al. 2005b).

3.3.6 Synergistic interaction between *Pleurotus eryngii* FAE and *Trichoderma viride* xylanase

PeFaeA (1.9 mU/12 mg DSWB) liberated 0.28 mM ferulic acid from DSWB after 16 h of incubation, whereas the xylanase alone (67 mU/12 mg DSWB) released only 0.17 mM of ferulic acid. The simultaneous incubation of both enzymes with DSBW resulted in a release of 7.64 mM ferulic acid from DSBW. Thus, PeFaeA alone released little ferulic acid from the natural substrate DSWB. In a synergistic action, the xylanase from *T. viride* increased the amount of released ferulic acid from DSWB more than 20-fold. Similar results were reported by Faulds & Williamson (1995) who showed that AnFaeA released a 24-fold higher level of ferulic acid from wheat bran when *T. viride* xylanase was added. Vardakou et al. (2003) showed that endoxylanases produced short-chain xylo-oligosaccharides which are more accessible substrates for FAEs. Consequently, an addition of endoxylanases seems mandatory, if the exploitation of ferulic acid from natural substrates is aimed (Garcia-Conesa et al. 2004; Moukouli et al. 2008; Topakas et al. 2003a).

3.4 Conclusion

An extracellular halotolerant FAE from *Pleurotus eryngii*, PeFaeA, was purified to homogeneity. The abundant occurrence of interfering laccases required the subsequent use of cation exchange, hydrophobic interaction, and size exclusion chromatography. Biochemical characterization of PeFaeA revealed unique properties, such as activity on a broad range of synthetic cinnamate and benzoate derivatives as well as natural substrates, such as feruloylated sugars and DSWB.

Good stability towards NaCl, metal ions, EDTA, and some organic solvents renders this enzyme an attractive candidate for biotechnological applications, particularly to release ferulic acid from agroindustrial waste materials. Free ferulic acid may be used as a precursor for the generation of flavour compounds, such as vanillin or vinyl guaiacol, or may be incorporated in antioxidant or photoprotecting preparations.

Acknowledgement

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4. Vorwort zur Publikation "A chlorogenic acid esterase with a unique substrate specificity from *Ustilago maydis*"

Nachdem Haase-Aschoff et al. (2013a) das Potential von Basidiomycota als FAE-Produzenten aufgezeigt haben (vgl. Kapitel 1.2.1), wurden 21 weitere Basidiomycota in analoger Weise hinsichtlich ihrer FAE-Aktivität analysiert (unveröffentlichte Arbeiten, Nieter et al.). Bei diesem Screening wurden drei weitere potentielle FAE-Produzenten identifiziert, welche Unterschiede bezüglich der prozentualen molaren Konversionsraten für FA-ME und ZA-ME aufwiesen: *Coprinus cinereus* (FA-ME 100 mol-%, ZA-ME 98 mol-%), *Ustilago maydis* (FA-ME 70 mol-%, ZA-ME 60 mol-%) und *Flammulina velutipes* (FA-ME 10 mol-%, ZA-ME 30 %). Durch den Zusatz von bekannten FAE-Induktoren wie Tween 80, Weizenkleie und Pektin zu den Submerskulturen der potentiellen FAE-Produzenten konnten differenzierte FAE-Aktivitäten induziert als auch teilweise Erhöhungen der Produktionsraten erzielt werden.

Die anschließenden Arbeiten fokussierten sich auf die FAE-Aktivität aus *U. maydis*, da aus *C. cinereus* bereits die codierende Gensequenz einer extrazellulären Esterase heterolog in *Pichia pastoris* exprimiert wurde und das rekombinante Enzym FA aus Weizenarabinoxylan freisetzten konnte (Hashimoto et al. 2010). Ferner schien die Identifikation der FAE-Aktivität aus *U. maydis* erstrebenswert, um das Wissen über FAE zu erweitern, da Benoit et al. (2008) in ihrem Sequenz-basierten FAE-Screening kein potentielles FAE-Gen im Genom von *U. maydis* lokalisieren konnten.

Die nachfolgende Publikation beschreibt eine Reinigungsstrategie für die FAE-Aktivität aus dem Kulturüberstand von *U. maydis* sowie die biochemische und molekularbiologische Charakterisierung des isolierten Enzyms. In einem umfassenden Substratscreening mit artifiziellen und natürlichen FAE-Substraten offenbarte sich eine signifikante Präferenz des Enzyms für Chlorogensäure gegenüber feruloylierten Substraten. Im Verlauf der Arbeiten stellte sich heraus, dass es sich bei dem FAE-aktiven Enzym um eine Chlorogensäureesterase mit FAE-Nebenaktivität handelte.

5. A chlorogenic acid esterase with a unique substrate specificity from *Ustilago maydis*

Abstract

An extracellular chlorogenic acid esterase from *Ustilago maydis* (UmChlE) was purified to homogeneity by using three separation steps, including anion-exchange chromatography on a Q Sepharose FF column, preparative isoelectric focusing (IEF), and, finally, a combination of affinity chromatography and hydrophobic interaction chromatography on polyamide. SDS-PAGE analysis suggested a monomeric protein of 71 kDa. The purified enzyme showed maximal activity at pH 7.5 and at 37 °C and was active over a wide pH range (3.5 to 9.5). Previously described chlorogenic acid esterases exhibited a comparable affinity for chlorogenic acid, but the enzyme from *Ustilago* was also active on typical feruloyl esterase substrates. Kinetic constants for chlorogenic acid, methyl *p*-coumarate, methyl caffeate, and methyl ferulate were as follows: K_m values of 19.6 µM, 64.1 µM, 72.5 µM, and 101.8 µM, respectively, and k_{cat}/K_m values of 25.83 mM^{-1} s^{-1}, 7.63 mM^{-1} s^{-1}, 3.83 mM^{-1} s^{-1} and 3.75 mM^{-1} s^{-1}, respectively. UmChlE released ferulic, *p*-coumaric, and caffeic acids from natural substrates such as destarched wheat bran (DSWB) and coffee pulp (CP), confirming activity on complex plant biomass. The full-length gene encoding UmChlE consisted of 1,758 bp, corresponding to a protein of 585 amino acids, and was functionally produced in *Pichia pastoris* GS115. Sequence alignments with annotated chlorogenic acid and feruloyl esterases underlined the uniqueness of this enzyme.

Keywords

Fungi; basidiomycete; feruloyl esterase; chlorogenic acid; feruloyl saccharides; coffee pulp; heterologous expression; *Pichia pastoris*

5.1 Introduction

Plant cell walls consist of a heterogeneous mixture of polymers, including cellulose, hemicellulose, pectin, and lignin. In addition to these main components, hydroxycinnamates play a key role in the formation of a complex network of these polymers. While hydroxycinnamates covalently cross-link plant cell wall polysaccharides to each other by ester bonds, links between polysaccharides and lignin are formed mainly by ether linkages (Ishii 1997). Thus, these phenolic acids (mainly ferulic, *p*-coumaric, and caffeic acids) not only influence the rigidity and mechanical properties of the cell wall but also play a role in plant defense (Cosgrove 2001). They are linked differently to sugar residues in plants: in grasses such as wheat, barley, or maize, ferulates and *p*-coumarates are mainly found esterified to the O-5 position of arabinose residues, whereas in pectin of dicotyledons, such as spinach or sugar beet, ferulic acid is esterified to the O-2 position of arabinose and the O-6 position of galactose residues (Ralet et al. 1994a). Furthermore, hydroxycinnamates also exist as soluble ester conjugates of quinic acid, the best-known example being chlorogenic acid [3-(3,4-dihydroxycinnamoyl) quinic acid] (Clifford 1999). Particularly high concentrations of chlorogenic acid are present in coffee, apple, pear, and potato tuber (Clifford 1999). The health, cosmetic, and pharmaceutical industries are especially interested in hydroxycinnamates due to their anticarcinogenic, anti-inflammatory, and antioxidant properties (Kroon et al. 1999). Consequently, enzymes that are able to release these phenolic compounds are not only essential for the degradation of plant biomass but also of interest for potential food and medical applications of the released products.

One group of these enzymes is the feruloyl esterases (FAEs) (EC 3.1.1.73), a subclass of the carboxylic ester hydrolases (EC 3.1.1.-). They hydrolyze the ester linkage between ferulic acid or related cinnamic acids and complex plant cell wall polysaccharides (Williamson et al. 1998). Over the years, a large number of FAEs, mainly from bacteria, yeasts, and fungi, were isolated and characterized (Crepin et al. 2004b; Fazary & Ju 2007; Topakas et al. 2007; Wong 2006). Crepin et al. (2004b) developed a classification system for FAEs, dividing them into four types (types A to D) based on their substrate specificity supported by primary sequence identity. Type A FAEs are active on methyl ferulate (FA-ME), methyl *p*-coumarate (*p*CA-ME), and methyl sinapate (SA-ME) but not on methyl caffeate (CA-ME). In contrast, type B FAEs hydrolyze FA-ME, *p*CA-ME, and CA-ME, while type C and D FAEs act on all four substrates. Only type A and D FAEs are able to release diferulates from complex substrates.

However, there have been only a few studies focusing on chlorogenic acid esterases (EC 3.1.1.42). To date, a small number of chlorogenic acid esterases have been reported, all

from *Aspergillus* sp. In 1980, Schöbel and Pollmann (1980a, 1980b) reported a specific chlorogenic acid esterase from a pectinolytic enzyme preparation of *Aspergillus niger*. More recently, Asther et al. (2005) demonstrated that another *A. niger* enzyme releases caffeic acid from industrial by-products such as coffee pulp (CP) and apple marc. The corresponding gene was homologously overexpressed by Benoit et al. (2007), and the properties of the recombinant and native enzymes were compared. Adachi et al. (2008) induced a chlorogenic acid esterase in mycelia of *Aspergillus sojae* with either instant coffee powder or CP. A hydroxycinnamic acid ester hydrolase from *Aspergillus japonicus* (Okamura & Watanabe 1982) and a cinnamate esterase from *A. niger* (Barbe & Dubourdieu 1998) also hydrolyzed chlorogenic acid. Additionally, activity toward chlorogenic acid was shown for the feruloyl esterase FAEB from *A. niger* (Levasseur et al. 2004), AoFaeB and AoFaeC from *Aspergillus oryzae* (Koseki et al. 2009b), TsFaeC from *Talaromyces stipitatus* (Vafiadi et al. 2006), and Fae1A from *Anaeromyces mucronatus* (Qi et al. 2011). Couteau et al. (2001) described human colonic bacteria acting on chlorogenic acid. This is the first study on the comprehensive biochemical characterization of a chlorogenic acid esterase from a basidiomycete. The activity of the enzyme (UmChlE) from the edible fungus *U. maydis* (corn smut; huitlacoche) toward different synthetic as well as complex natural substrates, such as destarched wheat bran (DSWB) and CP, indicates a possible key role in the decomposition of the cell walls of its host plant, *Zea mays*, and presumably related monocotyledons. In addition, the full sequence of UmChlE was amplified, and the amino acid residues responsible for the catalytic activity of the protein were identified.

5.2 Materials and methods

5.2.1 Chemicals and substrates

All chemicals used where of the highest purity grade and were purchased from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), or Sigma-Aldrich (Taufkirchen, Germany), unless otherwise noted. Methyl and ethyl ferulates were obtained from Alfa Aesar (Karlsruhe, Germany). Methyl caffeate, *p*-coumarate, and sinapate were synthesized according to methods described previously by Borneman et al. (1990). Feruloylated saccharides (5-O-*trans*-feruloyl-L-arabinofuranose (F-A); *β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AX); *α*-L-galactopyranosyl-(1→2)-*β*-D-xylo-pyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AXG)) prepared from destarched corn bran were kindly supplied by M. Bunzel (KIT, Karlsruhe, Germany). These esterase substrates were purified and identified as described previously (Allerdings et al. 2006; Linke et al. 2013). Coffee was obtained from Tchibo (Hamburg, Germany), and wheat bran was obtained

from Alnatura (Lorsch, Germany). PCR primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

5.2.2 Cultivation of *Ustilago maydis*

The basidiomycete *U. maydis* was purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany; No. 17144). The strain was grown on standard nutrition liquid (SNL) agar and maintained at 4 °C. SNL medium contained 30.0 g L^{-1} D-(+)-glucose monohydrate, 4.5 g L^{-1} L-asparagine monohydrate, 3.0 g L^{-1} yeast extract, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$; 1.0 mL L^{-1} trace element solution (0.08 g L^{-1} $FeCl_3$ x 6 H_2O, 0.09 g L^{-1} $ZnSO_4$ x 7 H_2O, 0.03 g L^{-1} $MnSO_4$ x H_2O, 0.005 g L^{-1} $CuSO_4$ x 5 H_2O, 0.4 g L^{-1} EDTA); the pH was adjusted to 6.0 with 1 M NaOH prior to autoclaving. For SNL agar plates, 15 g L^{-1} agar was added to SNL.
Starter cultures were grown on 10 % (v/v) SNL with 5 % (w/v) wheat bran. Main cultures of *U. maydis* were cultivated in 10 % (v/v) SNL with 1 % (w/v) wheat bran as inductor for esterase production (Garcia-Conesa et al. 2004). After 13 days of cultivation at 24 °C and 150 rpm on a rotary shaker (Infors, Bottmingen, Switzerland) cultures were harvested and stored at -20 °C until further processing.

5.2.3 Enzyme purification

The culture supernatant of *U. maydis* was separated from frozen cells by centrifugation (9,600 x *g* for 30 min at 4 °C), followed by filtration under reduced pressure using a 0.45-µm polyethersulfone (PES) membrane (Merck Millipore, Billerica, MA, USA). A total of 0.8 L of filtered supernatant was diluted one time with buffer A (50 mM Bis-Tris [pH 6.5]) and applied onto a 25 mL self-casted Q Sepharose FF column (GE Healthcare, Buckinghamshire, England) preequilibrated with buffer A. Fractions of 6.0 mL were collected at a flow rate of 4 mL min^{-1} and monitored for esterase activity. Elution was achieved by increasing the buffer B (buffer A with 1 M NaCl) concentration stepwise (5, 10, 50, 60, and 100 % buffer B). Active fractions eluted with 50 % buffer B were pooled and desalted by using an ultrafiltration module (30-kDa cutoff, PES; Sartorius, Göttingen, Germany). Afterwards, preparative isoelectric focusing (IEF) was performed with a Rotofor preparative IEF cell (Bio-Rad, Hercules, CA, USA). The desalted sample (50 mL) was mixed with 2 % ampholyte (pH 2 to 4; Serva, Heidelberg, Germany). Two percent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and proline were added to the sample to reduce protein precipitation during the run. The solution was focused at a constant power of 12 W for 4 h at 4 °C. Twenty fractions were collected and

analyzed for their pH and esterase activity. Active fractions from preparative IEF were pooled and washed with an ultrafiltration module (30-kDa cutoff, PES; Sartorius, Göttingen, Germany) to remove additives. Final purification of the enzyme was achieved with a combination of affinity chromatography and hydrophobic interaction chromatography using a 15 mL self-casted polyamide column (MP Biomedicals, Eschwege, Germany). The active sample (7.5 mL) was diluted one time with buffer [50 mM acetate (pH 6.5) with 4 M $(NH_4)_2SO_4$] and loaded onto the column. UmChlE was eventually eluted with 65 % 50 mM acetate buffer (pH 6.5), using a stepwise elution (78 %, 65 %, and 20 %).

5.2.4 Enzyme assay

The standard reaction mixture contained 25 µL of the sample, 10 µL Tris buffer (500 mM; pH 7.5), and 65 µL of the substrate (3 mM). Chlorogenic acid and methyl ferulate were used as standard substrates, unless otherwise stated. After incubation for 1 h at 37 °C, the reaction was terminated by doubling the reaction volume with acetonitrile, and the release of the corresponding free acids was analyzed by a high performance liquid chromatography (HPLC)-UV method. If appropriate, the hydrolysis of other benzoic and cinnamic acid ester derivatives as well as feruloylated saccharides was measured by using the same method. Samples were centrifuged (15 min at 15,000 x *g*) before injection into the HPLC column (Chromolith Performance RP-18e reverse-phase column, 100 by 4.6 mm; Merck, Darmstadt, Germany). Substrates and products were separated by using a stepwise gradient at a flow velocity of 1.5 mL min^{-1} as follows: 10 µL of sample loaded in 90 % buffer A (0.1 % formic acid) and 10 % buffer B (acetonitrile), 10 to 36 % buffer B in 8 min, 36 to 50 % buffer B in 1.5 min, 55 to 96 % buffer B in 0.5 min, 96 to 99 % buffer B in 2.5 min, 99 to 15 % buffer B in 0.5 min, 15 to 10 % buffer B in 1 min, and reequilibration with 10 % buffer B for 1 min, for a total run time of 14 min. Substance elution was detected at 232 nm (benzoic and 3-hydroxybenzoic acids), 254 nm (4-hydroxybenzoic and vanillic acids), 275 nm (cinnamic and gallic acids), 306 nm (*p*-coumaric acid), and 323 nm (ferulic, caffeic, chlorogenic, and sinapic acids) by using a Shimadzu UV-visible (UV-Vis) detector (SPD-10A VP; Shimadzu Deutschland GmbH, Duisburg, Germany). One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of free acid per minute under the specified conditions. All measurements were performed at least twice.

5.2.5 Biochemical characterization of UmChlE

5.2.5.1 Determination of temperature and pH optima

The optimal pH was determined by measuring the esterase activity at 37 °C with a pH in the range of 2 to 10 by using 50 mM solutions of glycine (pH 2 to 3.5 and pH 9 to 10), acetate (pH 3.5 to 5.5), Bis-Tris (pH 5.5 to 7), and Tris (pH 7 to 9). For the determination of the temperature optimum, an activity assay was performed for 60 min at various temperatures (20 °C to 90 °C).

5.2.5.2 Temperature and pH stability

For the determination of temperature stability, the enzyme solution was incubated for 60 min at different temperatures (20 °C to 60 °C). Subsequently, the substrate and 50 mM Tris buffer (pH 7.5) were added, and the activity assay was performed for 60 min at 37 °C. pH stability was determined by mixing 50 µL of the enzyme solution with 3 µL of the above-described buffer systems (500 mM; pH 2 to 10). After incubation for 60 min at 37 °C, 20 µL of the preincubated enzyme solution was mixed with 60 µL of the substrate and 25 µL 500 mM Tris buffer (pH 7.5). Finally, the residual enzyme activity was analyzed after further incubation (60 min at 37 °C).

5.2.5.3 Effect of solvents, metal ions, and inhibitors on enzyme activity

Enzyme tolerance to different solvents was investigated in the presence of 50 % (v/v) acetone, acetonitrile, diethyl ether, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), ethanol, isoamyl alcohol, isobutyl alcohol, isopropanol, methanol, *n*-hexane, *n*-heptane, *tert*-butyl methyl ether, or toluene. The reaction mixture contained 20 µL of the enzyme solution, 12 µL 500 mM Tris buffer (pH 7.5), 28 µL of the substrate (3 mM), and 60 µL organic solvent. After incubation (60 min at 37 °C at 900 rpm), the mixture was applied for HPLC analysis as described above. The effect of inhibitors on enzyme activity was tested with cetyltrimethylammoniumbromide (CTAB), EDTA, iodoacetamide, phenylmethylsulfonyl fluoride (PMSF), SDS, and various metal ions, such as Cs^+, K^+, Li^+, Sr^+, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Al^{3+}, Fe^{3+} and Cr^{3+}. Apart from PMSF (1 mM), all other potential inhibitors were added at concentrations of 5 mM. The effect of high salt concentrations (0.1 to 3 M NaCl) on enzyme activity was also investigated. NaCl was directly dissolved in a buffer-substrate mixture at the ratio and with the components used for the standard assay (1:6.5, v/v), to use as high an NaCl concentration as possible. Subsequently, 75 µL of the NaCl-buffer-substrate solution was incubated with 25 µL of the enzyme solution for 60 min at 37 °C and analyzed for residual enzyme activity by HPLC.

5.2.5.4 Substrate specificity and kinetic parameters

The substrate specificity of UmChlE toward different cinnamate and benzoate esters as well as natural substrates such as F-A, F-AX, and F-AXG was determined by activity assays, as described above. Stock solutions (3 mM) of all substrates were prepared in water. Kinetic parameters were determined from the initial rate of hydrolysis of 0.005 to 1 mM chlorogenic acid, methyl ferulate, methyl caffeate, and methyl *p*-coumarate (60 min at 37 °C at pH 7.5). K_m and v_{max} values were calculated with nonlinear regression using SigmaPlot 10.0 (Systat Software Inc., Chicago, IL, USA). Blank experiments were carried out for all assays by using water instead of enzyme. For the calculation of k_{cat}, the molecular mass was determined by using denaturing SDS-PAGE.

5.2.5.5 Enzymatic hydrolysis of natural substrates

DSWB was prepared as described previously by Johnson et al. (1988). The total content of ferulic, *p*-coumaric, and caffeic acids in DSWB and CP was determined after alkaline hydrolysis with 2 M NaOH (4 h at 50 °C at 220 rpm). The phenolic acid content of each sample was analyzed by HPLC after acidification with acetic acid. The concentration of chlorogenic acid in coffee was determined after incubation with 80 % (v/v) methanol. Samples of DSWB and CP (20 mg) were incubated (37 °C for 20 h at 650 rpm) with purified UmChlE (0.02 U) in a final volume of 400 µL containing 125 mM Tris buffer (pH 7.5). To investigate synergism with other carbohydrases, the release of ferulic acid was also analyzed after parallel incubation of UmChlE with *Trichoderma viride* xylanase (DSWB, 0.025 U; Fluka, Buchs, Switzerland). The amount of phenolic acids released from the natural substrates was quantified by using HPLC.

5.2.6 Denaturing SDS-PAGE

The enzyme purity and the molecular mass were determined by SDS-PAGE analysis (12 % resolving gel and 4 % stacking gel) according to the method of Laemmli (1970). Samples were prepared by mixing the samples 1:1 (v/v) with sample buffer (0.1 M Tris-HCl [pH 6.8], 0.2 M dithiothreitol [DTT], 4 % SDS, 20 % glycerol, 0.2 % bromophenol blue) and boiling the mixture for 10 min. After electrophoresis at 15 mA per gel, gels were stained with InstantBlue (Expedeon, Cambridgeshire, Great Britain).

5.2.7 Peptide mass fingerprinting

Amino acid sequences of tryptic peptides of UmChlE were deduced by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) using a maXis quadrupole time of flight (QTOF) mass spectrometer (Bruker, Bremen, Germany). The procedure was described in detail previously (Linke et al. 2013). NCBI BLAST (blastp program) searches were performed with the obtained amino acid sequences.

5.2.8 Amplification of the chlorogenic acid esterase sequence

Isolation of total RNA from the mycelium of *U. maydis*, grown for 13 days in 10 % (v/v) SNL supplemented with 1 % (w/v) wheat bran, as well as cDNA synthesis were performed as described previously by Linke et al. (2013). Amplification of the complete *CHLE* gene sequence was achieved by using untranslated-region (UTR) primers Uma_fw_UTR (TCCGTTCTTAGAAGCCAAACA) and Uma_rev_UTR (GAAAGCCAAGCAACAAGG TT), which were deduced based upon the available Um00182.1 gene sequence (GenBank accession no. XP_756329.1). PCRs were performed by mixing 10 µL 5x Phusion high-fidelity DNA polymerase buffer, 0.4 µL deoxynucleoside triphosphate (dNTP) mix (10 mM each), 25 pmol forward primer, 25 pmol reverse primer, 0.02 U Phusion high-fidelity DNA polymerase (Fermentas, St. Leon-Roth, Germany), 100 ng single-stranded cDNA, and double-distilled water (ddH_2O) to 50 µL. PCR was performed with a MasterCycler gradient (Eppendorf, Hamburg, Germany) by incubating the reaction mixture at 98 °C for 2 min and then for 35 cycles at 98 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. A final elongation step was performed at 72 °C for 10 min. All further steps, including analysis of PCR products, ligation, transformation in *Escherichia coli*, and colony PCR, were performed as described previously (Nieter et al. 2014).

5.2.9 Heterologous expression in *Pichia pastoris*

The *CHLE* gene with and without the native 21-amino acid signal peptide sequence was amplified by using primers UmChlE_Sfw (TTT TTC TCG AGA AAA GAG AGG CTA GGC TGC CTA ATC TG), UmChlE_Ofw (TTT TTC TCG AGA AAA GAG AGG CTG AAG CTC TTC CAC AAG TCT C), and UmChlE_rv (TTT TTG CGG CCG CTT AAT GAT GAT GAT GAT GAT GGA AGC CAA ACA C) (underlined bases are the XhoI and NotI restriction sites, respectively) and inserted into the XhoI-NotI site of the *Pichia pastoris* expression vector pPIC9 (Invitrogen, Karlsruhe, Germany). *P. pastoris* GS115 was transformed with the PmeI-linearized expression construct pPIC9-*HIS-CHLE* to achieve *CHLE* gene insertion at the *AOX1*

locus by electroporation (MicroPulser electroporator; Bio-Rad, Munich, Germany) (Lin-Cereghino et al. 2005). After selection of *P. pastoris* transformants with the ability to grow on histidine-deficient agar plates, 48 transformants of each expression construct were screened for chlorogenic acid esterase activity. The transformants were cultivated in 96-well plates as described previously by Sygmund et al. (2012). Culture supernatants were analyzed for enzyme activity after 24, 48, 72, 96, and 120 h by using the assay described above. To maintain stable expression conditions, 0.5 % (v/v) methanol was added every 24 h.

5.2.10 Nucleotide sequence accession number

The sequence of UmChlE has been deposited in the GenBank database under accession no. HG970190.

5.3 Results and discussion

5.3.1 Purification and general properties of UmChlE

When *U. maydis* was cultivated in SNL medium, esterase activity was detected, but the cultures turned deep black, impeding chromatographic purification. The composition of SNL medium promoted the synthesis of melanins. Melanin production by *U. maydis* was described previously (Nicolaus & Piattelli 1965; Wheeler 1983). Production was suppressed by reducing the concentration of all components of SNL to 10 % (v/v) and adding 1 % (w/v) wheat bran. These conditions resulted in even doubled esterase activities. Purification of the chlorogenic acid esterase UmChlE from the culture supernatant of *U. maydis* was accomplished by three complementary separation steps, including anion-exchange chromatography on a Q Sepharose FF column, preparative IEF, and, finally, a combination of affinity chromatography and hydrophobic interaction chromatography on polyamide (**Tab. 5-1**).

Tab. 5-1 Purification of UmChlE from *U. maydis*.

	Volume [mL]	Protein [mg]	Total activity [U][a]	Specific activity [mU mg^{-1}]	Recovery [%]	Purification [fold]
Culture supernatant	800	1272	2.39	1.9	100	1
Q Sepharose FF	50	4.9	0.32	64.4	12.1	34
Preparative IEF	15	0.97	0.05	49.8	2	27
Polyamide	5	0.03	0.01	237.6	0.3	127

[a] Activity was measured by the standard method in 50 mM Bis-Tris buffer pH 6 using 1.95 mM methyl ferulate as substrate.

The molecular mass of chlorogenic acid esterase as determined by denaturing SDS-PAGE was 71 kDa (**Fig. 5-1**, **lane 2**). The apparent mass of the native enzyme estimated by size exclusion

chromatography suggested that UmChlE was a monomer. These results demonstrated that UmChlE differed from other chlorogenic acid esterases described in the literature. While Asther et al. (2005) and Adachi et al. (2008) identified the native chlorogenic acid esterases as homodimers with subunits of about 80 kDa each, Benoit et al. (2007) suggested that the recombinant *A. niger* enzyme exists in a tetrameric form. Schöbel and Pollmann (1980a) also characterized the second known chlorogenic acid esterase from *A. niger* as a tetramer (four 60-kDa subunits), whereas Okamura and Watanabe (1982) described the hydroxycinnamic acid ester hydrolase of *A. japonicus* as a monomer of 145 kDa. Preparative IEF, used as a second purification step, yielded a pI of about 3.0 for UmChlE.

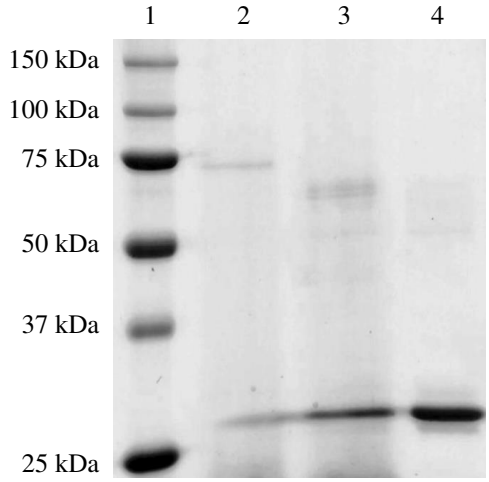

Fig. 5-1 SDS-PAGE analysis of the purified chlorogenic acid esterase (UmChlE) from *U. maydis*. Lane 1, Precision plus Protein™ Standard; lane 2, purified UmChlE after polyamide column; lane 3, deglycosylated UmChlE after treatment with endoglycosidase H for 1 h at 37 °C; lane 4, endoglycosidase H.

5.3.2 Characterization of the purified chlorogenic acid esterase

To date, only a few chlorogenic acid esterases (EC 3.1.1.42) have been well characterized (Adachi et al. 2008; Asther et al. 2005; Benoit et al. 2007; Schöbel & Pollmann 1980a, 1980b), while numerous feruloyl esterases (FAEs) (EC 3.1.1.73), a subclass of the carboxylic ester hydrolases, from a wide range of bacteria and fungi have been purified and characterized (Crepin et al. 2004b; Fazary & Ju 2007; Topakas et al. 2007; Wong 2006). Some feruloyl esterases showed the ability to hydrolyze chlorogenic acid, such as *A. niger* FAEB (Benoit et al. 2006b) and a type B feruloyl esterase from *Neurospora crassa* (Crepin et al. 2003b). As UmChlE accepted chlorogenic acid as the most preferred substrate but also hydrolyzed feruloyl esterase substrates efficiently, the enzyme was compared with both chlorogenic acid esterases and feruloyl esterases. As shown in **Fig. 5-2**, the influence of pH on esterase activity and stability was investigated for a pH range of 2 to 10. Maximum activity for the hydrolysis of

methyl ferulate was observed at pH 7.5, and 50 % residual activity was detected in a pH range of 5.5 to 9.5 (**Fig. 5-2A**).

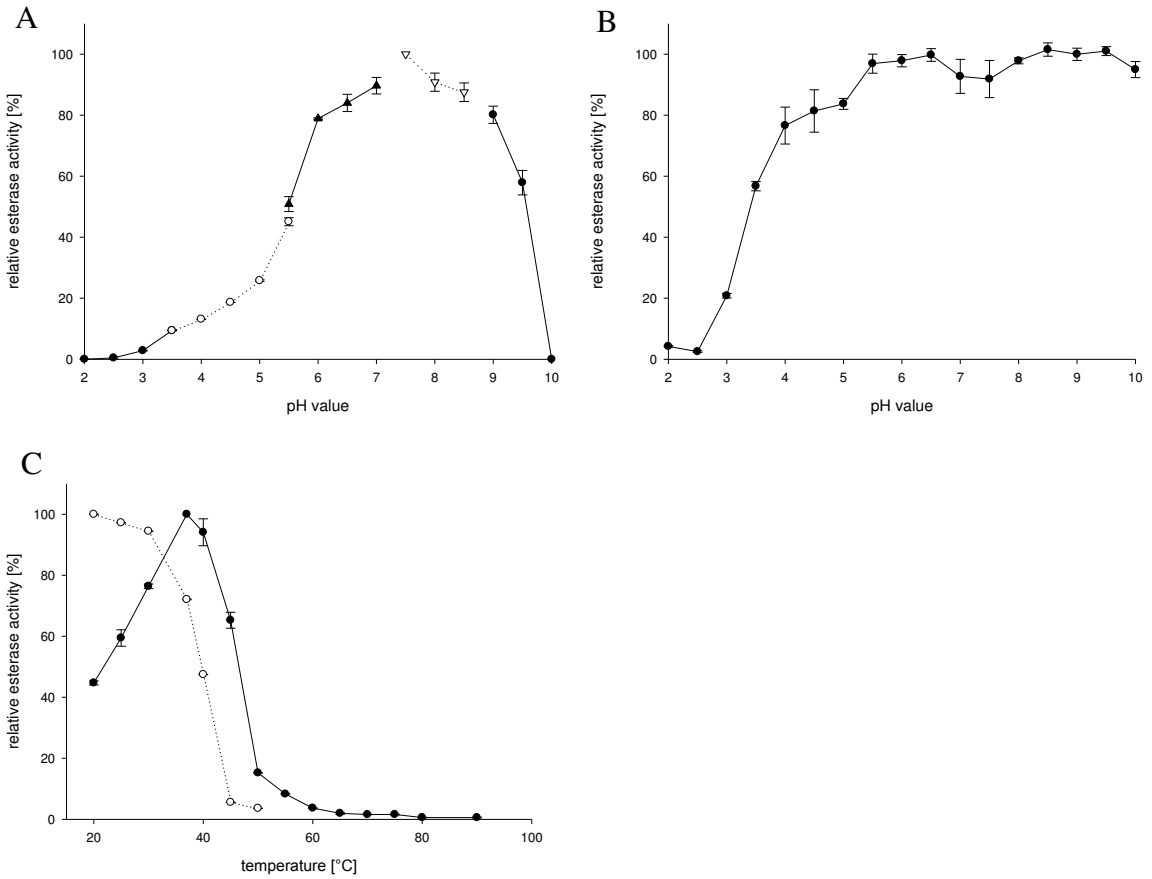

Fig. 5-2 Effect of pH and temperature on UmChlE activity. **(A)** The pH optimum was determined by using glycine buffer (filled circles), acetate buffer (open circles), Bis-Tris buffer (filled triangles), and Tris buffer (open inverted triangles), **(B)** pH stability, **(C)** Temperature optimum (filled circles) and thermostability profile (open circles). For pH and thermostability measurements, residual activities were determined after preincubating the enzyme for 1 h with the buffer systems mentioned above (pH 2 to 10) and at temperatures in the range of 20 °C to 60 °C, respectively. The remaining enzyme activity was determined after further incubation (1 h at 37 °C) with 1.95 mM methyl ferulate. Relative enzyme activity [%] is defined as the percentage of activity detected with respect to the maximum observed esterase activity in each experiment. Values are the averages of data from triplicate experiments, with standard deviations shown as error bars.

After 1 h of incubation at 37 °C, the enzyme was more stable under neutral to alkaline than under acidic conditions (**Fig. 5-2B**). UmChlE retained 80 % of its maximum activity between pH 4.5 and 10. The determined pH optima of the two previously reported chlorogenic acid esterases from *A. niger* (Asther et al. 2005; Schöbel & Pollmann 1980b) and of type B FAEs described previously by Koseki et al. (2009a) were between pH 6 and 7. Especially, the pH stability of UmChlE was different from that of the characterized chlorogenic acid esterases from *A. niger*, which were more stable under acidic than under alkaline conditions. The chlorogenic acid esterase isolated by Schöbel and Pollmann (1980a) was stable in a pH range of 3 to 8.5 after 10 min of incubation at an undefined temperature. Thus, the significant pH stability of

UmChlE under alkaline conditions makes the enzyme an attractive candidate for biotechnological applications, for instance, the treatment of pulp in the paper industry (Sigoillot et al. 2005). The optimal reaction temperature for UmChlE was determined to be 37 °C (**Fig. 5-2C**).

Enzyme activity started to decrease rapidly at 45 °C and was nearly zero at 60 °C. After preincubation at pH 7.5 and at 37 °C for 1 h, the enzyme lost 28 % of its activity. The thermostability of UmChlE decreased rapidly at temperatures above 40 °C. The determined temperature optimum was lower than those reported previously for chlorogenic acid esterases and most of the feruloyl esterases (Koseki et al. 2009a). Furthermore, the purified enzyme was less thermostable than those previously described (Asther et al. 2005; Benoit et al. 2007; Schöbel & Pollmann 1980a). The applicability of UmChlE for ester synthesis was examined by using 1:1 (v/v) buffer emulsions with different organic solvents (**Tab. 5-2**). Relative enzyme activities were calculated based on a sample incubated in buffer without organic solvent (37 °C for 1 h at pH 7.5 and at 900 rpm). Some of the tested solvents are water immiscible, but a large interfacial area between the organic and polar phases was ensured by vigorous shaking of the reaction mixture. UmChlE showed good stability in *n*-hexane and *n*-heptane, with relative activities of 87 % and 84 %, respectively. Moderate to slight activity was detected in isoamyl alcohol (64 %) and isobutyl alcohol (23 %). In all other systems, the residual enzyme activity was 7 %.

Tab. 5-2 Effect of organic solvents on UmChlE activity

Organic solvent	**Relative esterase activity [%]**
n-hexane	87 (2.12)
n-heptane	84 (0.89)
isoamyl alcohol	64 (2.43)
isobutyl alcohol	23 (6.53)
toluene	7 (1.67)
isopropanol	6 (0.37)
methanol	5 (0.23)
acetone	3 (2.59)
acetonitrile	3 (3.18)
DMF	1 (0.76)
diethyl ether	ND
DMSO	ND
ethanol	ND
tert-butyl methyl ether	ND

Shown are residual activities of UmChlE for the hydrolysis of 0.84 mM methyl ferulate (1 h, 37 °C, 900 rpm) in a binary system composed of Tris buffer pH 7.5 and organic solvents at a ratio 1:1 (v/v). The esterase activity determined without organic solvent was set to 100 %. Numbers in parentheses are the estimates of the standard error. ND, not detectable.

The good stability of UmChlE in *n*-hexane and *n*-heptane indicated the potential of this enzyme for reverse-synthetic activity. Indeed, Topakas et al. previously described the synthesis of phenolic acid esters with feruloyl esterases of *Fusarium oxysporum* (2003a) and *Sporotrichum thermophile* (2004) by using a surfactantless microemulsion formed in a ternary water-organic solvent mixture consisting of *n*-hexane, 1-propanol/1-butanol, and water as a reaction system.

5.3.3 Inhibition studies

Various metal ions and enzyme inhibitors were examined for their effect on chlorogenic acid esterase activity (**Tab. 5-3**). Residual enzyme activity was measured by using a standard activity assay in the presence of 5 mM metal ions/inhibitors (except for PMSF [1 mM]).

Tab. 5-3 Effect of metal ions, inhibitors and NaCl on UmChlE activity.

Concentration [mM]	Substance	Relative esterase activity [%]
5	KCl	108 (0.57)
5	$NiSO_4$	108 (0.87)
5	$MgCl_2$	106 (0. 53)
5	LiCl	106 (1.39)
5	CsCl	105 (1.21)
5	$SrNO_3$	104 (0,18)
5	$CaCl_2$	103 (2.76)
5	$FeSO_4$	102 (0.50)
5	$Cu(NO_3)_2$	96 (0.53)
5	$MnSO_4$	90 (0.09)
5	$ZnSO_4$	76 (1.26)
5	$Cr(NO_3)_3$	69 (3.54)
5	$CoCl_2$	67 (0.11)
5	$FeCl_3$	66 (0. 65)
5	$Al_2(SO_4)_3$	53 (0.12)
5	$HgAc_2$	ND
5	EDTA	105 (1.14)
5	CTAB	104 (0.70)
5	Iodacetamide	102 (0.81)
5	SDS	59 (0.83)
1	PMSF	36 (2.01)
100	NaCl	105 (0.02)
500	NaCl	121 (0.01)
1000	NaCl	125 (0.02)
2000	NaCl	142 (0.06)
3000	NaCl	150 (0.04)

The remaining enzyme activity was measured after 1 h incubation with 1.95 mM methyl ferulate and different metal ions as well as inhibitors at the concentrations listed (at 37 °C, 50 mM Tris buffer pH 7.5). Numbers in parentheses are the estimates of the standard error. A sample without additive was used for the calculation of relative enzyme activities. ND, not detectable.

Among the tested metal ions, a significant decrease in UmChlE activity to residual activities of 50 to 76 % was detected in the presence of Al^{3+}, Co^{2+}, Cr^{3+}, Fe^{3+}, and Zn^{2+}, while Mn^{2+} decreased the enzyme activity slightly to 90 %. Furthermore, the addition of Hg^{2+} resulted in a complete loss of enzyme activity. Hg^{2+} reacts with thiol groups and can also reduce disulfide bonds (Vallee & Ulmer 1972).

The inhibition of UmChlE activity by Zn^{2+}, Hg^{2+}, and Al^{3+} was consistent with the properties of a feruloyl esterase from *Russula virescens* (Wang et al. 2014b). This feruloyl esterase was also inhibited by Fe^{2+}, whereas UmChlE was inhibited only by Fe^{3+}. The negative effect of Fe^{3+} on esterase activity was also described previously by Donaghy and McKay (1997) and Yao et al. (2013). Some esterases were also inhibited by Cu^{2+} (Donaghy & McKay 1997; Yao et al. 2013) and Cd^{2+} (Wang et al. 2014b).

Enzyme activity was inhibited 41 % by SDS and 64 % by PMSF, while incubation with CTAB, EDTA, and iodoacetamide showed no effect (**Tab. 5-3**). The almost complete loss of activity in the presence of PMSF, a known esterase inhibitor, suggested that the catalytic triad of UmChlE contained a serine residue (Gold & Fahrney 1964). In contrast, enzyme activity was not influenced by CTAB, which reacts with tryptophan and thiol groups of cysteine, indicating that these amino acid residues were not involved in catalysis (Anson 1940).

Furthermore, the tolerance of UmChlE to various NaCl concentrations (0.1 to 3 M) was investigated. Enzyme activity increased with increasing salt concentrations, up to 150 % in the presence of 3 M NaCl (**Tab. 5-3**). A stimulatory effect of NaCl on esterase activity was previously reported by Nieter et al. (2014) and Kumar et al. (2012).

5.3.4 Hydrolytic specificity and kinetic properties of UmChlE

A comprehensive set of substrates was examined for hydrolysis by UmChlE. These substrates included cinnamic and benzoic acid esters as well as natural substrates such as F-A, F-AX, F-AXG, and chlorogenic acid (**Tab. 5-4**). Chlorogenic acid was found to be hydrolyzed most effectively. The determined specific activity of 2.8 U mg^{-1} for chlorogenic acid was set to 100 % and used for the calculation of relative activities for all other substrates. UmChlE hydrolyzed methyl *p*-coumarate (*p*CA-ME) (52 %), methyl ferulate (FA-ME) (46 %), and methyl caffeate (CA-ME) (18 %) but not methyl sinapate (SA-ME). These four typical synthetic feruloyl ester substrates are commonly used to classify FAEs. Crepin et al. (2004b) divided FAEs into four types (types A to D) based on their substrate specificity, their sequence homology, and biochemical analysis. The lack of hydrolysis of SA-ME would result in type B FAE according to Crepin et al. (2004b). This classification system has been extended by a phylogenetic analysis

of fungal FAEs by Benoit et al. (2008), while a descriptor-based computational analysis with pharmacophore modeling (Udatha et al. 2011) offers an alternative. UmChlE was also able to release ferulic acid from ethyl ferulate but about 50 % less than from FA-ME. Consequently, elongation of the ester-linked alkyl chain resulted in decreased enzyme activity (de Vries et al. 2002). While the presence of one methoxy group at the *meta* position of the phenyl ring lowered the enzyme activity marginally (52 % for *p*CA-ME versus 46 % for FA-ME), a second methoxy group caused a total loss of activity (SA-ME). Moreover, UmChlE hydrolyzed no substrate without a hydroxyl or methoxy group on the phenyl ring (methyl/ethyl cinnamate). The fact that UmChlE showed no hydrolytic activity against any benzoate derivative indicates the necessity for a certain distance between the aromatic ring and the ester bond. This requirement for the catalytic activity of feruloyl esterases was also reported previously by Topakas et al. (2005a) and Kroon et al. (1997).

Tab. 5-4 Substrate specificity of purified UmChlE.

Substance	**Relative esterase activity [%]**	**Specific esterase activity [U mg^{-1}]**
chlorogenic acid	100	2.76
F-A	75 (0.16)	2.06
F-AX	59 (0.36)	1.63
methyl *p*-coumarate	52 (0.06)	1.43
methyl ferulate	46 (0.16)	1.27
F-AXG	33 (0.09)	0.92
ethyl ferulate	28 (0.45)	0.78
methyl caffeate	18 (0.03)	0.49
methyl benzoate	ND	ND
ethyl benzoate	ND	ND
methyl 3-hydroxy benzoate	ND	ND
methyl 4-hydroxy benzoate	ND	ND
ethyl 4-hydroxy benzoate	ND	ND
propyl 4-hydroxy benzoate	ND	ND
butyl 4-hydroxy benzoate	ND	ND
methyl cinnamate	ND	ND
ethyl cinnamate	ND	ND
methyl gallate	ND	ND
methyl sinapate	ND	ND
methyl vanillate	ND	ND
ethyl vanillate	ND	ND

Purified UmChlE was incubated with 1.95 mM of different substrates for 1 h (at 37 °C, 50 mM Tris buffer pH 7.5). Relative enzyme activities were calculated based on the substance most preferred by UmChlE, chlorogenic acid which was set to 100 %. Numbers in parentheses are the estimates of the standard error.

Abbreviations: F-A: 5-O-*trans*-feruloyl-L-arabinofuranose, F-AX: *β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose, F-AXG: *α*-L-galactopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose and ND: activity not detectable.

UmChlE also hydrolyzed small natural substrates such as feruloylated mono-, di-, and trisaccharides (F-A, F-AX, and F-AXG). F-A was hydrolyzed most efficiently (2.0 U mg^{-1}),

followed by F-AX (1.6 U mg^{-1}) and F-AXG (0.9 U mg^{-1}). Likewise, *A. niger* FAEA (Benoit et al. 2006b) preferred the natural FAE substrates in the same order, whereas PeFaeA of *Pleurotus eryngii* (Nieter et al. 2014) and Est1 of *Pleurotus sapidus* (Linke et al. 2013) preferred the natural substrate F-A less than they preferred F-AX. In contrast, FAE of *Penicillium expansum* had higher specific activities for methyl ferulate (14.4 U mg^{-1}) than for F-AX (5.3 U mg^{-1}) (Donaghy & McKay 1997).

Kinetic properties were determined from the initial rates of hydrolysis of the most preferred substrates chlorogenic acid, FA-ME, *p*CA-ME, and CA-ME. CA-ME was chosen as it contains caffeic acid as a structure element in common with the best substrate, chlorogenic acid. Purified UmChlE possessed K_m, k_{cat}, and k_{cat}/K_m values of 72.5 µM, 0.28 s^{-1}, and 3.83 mM^{-1} s^{-1}, respectively, for CA-ME; 101.8 µM, 0.38 s^{-1}, and 3.75 mM^{-1} s^{-1}, respectively, for FA-ME; 64.1 µM, 0.49 s^{-1}, and 7.63 mM^{-1} s^{-1}, respectively, for *p*CA-ME; and 19.6 µM, 0.51 s^{-1}, and 25.83 mM^{-1} s^{-1}, respectively, for chlorogenic acid. Determination of K_m values demonstrated that the enzyme had a 3-fold higher affinity for chlorogenic acid than for *p*CA-ME and CA-ME. Among the four tested substrates, UmChlE possessed the lowest affinity for FA-ME. Furthermore, the k_{cat}/K_m values showed that UmChlE hydrolyzed chlorogenic acid 3- to 7-fold more efficiently than it hydrolyzed *p*CA-ME, FA-ME, and CA-ME. Consequently, the catalytic efficiency (k_{cat}/K_m), a good indicator of enzyme efficiency and specificity, was better for chlorogenic acid than for the three synthetic substrates. The better kinetic properties of UmChlE for chlorogenic acid than for CA-ME demonstrated the great impact of the linkage of quinic acid to caffeic acid. Quinic acid seemed to affect not only substrate binding and the formation of enzyme-substrate complexes but also the turnover rate (0.51 s^{-1} for chlorogenic acid versus 0.28 s^{-1} for CA-ME).

The substrate profile of UmChlE classified it as a feruloyl esterase, but the kinetic constants clearly showed an affiliation with the family of chlorogenic acid esterases. Here, the previously reported K_m values for chlorogenic acid as the substrate were 6.5 to 10 µM (Asther et al. 2005; Benoit et al. 2007) and 0.7 mM (Schöbel & Pollmann 1980b) for the second reported chlorogenic acid esterase from *A. niger*. There are few other reports available dealing with the enzymatic hydrolysis of chlorogenic acid. The following K_m values were determined for three type B feruloyl esterases for the substrate chlorogenic acid: 245 µM for *A. niger* FAEB, 180 µM for type B feruloyl esterase from *N. crassa* (Crepin et al. 2003b), and 630 µM for AoFaeB from *A. oryzae* (Koseki et al. 2009b). The feruloyl esterases mentioned above exhibited not only a 10-fold lower affinity for chlorogenic acid than did UmChlE but also possessed a 3-fold lower

K_m for *p*CA-ME (20 µM) than did UmChlE (64 µM). These feruloyl esterases also showed a higher specificity for CA-ME than for FA-ME.

Finally, UmChlE was clearly distinguished from the two other previously reported enzymes by its broad substrate profile. Previously identified chlorogenic acid esterases were active solely on chlorogenic acid. Asther et al. (2005) tested the activity of the native chlorogenic acid esterase for the methyl esters of hydroxycinnamates, while Benoit et al. (2007) investigated F-A and F-AX as the substrates for the recombinant enzyme, but none of these substrates was hydrolyzed.

5.3.5 Release of phenolic acids from the natural substrates DSWB and CP

The purified enzyme was investigated for its ability to release phenolic acids from agroindustrial waste material. Besides the classical esterase substrate DSWB, the agroindustrial by-product CP was selected due to its high content of chlorogenic acid. After alkaline hydrolysis, total caffeic, ferulic, and *p*-coumaric acids were quantified by HPLC for CP (1.65 mg g^{-1}, 0.53 mg g^{-1}, and 0.07 mg g^{-1}, respectively) and DSWB (none, 4.45 mg g^{-1}, and 0.18 mg g^{-1}, respectively). Furthermore, CP contained 2.66 mg g^{-1} of chlorogenic acid in total. After incubation of CP with UmChlE (0.02 U for 20 h at 37 °C and at 650 rpm), 68 % of the initial chlorogenic acid amount was hydrolyzed. The native as well as the recombinant chlorogenic acid esterases from *A. niger* released 100 % of the initial chlorogenic acid content of CP after incubation overnight, but the enzymes were used at 2- to 6-fold higher concentrations than that of UmChlE (Asther et al. 2005; Benoit et al. 2007). UmChlE also liberated significant amounts of phenolic acids from CP. Thus, 18 %, 15 %, and 26 % of alkali-extractable caffeic, ferulic, and *p*-coumaric acids were released, respectively. The high content of alkali-extractable caffeic acid compared to the total amount of chlorogenic acid can be explained by the existence of caffeic acid conjugates other than chlorogenic acid, such as dicaffeoyl quinic acid or esters of feruloyl-caffeoyl quinic acid (Clifford 1999).

UmChlE (0.02 U) liberated small amounts of ferulic acid from DSWB. Simultaneous incubation with *T. viride* xylanase (0.025 U) increased the yield of released ferulic acid from DSWB 12-fold, whereas incubation with *T. viride* xylanase liberated no ferulic acid at all. The observed synergistic action of xylanases and feruloyl esterases was reported previously (Faulds & Williamson 1995; Faulds et al. 2002; Garcia-Conesa et al. 2004; Moukouli et al. 2008). This result can be explained by the action of endoxylanases producing shorter-chain xylooligosaccharides, which are more accessible substrates for esterases.

5.3.6 Amplification and genetic characterization of the UmChlE sequence

Seven tryptic peptides of the purified chlorogenic acid esterase were obtained by ESI-MS/MS: ADATASAPTVK (P1), FAKPQPLGPASSHK (P2), HPTVEQSFKR (P3), LANGVGCTGG SLLR (P4), VWSYEFQQNDK (P5), EAMDALTNR (P6), and AKPPVGSLR (P7). A homology search with BLASTP (NCBI BLAST) identified these peptides (P1 to P7) as being part of the hypothetical protein Um00182.1 from *U. maydis* 521 (GenBank accession no. XP_756329.1). The hypothetical protein Um00182.1, annotated via a genome project, was classified as a member of the esterase_lipase superfamily containing a region specific for carboxylesterases.

After RNA isolation from *U. maydis* cultures grown for 13 days in 10 % (v/v) SNL medium supplemented with 1 % (w/v) wheat bran, cDNA was synthesized, and the sequence of the UmChlE (GenBank accession no. HG970190) was successfully amplified by using UTR primers. The complete coding sequence of UmChlE was 1,758 bp, corresponding to a protein of 585 amino acids (aa). The full amino acid sequence of UmChlE possessed three differences at the nucleotide level, which led to one change at the amino acid level at position 492 (proline to leucine) compared to the sequence of Um00182.1. The UmChlE sequence showed the highest similarity (89 %) and identity (81 %) to a carboxylesterase from *Pseudozyma hubeiensis* SY62 (GenBank accession no. GAC96757). This organism is an anamorph member of the order *Ustilaginales* (Basidiomycota). Only 29 % identity was obtained with an amino acid sequence of the chlorogenic acid esterase ChlE from *A. niger* (GenBank accession no. DQ993161). A signal peptide of 21 aa was identified at the N-terminus of the UmChlE sequence based upon prediction using SignalP 4.01. The predicted molecular mass of the mature protein was calculated to be 63.7 kDa by using the Compute pI/*Mw* tool of ExPASy. The discrepancy in the molecular mass deduced from denaturing SDS-PAGE (71 kDa) indicated a glycosylated protein. Nine potential *N*-glycosylation sites were identified, at positions 5, 109, 151, 298, 308, 370, 387, 487, and 558 (http://www.cbs.dtu.dk/services/NetNGlyc/). Treatment with endoglycosidase H resulted in a deglycosylated protein of 63 kDa (**Fig. 5-1**, **lane 3**), suggesting that UmChlE contained Asn-linked high-mannose oligosaccharides. Similar results were described for the chlorogenic acid esterase ChlE from *A. niger* (Benoit et al. 2007). For ChlE, a mass difference of 25 kDa was caused by glycosylation. Consequently, UmChlE had a lower degree of *N*-glycosylation than did ChlE. This may explain the relative thermolability of UmChlE, as it has been reported that *N*-glycosylation is important not only for protein folding but also for thermostability (Benoit et al. 2006a).

The sequence of UmChlE contained a G-X-S-X-G motif (**Fig. 5-3**) characteristic of the serine esterase family (Brenner 1988; Dodson & Wlodawer 1998).

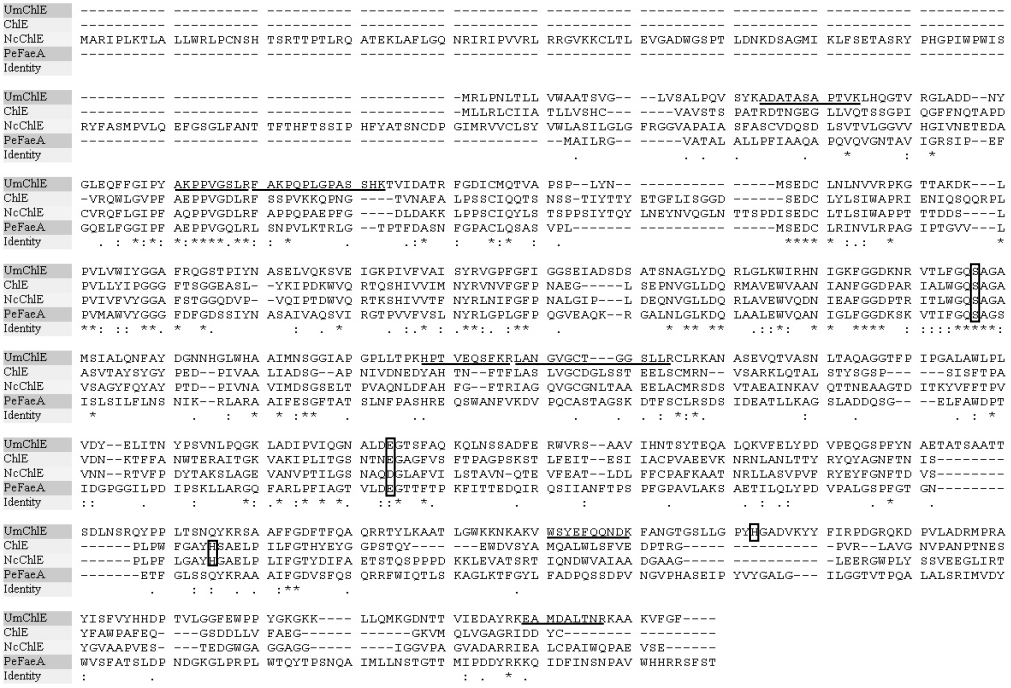

Fig. 5-3 Amino acid sequence alignment of *Ustilago maydis* UmChlE (GenBank accession no. HG970190 [this work]) with the chlorogenic acid esterase from *Aspergillus niger* (ChlE) (GenBank accession no. DQ993161), the chlorogenic acid esterase from *Neurospora crassa* (NcChlE) (GenBank accession no. EAA32507.3), and the feruloyl esterase from *Pleurotus eryngii* (PeFaeA) (GenBank accession no. CDI44666.1). Peptide fragments identified by ESI-MS/MS analysis are underlined. The catalytic triad of serine, glutamic/aspartic acid, and histidine is highlighted by boxes.

Here, the motif G-Q-S-A-G was the same as those in the sequences of ChlE from *A. niger* (GenBank accession no. DQ993161) and NcChlE from *Neurospora crassa* (GenBank accession no. EAA32507.3). This motif was also found in feruloyl esterases of *P. eryngii* PeFaeA (Nieter et al. 2014) and *P. sapidus* Est1 (Linke et al. 2013), while other feruloyl esterases often possess G-H-S-L-G and G-C-S-T-G as conserved motifs (Koseki et al. 2009b; Shin & Chen 2007). The catalytic triad of UmChlE was predicted to consist of Ser228, Glu360, and His497 (http://pfam.sanger.ac.uk/). Likewise, ChlE contained a catalytic triad of Ser-Glu-His, while the annotated chlorogenic acid esterase from *N. crassa* (GenBank accession no. EAA32507.3) showed the glutamic acid being replaced by aspartic acid (**Fig. 5-3).** Udatha et al. (2011) reported a catalytic triad of Ser-Asp-His for 324 analyzed feruloyl esterase sequences. Nonetheless, two feruloyl esterases containing a catalytic triad of Ser-Glu-His (Linke et al. 2013; Nieter et al. 2014) are also known.

5.3.7 Heterologous expression of UmChlE in *P. pastoris*

To confirm that the annotated sequence (GenBank accession no. HG970190) encodes the characterized chlorogenic acid esterase UmChlE from *U. maydis*, the putative sequence was cloned in frame with the *Saccharomyces cerevisiae* α-factor secretion signal sequence under the transcriptional control of the alcohol oxidase (*AOX1*) promoter and integrated into *P. pastoris* GS115. Expression constructs were generated with and without the native signal sequence. A hexahistidine tag was incorporated at the C-terminus. Only the transformants carrying the expression construct without the native signal sequence showed chlorogenic acid esterase activity. After induction with 0.5 % (v/v) methanol, these *P. pastoris* transformants expressed the recombinant gene successfully and secreted the active enzyme into the culture medium. After methanol induction for 96 h, the chlorogenic acid esterase activity of the culture medium (0.8 U L^{-1}) was 8-fold higher than that in control cultures of *P. pastoris* GS115.

5.4 Conclusion

An extracellular chlorogenic acid esterase from *U. maydis*, UmChlE, was purified to homogeneity and characterized. Compared to previously described chlorogenic acid esterases, UmChlE displayed a surprisingly broad substrate profile. The enzyme's unique properties may contribute to the specific environmental interaction between the pathogenic fungus and its host, *Zea mays*. Additionally, the release of caffeic, *p*-coumaric, and ferulic acids from agroindustrial by-products such as DSWB and CP renders this enzyme an attractive candidate for the generation of natural aroma precursors and antioxidants.

A recent study in mice did not confirm some of the supposed beneficial effects of dietary chlorogenic acid (Mubarak et al. 2013). Depending on the outcome of this debate, UmChlE could provide a general and efficient tool for reducing dietary risks involved in the consumption of higher levels of chlorogenic acid. The successful heterologous expression of active UmChlE in *P. pastoris* opens up new opportunities for future studies, e.g., for the production of sufficient enzyme for X-ray crystallography, which could help to explain the exceptional substrate specificity, and for future enzyme engineering.

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6. Vorwort zur Publikation "Heterologous production and characterization of a chlorogenic acid esterase from *Ustilago maydis* with a potential use in baking industry"

Mit einem jährlichen durchschnittlichen Pro-Kopf-Verbrauch in Europa von etwa 65 kg stellt Brot eines der wichtigsten und verbreitetsten Lebensmittel dar (Hoseney et al. 2008). Studien zufolge bedienen Europäer etwa die Hälfte ihres Kohlenhydrat- und etwa ein Drittel ihres Proteinbedarfs aus Brot und Backwaren (Uhlig 1998). Um diese Nachfrage zu decken, werden weltweit über 6 x 10^6 t Weizen pro Jahr angebaut (Shewry & Tatham 2000). Bedingt durch die Getreidesorte, den Standort und die klimatischen Begebenheiten ist die Fähigkeit einiger Länder, hochqualitative Mehle zu produzieren, limitiert. Um Schwankungen in der Mehlqualitität auszugleichen und Mehle mit konstant guten Backeigenschaften zu erzeugen, werden seit Jahrzehnten Zusatzstoffe wie Oxidantien und Emulgatoren den Teigen beigefügt. Im Zuge der fortschreitenden Mechanisierung in der Backwarenindustrie und der resultierenden erhöhten Nachfrage nach Teigen mit einer hohen Verarbeitungstoleranz und Fermentationsstabilität sowie als Reaktion auf die Entwicklung der Verbrauchermentalität nach „natürlichen" Produkten, haben Enzyme einen hohen Stellenwert in der Backwarenindustrie erlangt (Caballero et al. 2007). Hierbei stellt die enzymatische Behandlung von Mehlen eine interessante Alternative dar, um die Struktur von Teigen zu verändern. Ferner bringen Enzyme den Vorteil mit sich, dass sie nach dem Backvorgang nicht mehr aktiv im Endprodukt vorliegen und dementsprechend nicht auf dem Etikett der Backwaren gelistet werden müssen, da es sich um nicht-deklarierungspflichtige Verarbeitungshilfsstoffe handelt (Art. 20 Buchst. b Verordnung (EU) Nr. 1169/2011 in Verbindung mit Art. 3 Abs. 2 Buchst. b Verordnung (EG) Nr. 1333/2008). Allerdings fanden Enzyme nicht vor dem 20. Jahrhundert als Mehlbehandlungsmittel Anwendung (Mutsaers 1997). Heutzutage steht den Bäckern eine große Auswahl an Enzymen zur Verfügung, um verschiedene Qualitätsaspekte der Backwaren, wie beispielsweise das Aroma, das spezifische Volumen, die Krumenstruktur oder die Haltbarkeit zu beeinflussen (Haros et al. 2002; Hilhorst et al. 1999; Romanowska et al. 2006). Die zuvor beschriebene Evolution in der Backwarenindustrie lässt sich anhand der weltweiten Entwicklung des Backenzymbedarfs belegen. Während der Marktanteil von Backenzymen im Jahr 2000 140 Mio. Dollar betrug, erhöhte er sich im Jahr 2010 bereits auf 360 Mio. Dollar und soll Prognosen zufolge bis zum Jahr 2020 auf 900 Mio. Dollar ansteigen (Miguel et al. 2013). Neben dem traditionellen Einsatz von Hydrolasen (α-Amylasen (EC 3.2.1.1), Xylanasen (EC 3.2.1.8), Lipasen (EC 3.1.1.3) und Peptidasen (EC 3.4.-.-)) werden mittlerweile auch

Enzyme zur Verknüpfung der Makromoleküle des Mehls wie Laccasen (EC 1.10.3.2) oder Peroxidasen (EC 1.11.1.7) in der Backwarenindustrie verwendet (vgl. **Tab. 6-1**).

Tab. 6-1 Übersicht über die bisherigen Hauptenzyme der Backwarenindustrie und deren Effekte (nach Kornbrust 2012).

Effekte	**Enzyme**				
	α-Amylase	Peptidase	Xylanase	Lipase	Glucose-oxidase
Erhöhung der Gasproduktion und Teigfermentation	x				
Verbesserung der Teigkonditionierung, Teigstabilität und Krumenstruktur	x		x	x	
Stärkung des Glutennetzwerkes, Ersetzen von oxidierenden Agenzien					x
Schwächung des Glutennetzwerkes, Verbesserung der Teigdehnbarkeit		x			
Erhöhung des Gebäckvolumens	x		x	x	x
Krustenfarbenentwicklung	x				
Aufhellung der Krume				x	x
Haltbarkeitsverlängerung	x				
Ersetzen von Emulgatoren				x	

Zur Veränderung von rheologischen Eigenschaften der Teige sind die teilweise mit FA-Resten veresterten AX ein zentraler Angriffspunkt. Dabei sind FA-Reste involviert in die oxidative Vernetzung von AX miteinander als auch von AX mit Glutenproteinen (vgl. Kapitel 1.1.1). AX werden in zwei Typen unterteilt. Während die Mehrheit der AX zur Gruppe der wasserunlöslichen AX (WUAX) gehört, werden ein Viertel bis zu einem Drittel aller AX den wasserlöslichen AX (WEAX) zugeordnet (**Fig. 6-1**). Der Grad der Wasserlöslichkeit der AX ist abhängig von ihrer molaren Masse und dem Substitutionsgrad der Seitenketten (Courtin & Delcour 2002). Die Wasserunlöslichkeit der WUAX ergibt sich aus ihrer Lokalisation. Infolge von kovalenten Bindungen und nicht-kovalenten Interaktionen mit benachbarten AX oder anderen Zellwandkomponenten wie Proteinen, Cellulose und Lignin verbleiben die WUAX mit der Zellwand verankert (Iiyama et al. 1994), wohingegen die WEAX relativ locker an der Zellwandoberfläche gebunden sind (Mares & Stone 1976). Neben ihrem Verzweigungsgrad unterscheiden sich WUAX und WEAX in ihrem Wasserbindevermögen (9,9 g Wasser/g WUAX und 6,3 g Wasser/g WEAX) (Courtin & Delcour 2002; Kulp 1968). Schätzungen zufolge halten AX ein Viertel des Wassers im Teigsystem (Atwell 1997). Obwohl AX nur in

geringen Mengen im Weizenmehl vorhanden sind, haben sie einen signifikanten Einfluss auf die funktionellen Teigeigenschaften. WUAX sind bekannt für eine Verringerung des Brotvolumens (Krishnarau & Hoseney 1994). Die Ursachen für den negativen Effekt der WUAX auf die Backprodukte sind noch nicht komplett verstanden. Ein möglicher Erklärungsansatz ist, dass durch die Konkurrenz um Wasser, dieses für die Ausbildung des Glutennetzwerkes unzugänglich wird. Weiterhin destabilisiert WUAX die Gaszellen im Glutennetzwerk durch die Ausbildung physikalischer Barrieren während der Teigentwicklung (Goesaert et al. 2005).

Um die Struktur und Eigenschaften der AX in den Teigen zu modifizieren, kam es in den 1970er Jahren zur Einführung von Xylanasen in der Backwarenindustrie (Miguel et al. 2013). Zahlreiche xylanolytische Enzyme sind mittlerweile für die Brotproduktion auf dem Markt verfügbar (Bsp. VERON® 191; 292; 393 von AB Enzymes oder Panzea® 10X; BG von Novozymes), und weitere Quellen für Xylanasen werden untersucht. Ognean et al. (2011) zeigten, dass, bedingt durch die zugesetzte Xylanase, unterschiedliche technologische Effekte im Backprodukt erzielt werden können. In Abhängigkeit vom Backprodukt und dessen angestrebter Qualität, der Teigverarbeitung und den Teigeigenschaften werden Xylanasen benötigt, die spezifisch für die WUAX oder WEAX sind (**Fig. 6-1**).

Für die Herstellung von Brot finden präferiert Xylanasen Anwendung, die WUAX solubilisieren. Studien zufolge führt die Umwandlung von WUAX in WEAX nicht nur zu einer erhöhten Teigstabilität (Rouau 1993), sondern auch zu einem verbesserten Backverhalten (Goesaert et al. 2006; Hilhorst et al. 2002). Eine umfassende Hydrolyse der WUAX bewirkt eine Umverteilung von Wasser aus dem AX-Netzwerk in die Gluten- und Stärkephase. Im Verlauf der Umwandlung der AX in ihre wasserlösliche Form wird das AX-Protein-Netzwerk aufgelockert und Wasser entlassen (Verjans et al. 2010). Gleichzeitig erhöht sich die Viskosität infolge der Zunahme an hochmolekularen solubilisierten AX (SAX). Aus dieser Modifikation des AX Gefüges resultieren weichere, viskosere und insgesamt leichter zu handhabende Teige (Laurikainen et al. 1998), woraufhin ein besserer Ofenbetrieb, eine Volumenzunahme sowie eine feinere, weichere und homogenere Krume im Backprodukt erzielt werden (Courtin et al. 1999; Heldt-Hansen 2005). Insbesondere das spezifische Volumen sowie eine weiche und einheitliche Krumenstruktur werden als Hauptqualitätsparameter von Brot betrachtet (Steffolani et al. 2012).

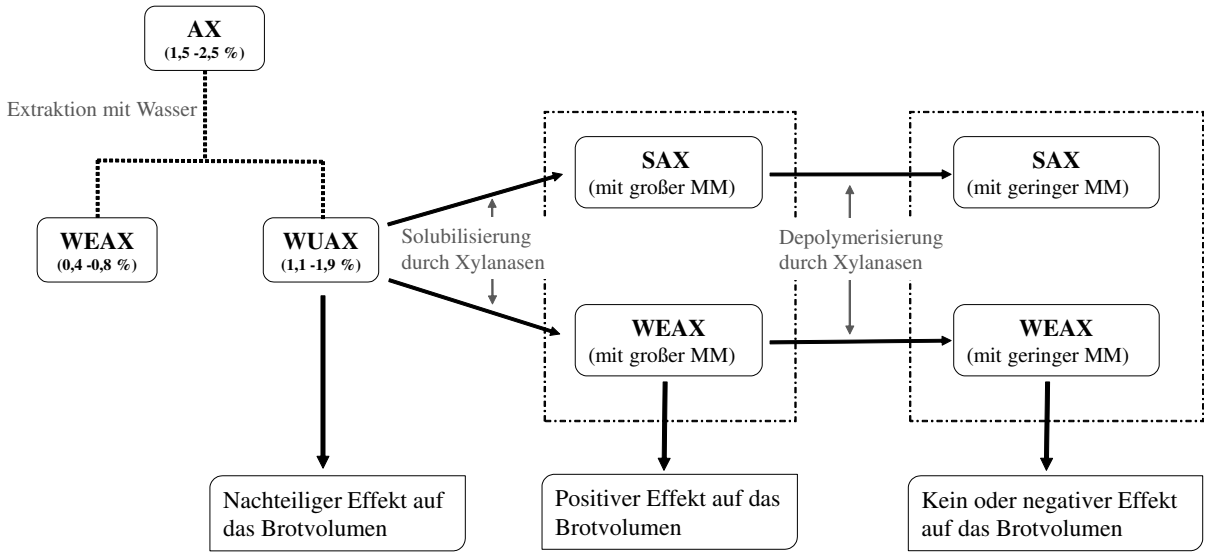

Fig. 6-1 Schematische Darstellung der enzymatischen Hydrolyse des Arabinoxylangefüges von Weizenmehlen durch Xylanasen und die Effekte verschiedener Arabinoxylanfraktionen auf das Brotvolumen (modifiziert nach Courtin et al. 2002 und Goesaert et al. 2005).

Romanowska et al. (2006) demonstrierten in ihrer Studie die Eignung einer Endoxylanase von *A. niger* IBT-90 für die Backwarenproduktion. Neben der Verbesserung der Kneteigenschaften des Teiges wurde eine Volumenzunahme, eine bessere Krumenporosität, eine erhöhte Feuchtigkeitsspeicherung und eine verlängerte Haltbarkeit erzielt. Vergleichbare Effekte sind beschrieben für eine Xylanase aus *Aspergillus* sp., *Penicillium occitanis* und *Thermotoga maritima*, welche das spezifische Brotvolumen um 30 % (Camacho & Aguilar 2003), 34,9 % (Driss et al. 2013) bzw. um 60,3 % (Jiang et al. 2005) erhöhten. Ferner wurde eine Reduktion der Krumenfestigkeit durch die Aktivität der Xylanasen aus *P. occitanis* (43,5 %), *T. maritima* (57,9 %) und *A. foetidus* (77 %) erzielt (Driss et al. 2013; Jiang et al. 2005; Shah et al. 2006).
Demgegenüber werden für die Produktion von trockenen Getreideerzeugnissen wie Knäckebrot, Kräckern, Waffeln und Keksen Xylanasen mit einer Spezifität für WEAX und SAX eingesetzt. Für die Herstellung dieser Backwaren bedarf es dehnbarer Teige mit einer verringerten Teigresistenz und Viskosität (Aehle 2007). Außerdem wird die erhöhte Wasserbindungskapazität der WUAX ausgenutzt, um eine zu schnelle Verdunstung von Wasser beim Backen zu verhindern. Im Verlauf dessen kommt es zu verlängerten Backzeiten und dunkleren Produkten (Aehle 2007). Eine Präferenz für die Hydrolyse von WEAX ist für die Xylanasen aus *A. aculeatus* (Frederix et al. 2003), *Trichoderma* sp. (Valeri et al. 2013) und A. *niger* (Biely et al. 1997) in der Literatur beschrieben.
Während Xylanasen zahlreich in der Backwarenindustrie eingesetzt werden, um die Kettenlänge der AX und damit die Qualität der Backwaren zu modifizieren (Miguel et al. 2013), existiert lediglich eine Studie, die das Potential von Ferulasäureester-spaltenden Enzymen für

die Backwarenindustrie verdeutlicht (Crepin 2003). Faulds et al. (2003) untersuchten die Spezifität von drei FAE (Repräsentanten der FAE Typen A, B und C) gegenüber feruloylierten wasserlöslichen AX (WEAX) und wasserunlöslichen AX (WUAX), um den aussichtsreichsten FAE Typ für Backversuche zu selektieren. Basierend auf der höchsten Freisetzung von FA aus WEAX durch die Typ C FAE aus *T. stipitatus* (TsFaeC) im Vergleich zur Typ A FAE aus *A. niger* (AnFaeA) und Typ B FAE aus *Neurospora crassa* (NcFae-1) wurden Backversuche mit TsFaeC durchgeführt (Faulds et al. 2003). Der Zusatz von TsFaeC zum Teig führte zu einem erhöhten Brotvolumen, zur Verbesserung der Krumenweichheit und der Krustenfarbe.

Nachdem die Chlorogensäureesterase UmChlE aus *U. maydis*, wie in Kapitel 5.3.7 beschrieben, heterolog in *P. pastoris* produziert werden konnte, beschreibt die nachfolgende Veröffentlichung die Optimierung der Produktionsbedingungen zur Steigerung der Esterase-Aktivitäten sowie ein *Up-Scaling* der Kultivierung, um entsprechende Mengen an rekombinanter UmChlE für Backversuche zu gewinnen. Außerdem erfolgte ein Vergleich der biochemischen Charakteristika des rekombinanten und des nativen Enzyms. Nachdem die Fähigkeit zur Hydrolyse von Ferulasäureestern aus komplexen natürlichen Substraten für die native UmChlE erfolgreich aufgezeigt wurde (Kapitel 5.3.4), sollte ferner der Einfluss der rekombinanten UmChlE auf die Teigrheologie und die Qualität der Backprodukte untersucht werden.

7. Heterologous production and characterization of a chlorogenic acid esterase from *Ustilago maydis* with a potential use in baking industry

Highlights

- Functional production of *Ustilago maydis* chlorogenic acid esterase in *Pichia pastoris*
- Distinguished from other chlorogenic acid esterases by its unique substrate profile
- The enzyme preferred substrates typical of graminaceous monocots
- Phenolic acids were released from complex side-streams of food processing
- The enzyme effectively modified the rheological properties of wheat dough

Abstract

Ustilago maydis, an edible mushroom growing on maize (*Zea mays*), is consumed as the food delicacy *huitlacoche* in Mexico. A chlorogenic acid esterase from this basidiomycete was expressed in good yields cultivating the heterologous host *Pichia pastoris* on the 5 L bioreactor scale (reUmChlE; 45.9 U L^{-1}). In contrast to previously described chlorogenic acid esterases, the reUmChlE was also active towards feruloylated saccharides. The enzyme preferred substrates with the ferulic acid esterified to the O-5 position of arabinose residues, typical of graminaceous monocots, over the O-2 position of arabinose or the O-6 position of galactose residues. Determination of k_{cat}/K_m showed that the reUmChlE hydrolyzed chlorogenic acid 18-fold more efficiently than methyl ferulate, *p*-coumarate or caffeate. Phenolic acids were released by reUmChlE from natural substrates, such as destarched wheat bran, sugar beet pectin and coffee pulp. Treatment of wheat dough using reUmChlE resulted in a noticeable softening indicating a potential application of the enzyme in bakery and confectionery.

Keywords

Basidiomycete; esterase; chlorogenic acid; feruloylated saccharides; coffee pulp; heterologous expression; *Pichia pastoris*; baking

7.1 Introduction

Chemical additives, such as emulsifiers or oxidants, are used in bread making for decades to adjust the variations of flour quality and processing parameters (Caballero et al. 2007). In recent years, the baking industry started to replace them by enzymes (Haros et al. 2002). Nowadays, due to increasing mechanization in the baking process and the increasing demand for natural products, enzymes have gained a key role. The treatment of flours with enzymes, such as α-amylases, transglutaminases, glucose oxidases, xylanases and peptidases results in a changed dough structure, improved dough handling, specific volume, water retention, crumb structure, fresh bread quality and shelf life (Haros et al. 2002; Hilhorst et al. 1999; Romanowska et al. 2006). Especially cereal flour pentosans are known to affect distinctly the quality of dough and bakery products (Rouau 1993). These non-starch polysaccharides mainly consist of arabinoxylans (AX) with a linear backbone of 1,4-linked β-D-xylopyranosyl units, of which up to 50 % are substituted at either O-2, O-3 or both positions by α-L-arabinofuranose residues (Faulds et al. 2003). Some of the arabinose residues on the AX backbone are further O-5 esterified with ferulic acid. Wheat AX are classified into two types: One-fourth to one-third are easily soluble in water (WEAX), while the remainder are water-insoluble AX (WUAX). WUAX were reported to decrease the bread volume (Krishnarau & Hoseney 1994), whereas the presence of WEAX affected the dough positively by increasing the viscosity due to their high water binding capacity (Rouau 1993). The reasons for the negative effect of WUAX on the baking products are not completely understood. One explanation is related to their detrimental effect on the gluten network formation caused by the competition for water and the destabilization of the gas cells during dough development (Goesaert et al. 2005). To modify the AX structure, xylanases (EC 3.2.1.8) were introduced in the 1970s in the baking sector (Miguel et al. 2013). They hydrolyze the AX backbone, causing a partial degradation of the AX network. Depending on the type of baked product, on dough processing and on dough properties, xylanases specific for WUAX or rather for WEAX are required. In bread-making, most commonly xylanases are used with preferential activity for WUAX. The solubilization of WUAX causes a redistribution of water to the gluten and starch phases and an increase in dough viscosity due to the formation of soluble AX (SAX). Consequently, these changes improve dough handling properties and stability, oven spring and specific loaf volume (Courtin & Delcour 2001; Hilhorst et al. 1999; Rouau 1993). In contrast, xylanases used in dry cereal products, such as biscuits, crackers and wafers, should be specific for WEAX and SAX to produce extensible doughs with a decreased resistance and viscosity (Aehle 2007).

Another possibility to change the AX structure and subsequently their water binding capacity is the hydrolysis of side chains of the AX. Over the last years, a number of enzymes have been identified in bacteria and fungi hydrolyzing the ester linkage between ferulic acid and complex plant cell wall polysaccharides, such as arabinose substituents on AX (Williamson et al. 1998). One group of these enzymes are feruloyl esterases (FAE; EC 3.1.1.73), a subclass of the carboxylic ester hydrolases (EC 3.1.1.-).

Recently, a chlorogenic acid esterase (EC 3.1.1.42) was isolated from the basidiomycete *U. maydis* (Nieter et al. 2015). The substrate screening showed that the UmChlE was not solely active on chlorogenic acid as previously published chlorogenic acid esterases (Asther et al. 2005; Benoit et al. 2007; Schöbel & Pollmann 1980a, 1980b), but hydrolyzed also typical FAE substrates, such as methyl ferulate, *p*-coumarate and caffeate, as well as destarched wheat bran (DSWB) and feruloylated saccharides. Furthermore, a synergistic interaction of UmChlE with carbohydrases, such as xylanases and pectinases, was observed for the enzymatic hydrolysis of DSWB and sugar beet pectin (SBP). The full length sequence of *CHLE* was amplified and the enzyme was recombinantly produced in *P. pastoris*.

In this study, the production of the reUmChlE was scaled up in a 5 L bioreactor. Subsequently, the enzyme was purified and characterized to compare its properties with the native enzyme. As the native enzyme released ferulic acid from AX in DSWB, the effect of this enzyme on dough rheology was investigated using the Kieffer dough and gluten extensibility rig. The reUmChlE was also combined with a xylanase to determine the effect on the specific loaf volume.

7.2 Material and methods

7.2.1 Chemicals and substrates

All chemicals were purchased in the required purity grade from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) or Sigma-Aldrich (Taufkirchen, Germany), unless otherwise stated. Methyl and ethyl ferulate (99 %; MFA and EFA, respectively) were obtained from Alfa Aesar (Karlsruhe, Germany). Methyl caffeate (CA-ME), *p*-coumarate (*p*CA-ME), and sinapate (SA-ME) were synthesized according to Borneman et al. (1990). Feruloylated saccharides (5-O-*trans*-feruloyl-L-arabinofuranose (F-A); 2-O-*trans*-feruloyl-*α*-L-arabinofuranosyl-(1→5)-L-arabinofuranose (Ara_2F); 6-O-*trans*-feruloyl-*β*-D-galactopyranosyl-(1→4)-D-galactopyranose (Gal_2F); *β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AX); *α*-L-galactopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AXG); D-xylopyranosyl-(1→4)-*β*-D-xylopyranosyl-

(1→3)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AXX)) were prepared from destarched corn bran (Allerdings et al. 2006; Schendel et al. 2015). Sugar beet pectin was provided from Herbstreith & Fox (Neuenbürg, Germany), coffee "Guatemala Grande" was from Tchibo (Hamburg, Germany), and wheat bran was from Alnatura (Lorsch, Germany). PCR primers were supplied by Eurofins MWG Operon (Ebersberg, Germany).

7.2.2 Production of recombinant UmChlE in *P. pastoris*

P. pastoris GS115 was used for heterologous expression of the *CHLE* gene that encodes for the chlorogenic acid esterase from *U. maydis* (GenBank accession no. HG970190). Expression constructs consisting of the *CHLE* sequence without its native signal sequence, but a C-terminal *HIS*-tag sequence, were cloned into the pPIC9 vector (Invitrogen, Karlsruhe, Germany), as described elsewhere (Nieter et al. 2015). After transformation and selection on histidine-deficient MD plates (13.4 g L^{-1} YNB without amino acids, 400 µg L^{-1} biotin, 20 g L^{-1} glucose, 20 g L^{-1} agar, 50 mg L^{-1} each of L-glutamic acid, L-lysine, L-leucine, and L-isoleucin), 48 *P. pastoris* transformants were screened for chlorogenic acid esterase activity in 96-well-plates. For the microscale cultivation, the transformants were cultured at first in 600 µL YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) for 36 h at 28 °C and 320 rpm. Subsequently, the cells were harvested by centrifugation (10 min, 4000 x g), washed twice with ddH_2O to remove the residual glucose from the YPD medium, and finally they were resuspended in 600 µL buffered complex BMMY medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 13.4 g L^{-1} YNB without amino acids, 400 µg L^{-1} biotin, 100 mM potassium phosphate pH 6.0, 0.5 % (v/v) methanol, 50 mg L^{-1} each of L-glutamic acid, L-lysine, L-leucine, and L-isoleucine) to start the expression. Culture supernatants were analyzed for enzyme activity after 24, 48, 72, 96 and 120 hours. To maintain stable expression conditions, 0.5 % (v/v) methanol was added every 24 hours. For the optimization of the expression conditions, buffered minimal medium (BMM; 13.4 g L^{-1} YNB without amino acids, 400 µg L^{-1} biotin, 100 mM potassium phosphate pH 6.0, 0.5 % (v/v) methanol, 50 mg L^{-1} each of L-glutamic acid, L-lysine, L-leucine, and L-isoleucine) was also used.

For up-scaling, *P. pastoris* precultures were cultivated in 300/500 mL shake flasks containing 50/100 mL YPD medium. Precultures were grown at 28 °C on an orbital shaker (Infors AG, Bottmingen, Switzerland) at 180 rpm for 48 h and were used for inoculation of the baffled flask and bioreactor cultures to an OD_{600} of 1.

Shake flask cultivation was performed in 500 mL baffled flasks containing 100 mL BMMY medium at 180 rpm and 18 °C for 72 h. These conditions were selected as a result of

optimization of the expression conditions, as described below. Daily, culture supernatants were screened for enzyme activity and 2 % (v/v) methanol was added to maintain induction.

For further up-scaling of the reUmChlE production in *P. pastoris*, a batch fermentation was carried out at 5 L scale in a stainless steel stirred tank reactor (KG5000, Medorex AG, Nörten-Hardenberg, Germany) with marine-type stirrer. Fermentation was performed in complex BMMY medium pH 6.0 containing 2 % (v/v) methanol, while the pH was not adjusted during fermentation. In the beginning, 0.1 mL L^{-1} antifoam agent (Tego KS800, Evonik Industries AG, Essen, Germany) was added to the medium. Following 16 h at 24 °C the temperature was kept at 18 °C until the end of the fermentation process (another 56 h). To maintain induction of gene expression 2 % (v/v) methanol was added every 24 h. The oxygen was supplied by using a constant flow of compressed air (5 L air/min), and dissolved oxygen saturation was monitored over the fermentation process using an oxygen electrode (Oxy Probe, Broadley James Corporation, Irvine, USA). By using a stirrer speed of 500 rpm the dissolved oxygen saturation was maintained throughout the process above 70 % of the initial value.

7.2.3 Purification of reUmChlE

The *P. pastoris* culture supernatant was separated from the cells by centrifugation (15,000 x g; 4 °C) and subsequently concentrated about 10-fold using a Sartocon slice cassette (Hydrosart, 10 kDa cut off, Sartorius, Göttingen, Germany). Afterwards, the His-tagged reUmChlE was purified under native conditions using a semi-batch method with Protino Ni-NTA Agarose (Macherey-Nagel, Düren, Germany). 18 mL concentrated culture supernatant were mixed with 2 mL of eightfold binding buffer (4 M NaCl, 40 mM imidazole, 160 mM Tris pH 7.9) and incubated with 3 mL Ni-NTA Agarose in a closed column for 60 min on a rocking shaker (Labnet, Edison, USA) at 4 °C. Purification was performed according to manufacturer's instructions with modified buffers. The column was washed three times (W0) with 10 mL of binding buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris pH 7.9), followed by 2 x 5 mL of wash buffer 1 (W1; 0.5 M NaCl, 15 mM imidazole, 20 mM Tris pH 7.9), 2 x 5 mL of wash buffer 2 (W2; 0.5 M NaCl, 30 mM imidazole, 20 mM Tris pH 7.9) and 5 mL of wash buffer 3 (W3; 0.5 M NaCl, 45 mM imidazole, 20 mM Tris pH 7.9). The target protein was eluted with 3 mL of elution buffer (0.5 M NaCl, 500 mM imidazole, 20 mM Tris pH 7.9). Eventually, the elution fractions were re-buffered to 25 mM Bis-Tris buffer pH 6.0 with PD-10 desalting columns (GE Healthcare, Little Chalfont, UK). The purification was monitored using denaturing SDS-PAGE analysis (12 % resolving gel, 4 % stacking gel) according to Laemmli (1970).

7.2.4 Peptide mass fingerprint

Amino acid sequences of tryptic peptides of the reUmChlE were deduced from ESI-MS/MS using a maXis QTOF mass spectrometer (Bruker, Bremen, Germany). The procedure was described in detail elsewhere (Linke et al. 2013).

7.2.5 Activity assay

The esterase activity was determined by HPLC as previously described (Nieter et al. 2015), using chlorogenic acid and methyl ferulate as standard substrates. The assay was terminated after 30 min incubation at 37 °C to avoid substrate limitation due to the high activity of the reUmChlE. Enzyme activities were expressed as units, and all measurements were performed at least twice.

7.2.6 Biochemical characterization of reUmChlE

The pH and temperature optimum or stability, substrate specificity and kinetic parameters of the reUmChlE were determined as described for the native enzyme (Nieter et al. 2015). For all measurements the incubation time with the substrate was shortened to 30 min.

7.2.7 Release of phenolic acids from natural substrates

DSWB was prepared as described by Johnson et al. (1988). Total ferulic, *p*-coumaric and caffeic acid content in SBP, DSWB and coffee pulp (CP) was quantified after alkaline hydrolysis using 2 M NaOH (4 h, 50 °C, 220 rpm). Phenolic acid content of each sample was analyzed after acidification with acetic acid using HPLC. The concentration of chlorogenic acid in CP was determined after treatment with 80 % (v/v) methanol. DSWB (20 mg), CP (20 mg) as well as SBP (2 mg) were incubated (37 °C, overnight, 650 rpm) with purified reUmChlE (10 mU) in a final volume of 400 µL containing 125 mM Tris buffer pH 7.5. To investigate synergistic interaction with carbohydrases, the release of ferulic acid was also analyzed after simultaneous incubation of reUmChlE with either *Trichoderma viride* xylanase (DSWB, 0.025 U, Fluka, Buchs, Switzerland) or pectinase preparations (SBP, SternEnzym, Ahrensburg, Germany). HPLC analysis was used to quantify the amount of phenolic acids released from the natural substrates.

7.2.8 Rheological measurements of reUmChlE treated dough

Rheological dough properties were measured using a Kieffer dough and gluten extensibility rig fitted onto a TA.XT2 Texture Analyzer (Stable Micro Systems Ltd., Godalming, United Kingdom). Microextension Kieffer tests were carried out according to Kieffer et al. (1998):

42 g flour was adjusted to 60 % water absorption capacity. Afterwards, 25 ml water containing 3.4 % sodium chloride and the appropriate dosage of reUmChlE (0.1, 1 and 10 U, respectively) were added. After kneading for 1 min (Y1-ETK laboratory fork kneader), the dough was rounded for 30 s as well as sheeted for 30 s using a rounding and sheeting station. Intermediate proofing of the dough took place for 3 min at 90 % humidity in a desiccator. For each sample at least nine parallel doughs were analyzed. Values were calculated as percentage change to a reference dough, which was prepared without additional enzyme.

7.2.9 Baking trials

For baking trials, the basic doughs were prepared as follows: 1000 g flour, 1.5 % yeast, 1.8 % NaCl, 2 % Biskin (sunflour oil, palm oil and palm kernel oil, partially hardened; Peter Kölln GmbH & Co. KGaA, Elmshorn, Germany), 40 ppm ascorbic acid and 600 mL water. The effect of reUmChlE (0.6 – 3 U) on bun volume was studied in combination with a xylanase from *Bacillus licheniformis* (14 – 48 U). Dough without enzyme treatment served as reference. The dough was kneaded with a DIOSNA SP 12 (DIOSNA Dierks & Söhne GmbH, Osnabrück, Germany) with frequency converter at 25 Hz (2 min) and 50 Hz (6 min). After 10 min of first proofing, the dough was divided into 250 g pieces. After further proofing for 20 min at room temperature the dough pieces were placed in a fermentation chamber at 32 °C for 120 min (normal fermentation) or 150 min (over fermentation), respectively. Finally the dough pieces were baked at 220 °C for 30 min. The bread volume was determined by a rapeseed displacement method (AACC standard 10-05.01) and the ratio volume/mass (specific volume) was calculated.

7.3 Results and discussion

7.3.1 Improved expression of the *CHLE* gene in *P. pastoris*

To produce the UmChlE heterologously in *P. pastoris*, the *CHLE* gene was cloned without its native signal sequence in the expression vector pPIC9. The vector pPIC9 contains a methanol inducible alcohol oxidase 1 promotor (P*AOX1*) and the yeast α-factor signal sequence for secreted production of recombinant proteins. Additionally, a C-terminal hexahistidine-tag coding sequence was incorporated into the *CHLE* gene to facilitate subsequent purification of the target protein. After transformation and selection of *P. pastoris* GS115 with the construct pPIC9-*CHLE-HIS*, a first screening for esterase activity was performed. Low esterase activity was detected in the culture supernatants after induction with 0.5 % (v/v) methanol, as previously described (Nieter et al. 2015).

To improve the enzyme production, the 16 transformants of the microscale cultivation showing highest esterase activities were used to investigate the effect of culture media, methanol concentration and induction period on enzyme production. Highest esterase activities were obtained in the buffered complex BMMY medium containing 2 % (v/v) methanol (**Supplement 1A**). Increasing the methanol concentration from 0.5 to 2 % (v/v) methanol led on average to a threefold higher production of reUmChlE. Similar observations were made for the heterologous production of a lipoxygenase (Kelle et al. 2014) and lipases (Kumari & Gupta 2014) in *P. pastoris*.

The strongest increase in reUmChlE production was detected between 48 and 96 h (**Supplement 1B**). An extension of the induction period to 120 h resulted only in a slight additional increase. One transformant (UmChlE 45) was distinguished from the 15 others showing a fourfold higher esterase activity after 48 h of induction. As gene copy number in *P. pastoris* was shown to relate to the level of recombinant protein produced (Romanos 1995; Vassileva et al. 2001), it is conceivable that multiple copies of the *CHLE* gene containing expression cassette were integrated into the genome of the transformant showing the highest esterase activity.

Cultivation of *P. pastoris* at lower temperatures resulted in an increased level of secreted recombinant protein (Cassland & Jonsson 1999; Hong et al. 2002). The most promising transformant was cultivated in baffled shake flasks in BMMY medium containing 2 % (v/v) methanol at various temperatures in the range from 16 to 28 °C. The esterase activities using methyl ferulate as substrate increased by lowering the incubation temperature from 1.02 U L^{-1} (28 °C) over 5.80 U L^{-1} (24 °C) to 11.30 U L^{-1} (20 °C). Hong et al. (2002) achieved higher laccase activities by lowering the cultivation temperature from 30 °C to 20 °C. Cassland and Jonsson (1999) obtained fivefold higher laccase activities when the *P. pastoris* cultures were kept at 16 – 19 °C instead of 28 °C. Reported explanations for this effect are poor stability of the recombinant protein at higher temperatures, higher release of peptidases from dead cells, and problems of protein folding at higher temperatures (Hong et al. 2002). As shown in **Fig. 7-1**, lowering the incubation temperature to 18 °C further increased the esterase activity, while incubation at 16 °C resulted in a decreased esterase activity compared to 20 °C after 48 and 72 h. Moreover, the comparison of the esterase activities at different cultivation temperatures depending on the induction periods (48, 72, 96 and 120 h) suggested that, above all, an increased protein stability at lower temperatures plays a key role for obtaining higher enzyme activities. While the esterase activities for the cultivation at 16 °C and 18 °C,

respectively, were almost the same for 72 to 120 h, the activity decreased significantly with increasing cultivation time at 20 °C.

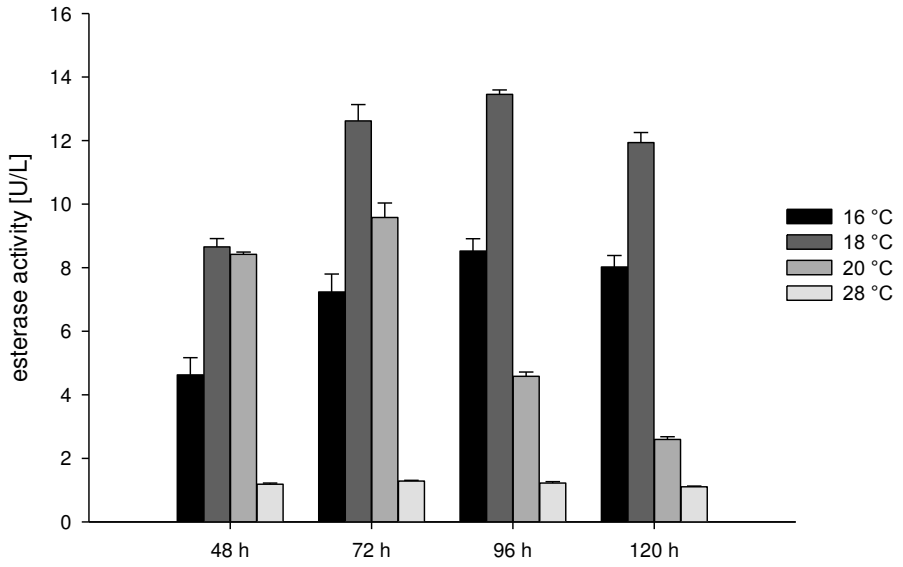

Fig. 7-1 Production of reUmChlE in *P. pastoris* depending on incubation temperature. Esterase activities of the culture supernatants were determined with HPLC using 1.95 mM methyl ferulate as substrate. Every 24 h the appropriate amount of methanol was added to maintain induction of gene expression in the *P. pastoris* pPIC9-*CHLE-HIS* transformants. The transformant showing highest esterase activity in the preliminary experiments was cultivated in baffled shake flasks at 16, 18, 20 and 28 °C, respectively. Cultivation took place in BMMY medium containing 2 % (v/v) methanol for 120 h. Error bars indicate standard deviations.

After optimization of the cultivation parameters and selection of UmChlE45 as the transformant producing the highest levels of reUmChlE, esterase activities of 12.6 U L^{-1} were found after 72 h of methanol induction in the culture supernatant which exceeded the productivity of the wild-type enzyme (2.9 U L^{-1} after 13 days of growth; (Nieter et al. 2015)) by a factor of 4.3.

7.3.2 Scale-up of recombinant chlorogenic acid esterase production

The scale-up of the reUmChlE production was necessary to obtain larger enzyme activities for the baking studies. The expression strain containing pPIC9-*CHLE-HIS* was cultivated in a 5 L bioreactor using batch fermentation. The bioreactor system allowed the monitoring of the oxygen saturation. Consequently, the most important parameter for efficient expression in *P. pastoris*, an adequate aeration during methanol induction, was continuously controlled. At the time of harvest the dissolved oxygen concentration was above 70 % indicating that there was no oxygen limitation. Esterase activities of 45.9 U L^{-1} confirmed the successful up-scaling of recombinant chlorogenic acid esterase production. On the 50-fold larger scale about fourfold higher esterase activities were achieved.

7.3.3 Purification of the reUmChlE

The purification of the reUmChlE containing a C-terminal His-tag was performed using immobilized metal ion affinity chromatography (IMAC). reUmChlE was eventually purified to homogeneity after four washing steps (5 – 45 mM imidazole). The last washing step (45 mM) was mandatory to remove remainders of unwanted host proteins (**Fig. 7-2**). Sequence analysis identified the protein band in the elution fraction (**Fig. 7-2, E1**) unambiguously as reUmChlE, with a molecular mass of about 87 kDa.

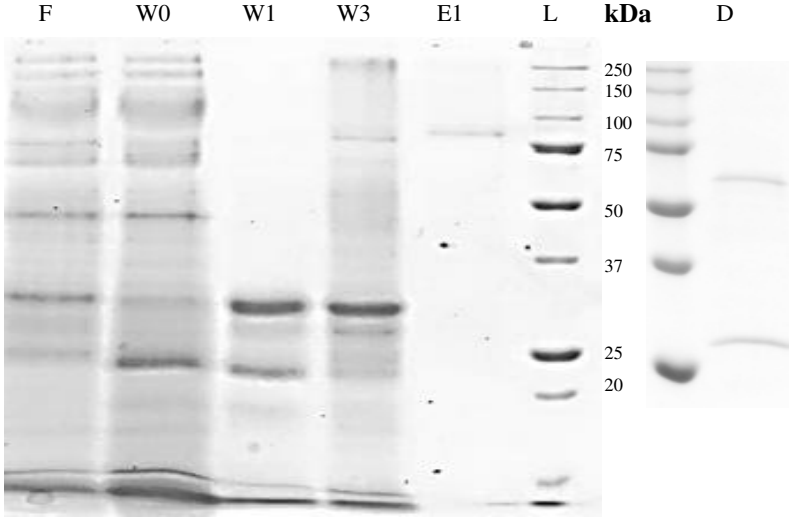

Fig. 7-2 Purification of recombinant chlorogenic acid esterase containing a C-terminal His-tag via IMAC using a Ni-NTA column. InstantBlue stained SDS-PAGE gel. Column flowthrough (F), first washing step with 5 mM imidazole (W0), second washing step with 15 mM imidazole (W1), last washing step with 45 mM imidazole (W3), elution fraction of Ni-NTA purification (E1), Precision plus Protein™ Standard (L), deglycosylated reUmChlE after treatment with endoglycosidase H for one hour at 37 °C (D).

As the Compute p*I*/Mw tool of ExPASy predicted a molecular mass of the His-tagged reUmChlE of 62.3 kDa, post-translational modifications were supposed. In particular, glycosylation is one of the most common modifications performed by *P. pastoris* (Eckart & Bussineau 1996). When purified reUmChlE was treated with Endoglycosidase H to remove N-linked glycosylations, the deglycosylated enzyme showed a molecular mass of about 63 kDa (**Fig. 7-2, D**). Likewise, the native UmChlE from the basidiomycete *U. maydis* was found to be glycosylated, but the molecular mass difference after treatment with Endoglycosidase H was only 8 kDa (Nieter et al. 2015). It is known that even if the target protein is glycosylated in the native host, *P. pastoris* may yield a different glycosylation pattern (Cereghino & Cregg 2000). A bioinformatical tool (http://www.cbs.dtu.dk/services/NetNGlyc/) identified eight potential *N*-glycosylation sites in the amino acid sequence of the reUmChlE. Recently, Fernández-Álvarez et al. (2010) studied *in silico* the glycosylation pathways in *U. maydis* and identified

distinct differences to yeast. While *U. maydis* typically attached five to eight mannose residues, *P. pastoris* commonly attached 14 mannose residues (Bretthauer & Castellino 1999).

7.3.4 Biochemical characterization of the reUmChlE

The pH optimum of the purified reUmChlE at pH 7.5 (**Fig. 7-3A**) was identical to that of the native enzyme (Nieter et al. 2015). However, the recombinant enzyme differed from the native UmChlE by exhibiting two- to threefold higher relative activity in the range from pH 3.0 – 5.0. While the native UmChlE was more stable under neutral to alkaline conditions than under acidic conditions after one hour incubation at 37 °C, the recombinant enzyme was stable over the entire pH range examined (pH 2.0 – 10.0). In contrast, the recombinant chlorogenic acid esterase from *Aspergillus niger* possessed only 50 % residual activity after one hour at pH 8.5 (Benoit et al. 2007). The improved pH stability of the reUmChlE may be explained by different glycosylation. Chu et al. (1978) reported a negative effect of deglycosylation on pH stability of an external invertase of *Saccharomyces cerevisiae* under acidic conditions.

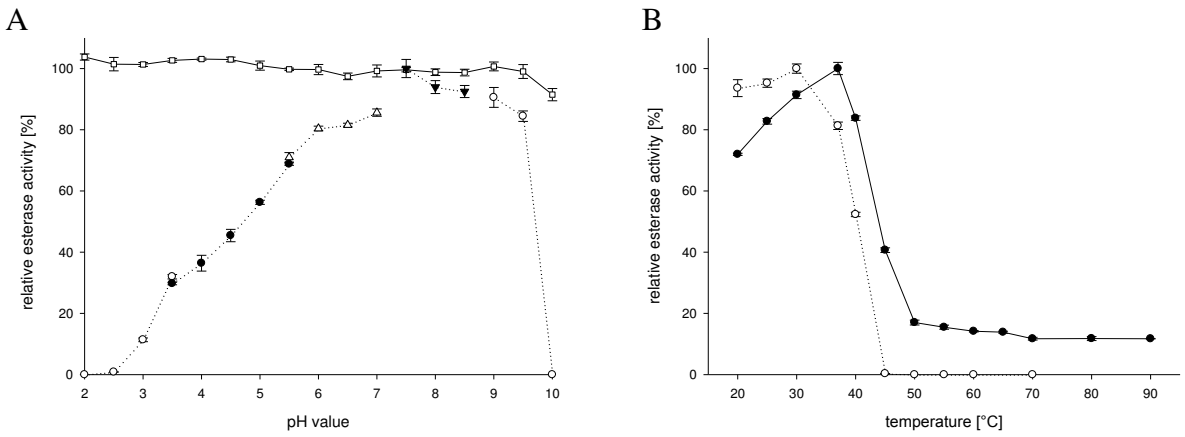

Fig. 7-3 Influence of pH and temperature on the enzyme activity of the reUmChlE. **(A)** pH stability (open square) und optimum was determined using glycine buffer (open circle), acetate buffer (filled circle), Bis-Tris buffer (open triangle) and Tris buffer (filled inverted triangle), **(B)** Temperature optimum (filled circle) and thermostability profile (open circle). For pH and thermostability measurements residual activities were determined after preincubating the enzyme for 1 h in the buffer systems above (pH 2 – 10) and at temperatures in the range of 20 – 70 °C, respectively. Remaining esterase activity was determined after further incubation (30 min, 37 °C) with 1.95 mM methyl ferulate as substrate. Relative enzyme activity [%] is defined as the percentage of activity detected with respect to the maximum observed esterase activity in each experiment. The presented values are the mean of data from triplicate experiments. Error bars indicate standard deviations.

The optimal reaction temperature of the reUmChlE at 37 °C (**Fig. 7-3B**) was equal to that of the native enzyme. The recombinant enzyme showed not only higher residual activities after incubation at 20 – 30 °C, but possessed approximately 15 % of its initial activity at 60 – 90 °C, where the native UmChlE lost its activity almost completely. No significant difference was found for the thermostability of both enzymes. After one hour of preincubation of the

reUmChlE at 37 °C, 20 % of initial activity was lost. While after preincubation at 40 °C only 50 % esterase activity was left, the enzyme was completely denatured at higher temperatures. Benoit et al. (2007) found that the recombinant *A. niger* chlorogenic acid esterase was more stable than the native enzyme and explained this with the higher degree of glycosylation of the recombinant enzyme. The native *A. niger* enzyme was completely inactivated after 10 min of incubation at 55 °C, whereas the recombinant enzyme showed 50 % residual activity after 90 min at 60 °C. In 2008, Shental-Bechor and Levy demonstrated that the thermostability depended mainly on the position of the glycosylation sites and to a lesser extent on the size of the glycans.

7.3.5 Substrate profile and kinetic parameters

To investigate the substrate specificity of reUmChlE, a comprehensive set of substrates was analyzed for hydrolytic activity of the enzyme. Besides the common substrate chlorogenic acid, various cinnamate and benzoate derivatives were tested as well as naturally occurring feruloylated saccharides. The recombinant enzyme possessed the same substrate preference as the native UmChlE (Nieter et al. 2015) (**Tab. 7-1**). Its broad substrate profile renders UmChlE unique in the family of chlorogenic acid esterases, as all previously identified chlorogenic acid esterases were solely active on chlorogenic acid (Asther et al. 2005; Benoit et al. 2007; Schöbel & Pollmann 1980b).

Tab. 7-1 Substrate profile of purified reUmChlE.

Substance	Relative esterase activity [%]	Specific esterase activity [U mg^{-1}]
chlorogenic acid	100	6.38
F-A	63 (0.27)	4.04
F-AX	54 (0.30)	3.46
F-AXX	38 (0.03)	2.40
methyl *p*-coumarate	37 (0.06)	2.35
methyl ferulate	35 (0.06)	2.26
F-AXG	27 (0.08)	1.75
ethyl ferulate	28 (0.03)	1.57
Ara_2F	18 (0.14)	1.17
methyl caffeate	15 (0.02)	0.97
Gal_2F	5 (0.04)	0.34
methyl benzoate	ND	ND
ethyl benzoate	ND	ND
methyl 3-hydroxy benzoate	ND	ND
methyl 4-hydroxy benzoate	ND	ND
ethyl 4-hydroxy benzoate	ND	ND
propyl 4-hydroxy benzoate	ND	ND
butyl 4-hydroxy benzoate	ND	ND
methyl cinnamate	ND	ND
ethyl cinnamate	ND	ND
methyl gallate	ND	ND
methyl sinapate	ND	ND
methyl vanillate	ND	ND
ethyl vanillate	ND	ND

Purified reUmChlE was incubated with 1.95 mM of different substrates for 30 min at 37 °C. Relative enzyme activities were calculated based on the most preferred substrate of the reUmChlE: chlorogenic acid which was set to 100 % to calculate relative enzyme activities. Numbers in parentheses are standard deviations. Abbreviations: F-A: 5-O-*trans*-feruloyl-L-arabinofuranose, Ara_2F: 2-O-*trans*-feruloyl-*α*-L-arabinofuranosyl-(1→5)-L-arabinofuranose, Gal_2F: 6-O-*trans*-feruloyl-*β*-D-galactopyranosyl-(1→4)-D-galactopyranose, F-AX: *β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose, F-AXG: *α*-L-galactopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose, F-AXX: D-xylopyranosyl-(1→4)-*β*-D-xylopyranosyl-(1→3)-5-O-*trans*-feruloyl-L-arabinofuranose and ND: activity not detectable.

Chlorogenic acid was hydrolyzed by far the best, and the determined specific activity of 6.38 U mg^{-1} was set to 100 % to calculate relative enzyme activities for all other substrates. While the calculated relative esterase activities were nearly the same as for the native UmChlE, the specific activities of the recombinant enzyme were approximately twofold higher. The higher specific activities of reUmChlE suggested that the more complex and time-consuming purification of the native UmChlE resulted in a stronger inactivation. A quite similar specific activity for chlorogenic acid (6.71 U mg^{-1}) was reported for the recombinant chlorogenic acid esterase from *A. niger* (Benoit et al. 2007).

To get more insight into the mechanism of action of UmChlE, the panel of natural feruloylated substrates was extended by F-AXX as well as Gal_2F and Ara_2F (**Fig. 7-4**).

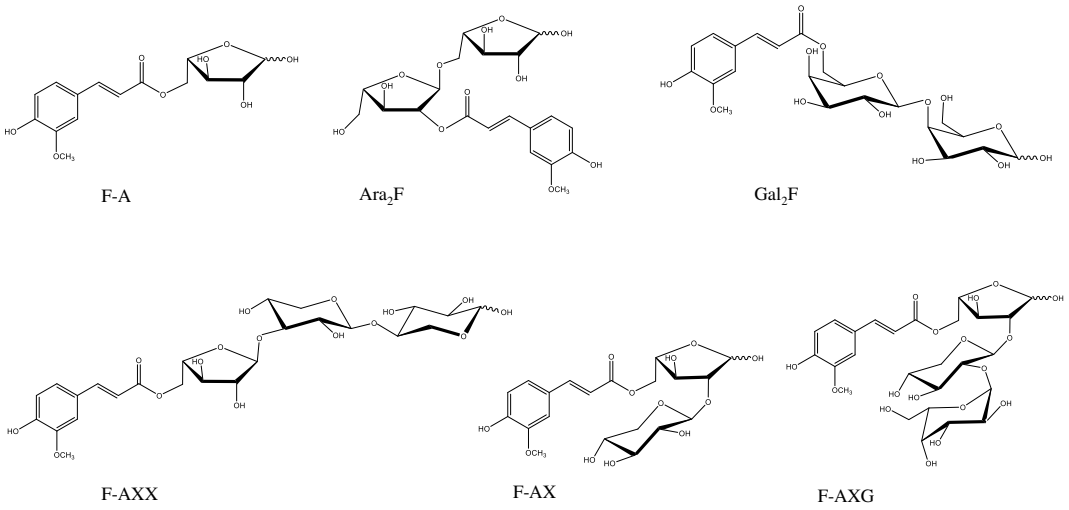

Fig. 7-4 Structures of feruloylated oligosaccharides. 5-O-*trans*-feruloyl-L-arabinofuranose (F-A); 2-O-*trans*-feruloyl-*α*-L-arabinofuranosyl-(1→5)-L-arabinofuranose (Ara_2F); 6-O-*trans*-feruloyl-*β*-D-galactopyranosyl-(1→4)-D-galactopyranose (Gal_2F); *β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AX); *α*-L-galactopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→2)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AXG); D-xylopyranosyl-(1→4)-*β*-D-xylopyranosyl-(1→3)-5-O-*trans*-feruloyl-L-arabinofuranose (F-AXX).

In Gal_2F, ferulic acid is esterified to the O-6 position of a galactopyranose residue, whereas in Ara_2F it is linked to the O-2 position of an arabinofuranose residue. Thus, these two substrates represented the linkage of ferulic acid mainly found in pectins of dicotyledons, such as spinach, sugar beet and in pseudocereals such as amaranth and quinoa (Ralet et al. 1994a; Wefers et al. 2015). In contrast, in graminaceous monocots such as wheat, barley or maize, ferulic acid is esterified to the O-5 position of arabinofuranose residues, represented by F-A; F-AX; F-AXG, F-AXX. The substrate profile revealed that the reUmChlE was more active towards oligosaccharides with arabinose O-5 esterified ferulic acid. With increasing complexity of the substrates, the efficiency of hydrolysis decreased (F-A 63 % > F-AX 54 % > F-AXX 38 % > F-AXG 27 %). The fact that the reUmChlE was more active on F-AXX (representing an AX backbone fragment) than on F-AXG (representing a complex feruloylated AX side-chain) could originate from different conformations of the substrates. While F-AXX is more or less a linear molecule with a straight aliphatic chain, F-AXG is a more bulky molecule due to its branched chain (**Fig. 7-4**). On the other side, reUmChlE released much less ferulic acid from Ara_2F and Gal_2F. Besides the conformation/structure of the enzyme, one reason for the higher specificity of reUmChlE towards the arabinose O-5 esterified substrates could be the natural habitat of *U. maydis*. To thrive on its preferred host plant, *Zea mays*, specific enzymes are required to degrade the cell walls, which as a graminaceous monocot mainly presents arabinose O-5-linked ferulates. Kinetic constants K_m and k_{cat} were measured, and catalytic efficiency (k_{cat}/K_m) was determined for the substrates chlorogenic acid (26.3 µM; 19.0 s^{-1}; 723.3 $mM^{-1}s^{-1}$), methyl

ferulate (179.6 µM; 7.1 s^{-1}; 39.3 $mM^{-1}s^{-1}$), methyl *p*-coumarate (234.2 µM; 7.3 s^{-1}; 31.0 $mM^{-1}s^{-1}$) and methyl caffeate (125.1 µM; 1.9 s^{-1}; 14.8 $mM^{-1}s^{-1}$). While the K_m value for chlorogenic acid of the reUmChlE (26.3 µM) and the native UmChlE (19.6 µM) were very close, the K_m values for the synthetic substrates investigated were up to fourfold higher for the reUmChlE than those determined for the native enzyme (Nieter et al. 2015). The lower substrate affinity of the reUmChlE compared to the wild-type enzyme could have originated from an altered protein folding of the recombinant enzyme due to the C-terminal His-tag or posttranslational modifications performed by *P. pastoris*. In contrast, homologous overexpression of the chlorogenic acid esterase gene from *A. niger* resulted in a slightly increased affinity for chlorogenic acid (recombinant ChlE K_m= 6.5 µM (Benoit et al. 2007) *vs* the wild-type enzyme K_m= 10 µM (Asther et al. 2005)). The more than tenfold increased catalytic efficiency of reUmChlE compared to the native UmChlE is attributed to the increased v_{max}.

7.3.6 Enzymatic hydrolysis of the natural substrates SBP, DSWB and CP

SBP and DSWB were used as complex natural substrates due to the different linkage positions of ferulic acid to cell wall polysaccharides (arabinose O-5 linkage (AX), arabinose O-2 linkage (pectic arabinans), or galactose O-6 linkage (pectic galactans)) (Ralet et al. 1994a), and the agricultural by-product CP was selected, as it is rich in chlorogenic acid. To quantify the phenolic acids released from the natural substrates, the initial contents of caffeic, ferulic and *p*-coumaric acid were determined after alkaline hydrolysis as follows: CP (1.77 mg g^{-1}; 0.47 mg g^{-1}; 0.05 mg g^{-1}), DSWB (– ; 4.45 mg g^{-1}; 0.18 mg g^{-1}) and SBP (– ; 6.78 mg g^{-1}; – ;). The initial chlorogenic acid content in CP was 2.66 mg g^{-1}.

After overnight incubation of CP with reUmChlE (10 mU), the chlorogenic acid was totally hydrolyzed. The same result was reported for the native and recombinant chlorogenic acid esterase from *A. niger* (Asther et al. 2005; Benoit et al. 2007). Both enzymes were used with five- to tenfold higher activities than reUmChlE, whereas the native UmChlE (20 mU) hydrolyzed only 68 % of the initial chlorogenic acid content of CP (Nieter et al. 2015). The fact that reUmChlE hydrolyzed the total chlorogenic acid of CP overnight is explained by its kinetic parameters; compared to the wild-type enzyme, the reUmChlE hydrolyzed chlorogenic acid not only 37-fold faster, but also 28-fold more efficiently.

The release of ferulic acid from the complex natural substrates DSWB and SPB was investigated, too. While 10 mU reUmChlE liberated less than one percent of the alkali-extractable ferulic acid from both substrates overnight, the released amount was increased by

the synergistic action of carbohydrases. The simultaneous incubation with *T. viride* xylanase (1 U) increased the amount of released ferulic acid from DSWB more than 14-fold, which is in accordance with the result obtained for the native UmChlE (Nieter et al. 2015). In contrast, the reUmChlE was less active on SBP, even in presence of different pectinase preparations. These findings are in compliance with the investigations on substrate specificity of the reUmChlE, where the enzyme was more active on O-5 ester linked ferulic acid, as present in wheat bran, than on O-2 or O-6 ester linked ferulic acid found in the pectins of dicotyledons.

7.3.7 Effect of reUmChlE on the rheological properties of dough

The effect of reUmChlE on the maximum resistance (force, R_{max}) and extensibility (distance, E_x) of dough was investigated as a function of enzyme dosage (0.1, 1 and 10 U reUmChlE). While the lowest reUmChlE activity resulted in no significant effect on the dough rheology, higher dosages decreased the maximum resistance (1 U: -12.6 % and 10 U: -19 %) noticeably. Gradual dough softening was observed with increasing reUmChlE activity (1 U vs. 10 U) compared to the reference dough without added enzyme. This softening effect was previously described in the literature for xylanases as a consequence of the degradation of the cross-linked AX network and the related redistribution of the water from AX to the gluten and starch phases (Selinheimo et al. 2006). In contrast, Selinheimo et al. (2006) observed a dough hardening after the addition of laccases. They explained this effect by the action of laccases to cross-link the esterified ferulic acid of the AX chains, thereby resulting in a strong AX network in the dough. The dough softening after treatment with reUmChlE might have resulted from a preferential activity of the enzyme for WEAX and SAX, which was described to cause a decreased resistance and viscosity (Aehle 2007). This is supported by the results of Faulds et al. (2003), who investigated the specificity of FAEs for feruloylated WEAX and WUAX and found for all three FAEs (representatives of the FAE types A, B and C) a preference for WEAX. As reUmChlE possessed a type B FAE side activity (like the native enzyme; (Nieter et al. 2015)), a comparable mode of action is suggested. Additionally, reUmChlE treated doughs were less sticky and showed a good workability compared to the reference sample. The improved dough handling properties can be explained by the reUmChlE-mediated depolymerisation of the cross-linked AX network. Partial removal of the ferulic acid linkages should result in lower molecular mass pentosans which possess a higher water holding capacity.

7.3.8 Baking trials with reUmChlE

As the specific loaf volume is one of the most important quality parameter, the purified reUmChlE was investigated for its possible effects using activities of 1 U and 5 U combined with a xylanase concentration of 25 ppm. Both experiments resulted in a decreased bread volume compared to the reference with xylanase (25 ppm) alone. To exclude an interference of the xylanase action on AX and reUmChlE activity on the one side as well a negative effect of a too extensive AX degradation on the other, a design of experiment (DOE) study was performed varying the activities of xylanase (14 – 48 U) or reUmChlE (0.6 – 3 U). A negative effect of reUmChlE on bun volume was found (**Fig. 7-5**).

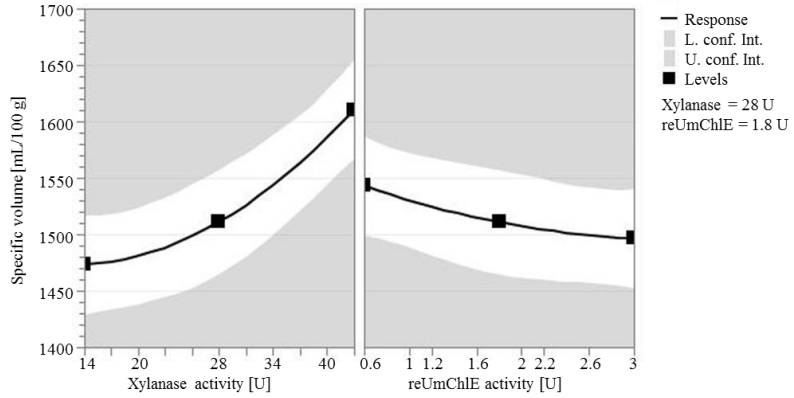

Fig. 7-5 Prediction plot of the design of experiment analysis. Effect of reUmChlE (using 28 U of xylanase) and xylanase (using 1.8 U reUmChlE) on specific loaf volume.

While increasing activities of reUmChlE decreased the bun volume, the opposite effect was observed by increasing the xylanase. The detrimental effect of reUmChlE on the specific loaf volume is visualized in **Fig. 7-6**.

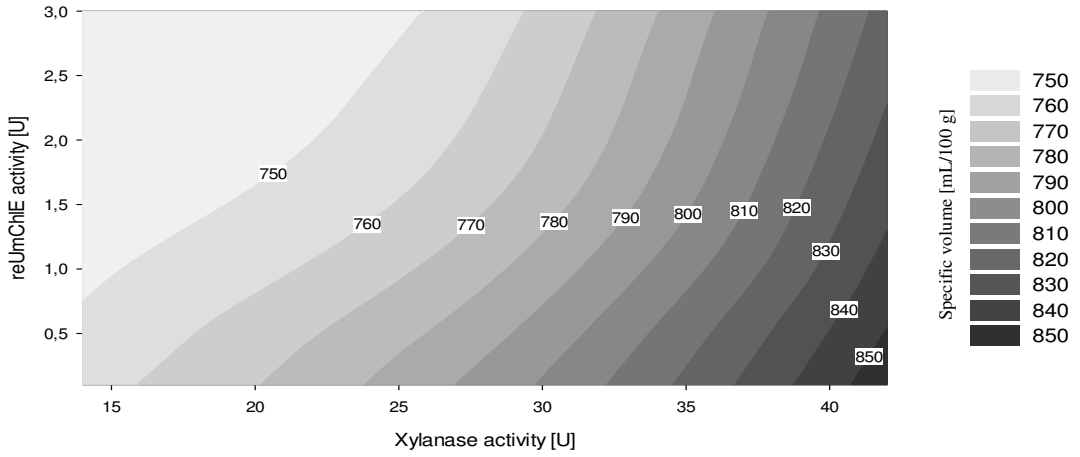

Fig. 7-6 Response Contour plot of the design of experiment. For the baking trials different activities of reUmChlE (0.6 – 3 U) and xylanase (14 – 48 U) were combined.

The highest specific loaf volumes were obtained by using the lowest reUmChlE concentration. An explanation for the decreased bun volume may be the redistribution of the water in the dough. Due to the improved water binding of the pentosans after depolymerisation by the action of the reUmChlE, less water is available for the formation of the three-dimensional viscoelastic gluten network. In breadmaking, a strong gluten network favors gas retention in the dough and the development of the bread volume (Goesaert, et al., 2005)

7.4 Conclusion

A chlorogenic acid esterase from *U. maydis* was heterologously produced in *P. pastoris*, purified to homogeneity and biochemically characterized. Lower temperatures improved the production of reUmChlE. A scale-up to a 5 L bioreactor generated sufficient enzyme for application studies. Substrate specificity and biochemical properties of the recombinant chlorogenic acid esterase were similar to those of the native enzyme. While the reUmChlE showed an improved pH stability compared to the native enzyme, it possessed no improved thermostability. The low thermostability of the reUmChlE may be an advantage for baking, as the enzyme will lose its activity during the thermal process. Rheological measurements showed that the treatment of the wheat dough with the reUmChlE caused a softening. While the reUmChlE did not increase the specific bread volume, the improved dough handling and decreased resistance suggested possible improvements in the production of crackers, wafers and biscuits. As chlorogenic acid, the major phenolic compound in roasted coffee beans, contributes to the acidity, astringency and bitterness in brewed coffee (Moon et al. 2009; Tai et al. 2014), the reUmChlE may moreover be effective to improve the taste and digestibility of coffee beverages.

Acknowledgements

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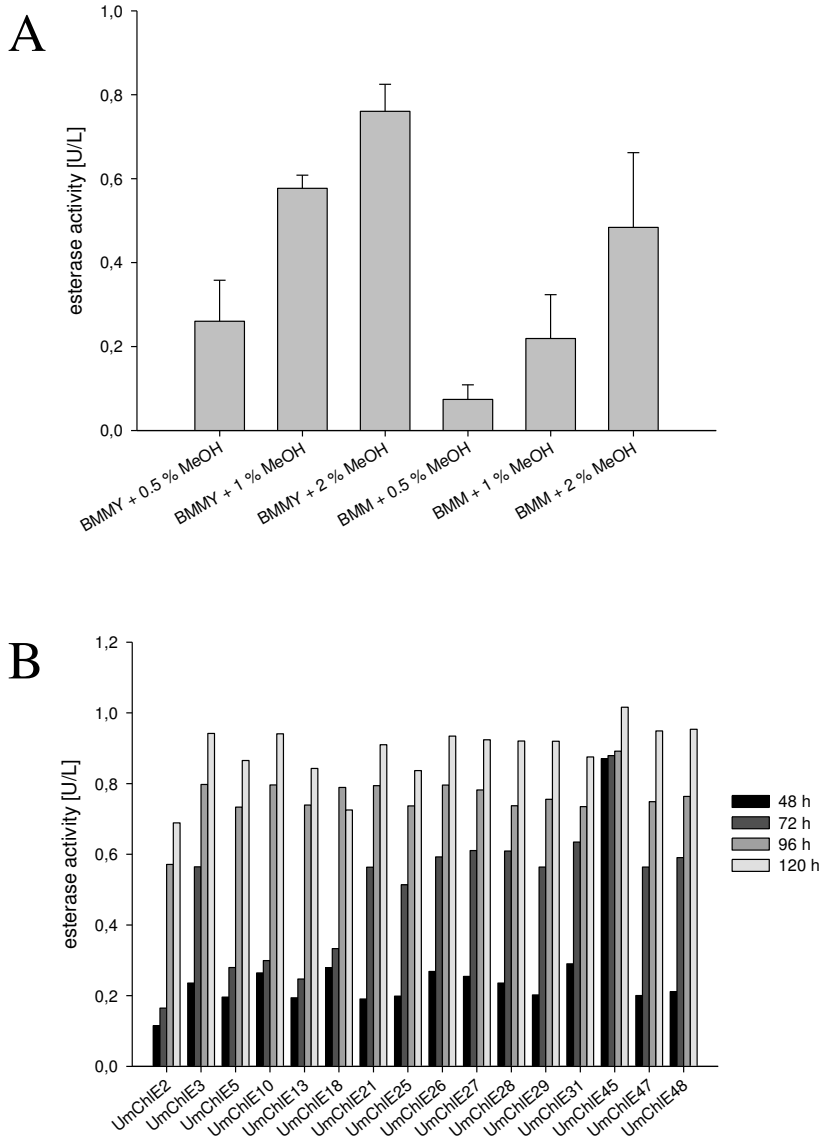
Appendix. Supplementary data.



Supplement 1 Production of reUmChlE in *P. pastoris* depending on cultivation parameters. Esterase activities of the culture supernatants were determined with HPLC using 1.95 mM methyl ferulate as substrate. Every 24 h the appropriate amount of methanol was added to maintain induction of gene expression in the *P. pastoris* pPIC9-*CHLE-HIS* transformants. A) Effect of culture medium and methanol concentration on reUmChlE production. Presented are the average values for 16 individual transformants. Cultivation was done in 96-well-plates for 96 h at 28 °C. B) Determination of the optimal induction period for production of reUmChlE. For the experiment 16 selected transformants were cultivated in BMMY medium containing 2 % (v/v) methanol for 120 h at 28 °C. Error bars indicate standard deviations.

8. Ausblick

Im Rahmen dieser Arbeit konnte eine Typ A Ferulasäureesterase (FAE) aus *Pleurotus eryngii* (PeFaeA) und eine Chlorogensäureesterase mit Typ B FAE-Nebenaktivität aus *Ustilago maydis* (UmChlE) isoliert, umfassend biochemisch charakterisiert und kloniert werden. Die erfolgreiche heterologe Produktion der zwei Enzyme in *Pichia pastoris* ermöglichte die Durchführung erster Applikationsstudien im Labormaßstab. Um die Proteinausbeuten zu erhöhen und ausreichende Enzymmengen für industrielle Anwendungen zu generieren, bieten sich weiterführende Arbeiten an, in denen eine Optimierung der Fermenterkultivierung durchgeführt wird. Denkbar wäre eine quasi-kontinuierliche Zuleitung von Methanol zur Aufrechterhaltung der Genexpression, anstatt der bisher praktizierten täglich einmaligen Zudosierung von 2 % (v/v) Methanol. Eine Etablierung dieser Prozessführung wäre ebenfalls für eine spätere industrielle Enzymproduktion erstrebenswert.

Die in dieser Arbeit erzielten wissenschaftlichen Erkenntnisse bestätigen das in Vorarbeiten aufgezeigte Potential von Basidiomycota zur Sekretion von FAE. Basidiomycota produzieren ein vielfältiges Portfolio an FAE. Neben den in dieser Arbeit untersuchten Typ A und B FAE konnten außerdem zwei Typ D FAE aus *Schizophyllum commune* und zwei Typ B FAE aus *Rizoctonia solani* kloniert und rekombinant produziert werden (unveröffentlichte Arbeiten, Nieter et al.). Insbesondere die Typ D FAE aus *S. commune* sollten in weiterführenden Arbeiten ausführlich charakterisiert und hinsichtlich ihres Effektes auf die Teigrheologie untersucht werden. Ein umfassendes Substratscreening mit einer Auswahl an Benzoesäure- und Hydroxyzimtsäureestern zeigte für die Typ D FAE aus *S. commune* ein außergewöhnliches Substratspektrum; neben der präferierten Hydrolyse von typischen FAE-Substraten, besaßen die *S. commune* Enzyme ebenfalls Aktivität gegenüber Benzoesäurederivaten (betrachtet wurden alle in **Tab. 5-4** aufgeführten Substrate). Dies stellt ein Novum dar; keine der in der Literatur veröffentlichten Typ C oder D FAE zeigt Aktivität gegenüber Benzoesäurederivaten.

Die Notwendigkeit der Erweiterung des Klassifizierungssystems von FAE wurde bereits in Kapitel 1.2.2 angeführt und wird durch die im Rahmen dieser Arbeit erzielten Erkenntnisse bestärkt. Sowohl PeFaeA und UmChlE als auch die Est1 aus *P. sapidus* weisen eine katalytische Triade bestehend aus Serin, Glutamat und Histidin auf, während alle anderen identifizierten FAE anstelle von Glutamat Aspartat im aktiven Zentrum besitzen. Dementsprechend müsste das von Udatha et al. (2011) veröffentlichte Klassifizierungssystem um zusätzliche FAE-Familien erweitert werden. Weiterhin wäre es von wissenschaftlichem Interesse, mittels zielgerichteter Mutagenese der katalytischen Triade (Glutamat → Aspartat) den Einfluss dieses

Aminosäureaustausches auf das Substratspektrum und die kinetischen Parameter der basidiomycetischen FAE zu charakterisieren. Für eine Verbesserung des Klassifizierungssystems wäre ebenfalls die Durchführung von Röntgenkristallstrukturanalysen erstrebenswert, um erste 3D-Strukturen basidiomycetischer FAE zugänglich zu machen. Im Zuge der heterologen Produktion der PeFaeA und UmChlE mit C-terminalem Hexa-Histidin-*tag* sollte dies zeitnah realisierbar sein.

In der vorliegenden Arbeit wurde zwischen der PeFaeA aus *P. eryngii* und der Est1 aus *P. sapidus* eine hohe Sequenzähnlichkeit (93 %) und -identität (87 %) gezeigt. Dementsprechend wäre eine Genom-basierte Identifizierung weiterer basidiomycetischer FAE durch den Einsatz von Gensonden denkbar.

Außerdem konnte im Rahmen dieser Arbeit das Potential der PeFaeA und der UmChlE für industrielle Anwendungen aufgrund der Freisetzung von industriell wertvollen Hydroxyzimtsäuren aus Nebenströmen der Lebensmittelindustrie sowie durch die Modifikation der rheologischen Eigenschaften von Weizenmehlteigen demonstriert werden. Fortführende Arbeiten könnten sich mit weiteren biotechnologischen Applikationsstudien wie der Synthese von bioaktiven phenolischen Verbindungen befassen. Die Fähigkeit zur Erzeugung von feruloylierten Mono- und Disacchariden durch Umesterung wurde kürzlich für die rekombinante FAE aus *P. sapidus* gezeigt (Kelle et al. 2015). Dass die Anwendung der UmChlE für diese Zwecke erfolgsversprechend sein kann, zeigten erste Vorversuche, in denen die UmChlE die Veresterung von Ferulasäure zu Ferulasäuremethylester katalysierte (unveröffentlichte Arbeiten, Nieter et al.).

Um den Einsatz der bearbeiteten basidiomycetischen Esterasen für industrielle Prozesse ökonomisch zu gestalten, ist die Etablierung heterologer Wirtssysteme für eine kostengünstige Überproduktion der Enzyme erstrebenswert. Weiterhin könnten sich fortführende Arbeiten mit dem Proteinengineering befassen, um robuste FAE-aktive Enzyme mit einem breiten Applikationsbereich zu generieren. Ausgehend von der Tatsache, dass für die heterologe Produktion der UmChlE in *P. pastoris* maximale FAE-Aktivitäten von 46 U L^{-1} erzielt wurden, während in analoger Weise durchgeführte heterologe Produktionen weiterer basidiomycetischer FAE aus *S. commune* und *R. solani*, ohne Optimierung der Kultivierungsparameter, 20-fach höhere Aktivitäten lieferten (unveröffentlichte Arbeiten, Nieter et al.), könnte eine Optimierung der Gensequenz zur Steigerung der Enzymausbeuten zielführend sein.

Im Zuge der Prozessoptimierung wurden in neueren Studien quervernetzte FAE-Aggregate (*cross-linked enzyme aggregates* - CLEAs) für die Synthese von bioaktiven phenolischen

Verbindungen eingesetzt (Vafiadi et al. 2008; Vafiadi et al. 2009). Die CLEAs zeichneten sich in den Versuchen durch eine höhere Stabilität und Aktivität als die freien Enzyme aus (Fazary et al. 2009).

Zusammenfassend untermauert die vorliegende Arbeit, dass es zukünftig weiterer Untersuchungen hinsichtlich der Struktur und des Substratspektrums der basidiomycetischen FAE bedarf. Erst nach Ermittlung dieser Grundlagen wird es gelingen, diese Biokatalysatoren zielführend für industrielle Applikationen zu verbessern.

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