Bioresource Technology 102 (2011) 4962–4967

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Influence of organic co-solvents on the activity and substrate specificity of feruloyl esterases

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ARTICLE INFO

Article history:
Received 20 October 2010
Received in revised form 27 January 2011
Accepted 28 January 2011
Available online 26 February 2011

Keywords:
Biomass utilization
Dimethylsulfoxide
Feruloyl esterase
Organic co-solvents
Polymer acylation

ABSTRACT

Organic co-solvents can expand the use of enzymes in lignocellulose deconstruction through making substrates more soluble and thus more accessible. In choosing the most adequate co-solvent for feruloyl esterases, hydrolysis of methyl *p*-hydroxycinnamates by three pure enzymes (and a multi-enzyme preparation) was evaluated. Low concentrations of dimethylsulfoxide (DMSO) enhanced hydrolysis by two of the enzymes while at levels >20%, activity was reduced. DMSO also enhanced acetyl esterase-type activity of the enzymes. The co-solvent effect was different for each enzyme-substrate couple, indicating that other factors are also involved. Kinetic studies with a *Talaromyces stipitatus* feruloyl esterase showed low concentrations of dimethylsulfoxide enhanced the hydrolytic rate while K_m also increased. Moreover, long-term incubation (96 h) of an *Aspergillus niger* feruloyl esterase in dimethylsulfoxide:water provided to the enzyme the ability to hydrolyze methyl *p*-coumarate, suggesting an active-site re-arrangement. Dimethylsulfoxide (10–30%) is proposed as an adequate co-solvent for feruloyl esterase treatment of water-insoluble substrates.

1. Introduction

The proposed exploitation of sustainable farm and forest lignocellulosic wastes and biomass crops into bioalcohol and high value-added products requires further insights into the nature of both the substrates and the processes involved in these bioconversions. If a 'white' or 'green' biotechnological process is desired, then the mode-of-action and accessibility of enzymes to the substrate should be considered. Recalcitrant barriers to enzyme accessibility to their substrates in the plant cell wall are due to the complexity of the interactions between the polymers and the presence of substitutions along the main polysaccharide chains. In the lignocellulosic cell wall, there are two types of substitutions on polysaccharides: those substitutions to limit access of enzymes to the polysaccharide backbone, and those which form cross-linkages between polysaccharides to increase the physical strength and integrity of the plant cell wall. Hemicelluloses and pectins have a variety of non-sugar side chains attached to the backbone. These include acetyl, methoxyl and feruloyl groups, all of which modify physical properties such as degradability, solubility and crystallinity of the polysaccharide. Furthermore, lignins from diverse herbaceous plants have been shown to be substituted with acetate and/or *p*-coumarate groups (del Rio et al., 2007, 2008; Ralph et al., 2002), as well as diferulates being proposed sites for lignin attachment to the hemicellulose (Grabber et al., 2002).

Feruloyl esterases (E.C. 3.1.1.73; synonym: cinnamoyl esterase) are serine esterases belonging to the carboxylic acid esterase family and cleave the ester bonds between hydroxycinnamic acids and hemicellulosic sugars in the plant cell wall (Faulds, 2010). They have been classified into four groups (A–D) based on biochemical specificity and sequence identity (Crepin et al., 2004). The type-A feruloyl esterase from *Aspergillus niger* structurally resembles a lipase (Faulds et al., 2005). Most feruloyl esterases also release esterified acetate from plant cell wall and it has been assumed that this acetate arose from the hemicellulosic fraction. Feruloyl esterases are becoming more important in the optimized breakdown of lignocellulosic biomass due to the presence of hydroxycinnamic acids on both hemicelluloses and lignin polymers. It has been shown that feruloyl esterases can cleave at least one of the two ester links of diferulic acids without the need to totally release free diferulic acid (Garcia-Conesa et al., 1999), and this is believed to facilitate loosening of the plant cell wall and thus better accessibility of glycoside hydrolases to the polysaccharides.

To exploit the components of the lignocellulosic biomass, the material needs to be pretreated with a variety of mechanical,

Abbreviations: DMSO, dimethylsulfoxide; AnFaeA, type-A feruloyl esterase from *Aspergillus niger*; MFA, methyl ferulate; MpCA, methyl *p*-coumarate; NcFaeB, type-B feruloyl esterase from *Neurospora crassa*; pNP, *p*-nitrophenyl; TsFaeC, type-C feruloyl esterase from *Talaromyces stipitatus*.

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doi:10.1016/j.biortech.2011.01.088

chemical and/or biochemical methods. Hemicelluloses can be removed through mild alkali treatments (Mandalari et al., 2005) or through steam-explosion (Han et al., 2010). Lignin, however, is insoluble in aqueous environment and thus requires the use of strong alkali or organic solvents at high temperature in processes such as organosolv. It has been reported that lipases and esterases can be used in organic solvents (Cherif and Gargouri, 2010), although enzymes in general get inactivated or give very low rates of reaction in non-aqueous media. The use of solvents can also be beneficial to an enzymatic reaction, as they can increase the thermal stability of enzymes, decrease water-dependent side-reactions or eliminate microbial contamination. Most esterase synthetic reactions are performed in organic solvents containing less than 1% water. However, in the absence of water, which acts as a molecular lubricant, enzymes become very rigid (Klibanov, 2001), thus affecting their utilisability. This will then affect the potential use of enzymes to process sustainable biopolymers such as lignin.

If the presence of co-solvents will aid in the enhanced solubilization of plant biomass, it is thus important to understand how key enzyme activities behave in the presence of such systems. In this paper, we describe the effect of organic co-solvents on the activity of three fungal feruloyl esterases representing different classes of this type of enzyme, together with those within a feruloyl esterase-containing commercial fungal multi-enzyme preparation, Ultraflo, which can be used for the breakdown of lignocellulosic biomass.

2. Methods

2.1. Materials

Recombinant feruloyl esterases, AnFaeA from *A. niger* (optimal temperature 55–60 °C, optimum pH 5.0), NcFaeB from *Neurospora crassa* (optimal temperature 55 °C, optimum pH 6.0), and TsFaeC from *Talaromyces stipitatus* (optimal temperature 60 °C, optimum pH 6–7), were heterologously expressed in *Pichia pastoris*, as previously described (Juge et al., 2001; Crepin et al., 2003a, 2003b). Ultraflo, a thermostable multi-enzyme preparation from *Humicola insolens* (FAE activity: optimal temperature 60–65 °C, Faulds et al., 2002), was kindly provided by Novozymes (Bagsvaerd, Denmark). Ultraflo was desalted through PD-10 or NAP-5 columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) into 100 mM MOPS buffer, pH 6.0 containing 0.05% (w/v) sodium azide, prior to use. Dimethylsulfoxide (DMSO) was obtained from Sigma–Aldrich Quimica (Madrid, Spain), acetone and methanol from Panreac Quimica (Barcelona, Spain), and 1,4-dioxane, glycerol, propanol and ethanol were purchased from Merck (New Jersey, USA). All solvents were of analytical grade. Methyl ferulate (MFA) and *p*-coumarate (MpCA) were purchased from Apin Chemicals Ltd. (Abingdon, UK). *p*-Nitrophenyl acetate was purchased from Sigma–Aldrich.

2.2. Determination of esterase activity

Feruloyl esterase activities were assayed using MFA and MpCA (60 μM) using the standard conditions for FAE of 37 °C in 100 mM MOPS (pH 6.0) and the release of the corresponding free acid measured spectrophotometrically at 335 nm (Ralet et al., 1994). Hydrolysis of 1 mM *p*-nitrophenyl acetate (pNP-acetate) was performed at 37 °C in 100 mM MOPS (pH 6.0) and the continual release of *p*-nitrophenol monitored at 410 nm. All assays were prepared and analyzed in duplicate. One unit of activity (1 U) is defined as the amount of enzyme releasing 1 μmol of product/min under the defined conditions. Organic solvents (DMSO, acetone, 1,4-dioxane, glycerol, methanol, ethanol, propanol) were slowly added to the buffer, mixed to insure homogeneity, then enzyme added. After brief equilibrium at 37 °C, substrate was added to initiate the reaction.

2.3. Inhibition kinetics

Kinetic constants (V_{max} and K_m) of TsFaeC against MFA, MpCA and *p*-nitrophenyl acetate were determined from Michaelis–Menten equation, using the Lineweaver–Burk double reciprocal plots in the presence and absence of DMSO. Values were estimated using a non-linear regression model (GraFit) that also gives an estimate of the standard error of each parameter.

3. Results and discussion

3.1. Effect of solvent on activity against methyl p-hydroxycinnamates

The activity of AnFaeA, NcFaeB, TsFaeC and Ultraflo on MFA was determined in the absence and presence of organic co-solvents at different concentrations. 1,4-Dioxane (Fig. 1c) inhibited all the

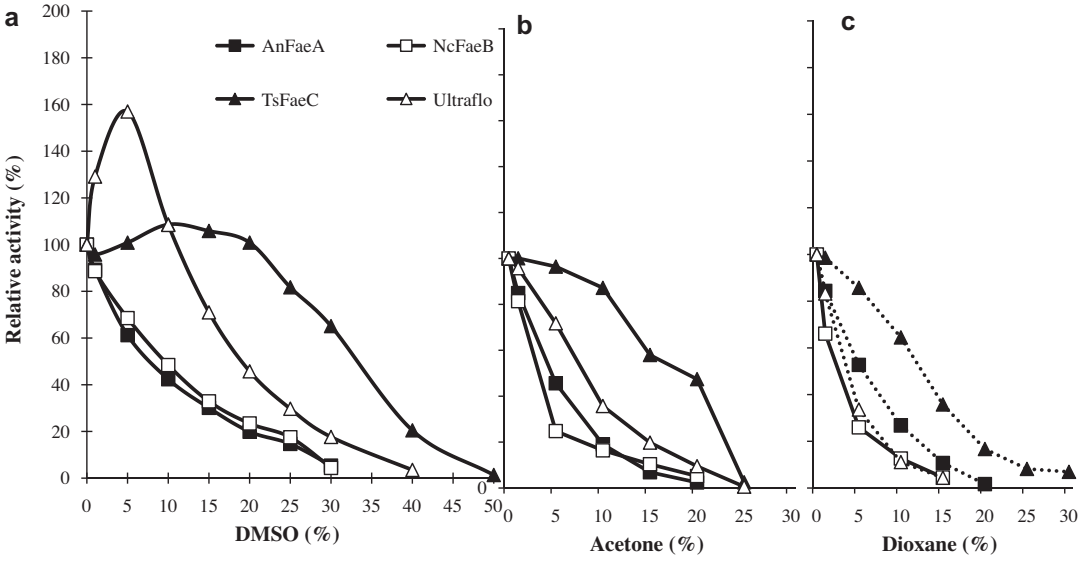

Fig. 1. Effect of (a) DMSO, (b) acetone, or (c) 1,4-dioxane on the activity of the feruloyl esterases from *A. niger* (AnFaeA), *N. crassa* (NcFaeB) and *T. stipitatus* (TsFaeC), and the commercial multi-enzyme preparation, Ultraflo, against MFA. Values are expressed as the percentage of residual activity against the percentage of organic co-solvent.

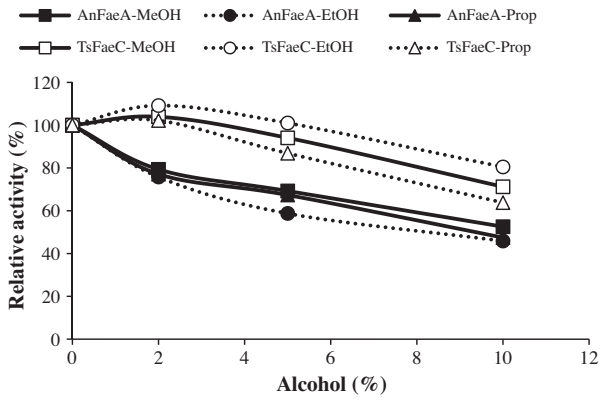

Fig. 2. Effect of methanol, ethanol and propanol on the activity of the feruloyl esterases from *A. niger* (AnFaeA) and *T. stipitatus* (TsFaeC) against MFA. Values are expressed as the percentage of residual activity against the percentage of organic co-solvent.

esterases in the presence of low concentrations of solvent, while the presence of acetone (Fig. 1b) also led to a decrease in activity, but the enzymes were able to cope with a higher concentration of this solvent. TsFaeC was the most stable esterase in both these solvents, while NcFaeB appeared to be the less stable. In the presence of low concentrations of DMSO (Fig. 1a), however, an increase in activity was measured for both Ultraflo and TsFaeC. An 1.6-fold increase in activity against MFA being measured with Ultraflo.

Dehydration of enzymes in organic solvents can causes minor structural perturbations (Griebenow and Klibanov, 1995) resulting in inactivation. The partial inactivation mechanism is not clearly understood for esterases but inactivation is independent of physicochemical properties of the solvent, hydration state and temperature (Castillo et al., 2006). A decrease in activity of the serine protease subtilisin Carlsberg in solvents was thought to involve a diminishing ability to stabilize the transition state or minor structural disorders, such as reorientation of the oxyanion hole, that force substrates to adopt a less catalytically active conformation (Fasoli et al., 2009). The fact that the different feruloyl esterases lost activity at different rates, and in some cases increased activity suggests that the effect is not based on a change in solubility of the substrate in the solvent, but in individual and different changes in the active site of each esterase.

Alcohol solvents – such as methanol, ethanol and propanol – also had an inhibitory effect on activity against methyl ferulate (Fig. 2). There was a slight stimulatory effect with 2% (v/v) methanol and ethanol on TsFaeC, but generally alcohols reduce the hydrolytic efficiency of the feruloyl esterases. This has implications for simultaneous saccharification and fermentation reactions for bioalcohol production, as the production of alcohol may begin to inhibit the activity of the hydrolyzing enzymes and reduce the yield of conversion.

The recognition of the different substitutions around the aromatic ring of the hydroxycinnamic acids is the basis of the classification of feruloyl esterases. AnFaeA has no activity against MpCA while the other esterases have high activity against this substrate. The effect of organic co-solvents to change the specificity of the esterases was examined by using MpCA (Fig. 3). The solvents, however, did not interact with AnFaeA sufficiently for the esterase to improve its ability to hydrolyze this substrate. Increased activity for Ultraflo was again measured in the presence of DMSO and acetone, with hydrolysis occurring at a high level in the presence of up to 40% DMSO and 30% acetone. NcFaeB was also not affected by the presence of up to 25% DMSO, but was more affected by acetone where a steady decrease in hydrolysis was observed with increasing acetone concentrations. Only the ability of TsFaeC to hydrolyze MpCA was reduced by DMSO and acetone, which was very different from this feruloyl esterase's ability to hydrolyze MFA in the presence of DMSO. TsFaeC was the most effected esterase by the co-solvents in its ability to hydrolyze MpCA and most stable in the hydrolysis of MFA.

The percentage of solvent present in the assay which caused a 50% reduction in activity, the IC_{50} values, is shown in Table 1. It is clear from these values that the effect of solvent on esterase activity is dependent on both the solvent and the substrate, although it is not due to substrate solubility in the solvent. While most of the literature regarding enzyme activity in organic solvents corresponds to the use of the enzymes in biosynthetic reactions, or as with lipases acting at solvent-aqueous interfaces, there are a few reports of increased activity in the presence of low concentrations of solvent (Schmidt-Dannert et al., 1996; Cherif and Gargouri, 2010).

3.2. Effect of solvent on activity against p-nitrophenyl acetate

All feruloyl esterases to date are able to hydrolyze model acetyl esters, such as pNP-acetate or α-naphthyl acetate, as well

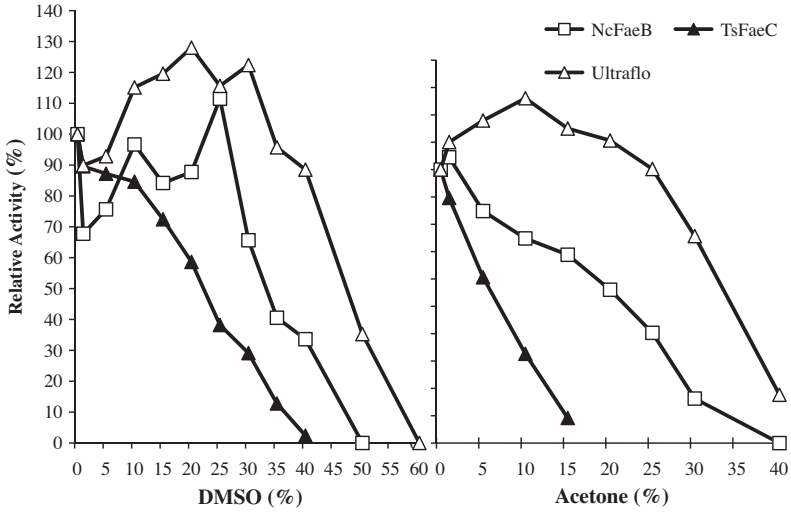

Fig. 3. Effect of (a) DMSO and (b) acetone on the activity of the feruloyl esterases from *N. crassa* (NcFaeB) and *T. stipitatus* (TsFaeC), and the commercial multi-enzyme preparation, Ultraflo, against MpCA. Values are expressed as the percentage of residual activity against the percentage of organic co-solvent.

Table 1
Solvent IC_{50} values (%) required to inflict a 50% decrease in the initial activity of the *A. niger* (AnFaeA), *N. crassa* (NcFaeB) and *T. stipitatus* (TsFaeC) feruloyl esterases, and commercial Ultraflo, against the given substrate at 37 °C, pH 6.0. The dash (–) signifies that no activity was detected for AnFaeA on MpCA.

	DMSO		Acetone		1,4-Dioxane
	MFA	MpCA	MFA	MpCA	MFA
AnFaeA	8	–	5	–	6
NcFaeB	10	34	4	22	3
TsFaeC	33	23	19	8	13
Ultraflo	19	48	9	35	4

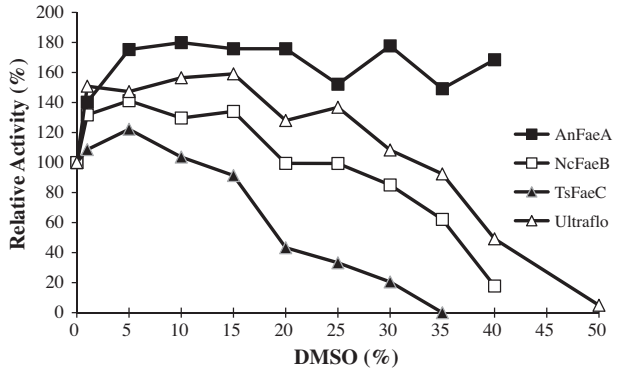

Fig. 4. Effect of DMSO on the hydrolysis of *p*-nitrophenyl acetate by the feruloyl esterases from *A. niger* (AnFaeA), *N. crassa* (NcFaeB) and *T. stipitatus* (TsFaeC), and the commercial multi-enzyme preparation, Ultraflo. Values are expressed as the percentage of residual activity against the percentage of organic co-solvent.

as releasing acetate from substituted polysaccharides (Altaner et al., 2003; Puchart et al., 2007; Faulds et al., manuscript in preparation). As it appears that most non-woody lignins are naturally acetylated (del Rio et al., 2007), the activity of the feruloyl esterases against pNP-acetate in the presence of DMSO was examined (Fig. 4), the results to be used in future deacylation studies in lignin dissolving solvents. The co-solvent addition at low concentrations ($\leqslant$15% v/v) led to an increase in hydrolytic activity for all four enzymes, but the activity of AnFaeA was particularly enhanced, with no inhibitory effect even at 40% DMSO (v/v). TsFaeC was the least stable esterase in DMSO against pNP-acetate. In general, when pNP-acetate is the substrate, the esterases are more stable to solvent deactivation. It is possible that the *p*-nitrophenol unit has a stronger interaction than the acetate and this stabilizes the active site due to its aromatic ring. The nitro-substituted ring is thus better than the phenol ring in protecting the active site residues.

3.3. Influence of viscosity on esterase activity

In aqueous media, enzymes pose sufficient conformational mobility to optimize catalysis, while organic media lack water's ability to engage in multiple hydrogen bonds. This leads to a more rigid protein structure and stronger intraprotein electrostatic interactions (Affleck et al., 1992). The activating effect of water can, to a certain extent, be mimicked by other solvents capable of forming multiple hydrogen bonds, such as glycerol and ethylene glycol (Zaks and Klibanov, 1988). To determine whether the effect observed with organic solvents was due to an increase in medium viscosity influencing the mobility of both substrate and enzyme, glycerol was added to the assay mixture and the effect on TsFaeC activity on both MFA and MpCA determined (Fig. 5). Glycerol is

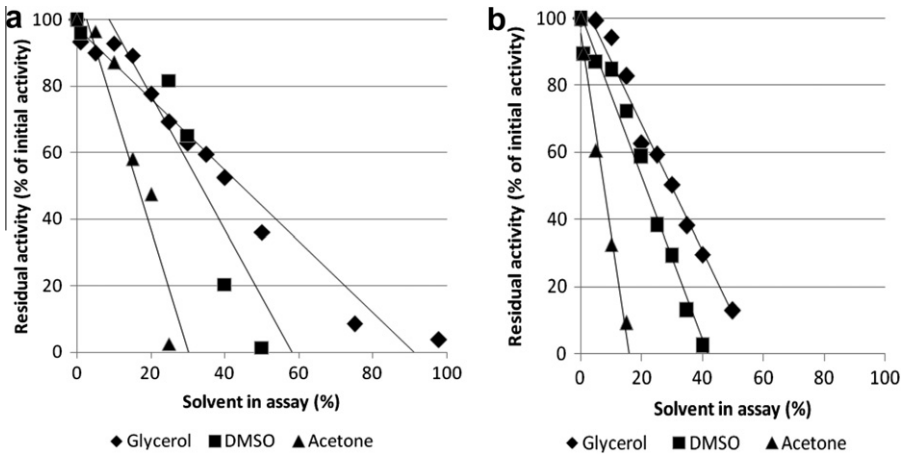

Fig. 5. Effect of solvent concentration on the activity of the *T. stipitatus* feruloyl esterase (TsFaeC) on methyl ferulate (a) and methyl *p*-coumarate (b). Values are expressed as the percentage of residual activity against the percentage of organic co-solvent.

Table 2
Effect of the presence of DMSO on the kinetic constants for the hydrolysis of MFA and MpCA by TsFaeC. Values in parenthesis represent standard error.

DMSO (%)	MFA			MpCA		
	K_m (μM)	V_{max} (U/mg)	(V_{max}/K_m)	K_m (μM)	V_{max} (U/mg)	(V_{max}/K_m)
0	2.22 (±0.31)	28,019 (±862)	12,621 (±1805)	5.61 (±0.41)	19,315 (±498)	3443 (±267)
1	3.40 (±0.43)	28,223 (±1150)	8301 (±1103)	5.74 (±0.64)	19,513 (±750)	3399 (±401)
3	4.28 (±0.19)	31,913 (±450)	7456 (±347)	6.81 (±0.92)	23,445 (±1183)	3443 (±496)
5	4.14 (±0.80)	37,400 (±2723)	9064 (±1865)	15.69 (±1.07)	42,835 (±1006)	2730 (±197)
10	7.29 (±1.51)	45,327 (±4187)	6218 (±1410)	33.20 (±1.92)	55,605 (±1252)	1675 (±104)
15	7.45 (±1.47)	38,839 (±3734)	5213 (±1144)	43.49 (±2.04)	54,254 (±1039)	1248 (±63)

Table 3
Effect of prolonged exposure to DMSO and acetone on the activity of the *A. niger* (AnFaeA), *N. crassa* (NcFaeB) and *T. stipitatus* (TsFaeC) feruloyl esterases, and commercial Ultraflo. Co-solvent was added at a concentration (%) indicated to give an IC_{50} value, as shown in Table 1. Values are expressed as U/mg. Values in parenthesis represent standard error.

Co-solvent esterase	MFA						MpCA					
	None	DMSO			Acetone		None	DMSO			Acetone	
		%	1 min	96 h	%	1 min	96 h		%	1 min	96 h	%	1 min	96 h
AnFaeA	6.06 (±0.15)	5	5.97 (±0.30)	7.16 (±0.05)	5	6.12 (±0.06)	7.37 (±0.04)	0	5	0	1.47 (± 0.25)	5	0	1.54 (±0.06)
NcFaeB	4.66 (±0.15)	10	4.41 (±0.05)	3.48 (±0.01)	10	4.35 (±0.63)	3.29 (±0.34)	42.12 (±0.81)	10	36.72 (±1.70)	28.88 (±0.12)	10	39.4 (±4.0)	23.31 (±8.0)
TsFaeC	1168 (±20)	25	1187 (±22)	795 (±20)	10	1276 (±71)	1036 (±17)	1223 (±0.7)	25	1178 (±13)	1112 (±31)	10	952 (±3)	1074 (±26)
Ultraflo	1055 (±10)	20	917 (±37)	914 (±55)	15	614 (±4)	573 (±144)	1744 (±112)	20	754 (±17)	439 (±3)	15	779 (±8)	768 (±37)

also considered an organic solvent (Xu et al., 1997) and because of its extremely high viscosity (over 1000 mPa s) it forms very viscous mixtures with water passing from 1.0 to 15.2 mPa s when glycerol concentration (w:v) increases from 0% to 65% (Segur and Oberstar, 1951). While activity was significantly reduced in the presence of glycerol, the effect was not as strong as with DMSO and acetone. However, the substrate also had a bearing on the influence of the solvent, with activity being reduced quicker with MpCA (IC_{50} of 30% glycerol) than with MFA (IC_{50} of 50% glycerol). There was still residual activity against MFA when the glycerol concentration was nearly 100%. TsFaeC is generally slightly more active against MpCA than MFA (Garcia-Conesa et al., 2004), so the normal specificity cannot explain the observed reduction in activity.

3.4. DMSO inhibition/stimulation kinetics

In general, the catalytic efficiency of an enzyme in an organic solvent is lower than in water. Hydrophilic solvents have the tendency to strip tightly bound water, which is essential for catalysis, from the enzyme molecule. Water is believed to increase the dielectric behavior at the enzyme's active site in anhydrous solvents (Affleck et al., 1992), possibly affecting electronic interactions known to be critical for transition state stabilization.

To determine whether the co-solvent is affecting the active site conformation, Michaelis–Menten kinetic values were determined against MFA and MpCA for TsFaeC in the presence of 0–15% DMSO (Table 2). For both substrates, both the K_m and V_{max} values increased with increasing DMSO in the assay. Moreover, the catalytic efficiency decreased suggesting that while the rate is enhanced by the presence of the organic co-solvent, there is also competitive inhibition taking place, hence the increased K_m. The general mechanism of serine esterases involves the formation of a substrate-enzyme complex followed by formation and breakdown of the first tetrahedral intermediate leading to the liberation of the first product and formation of an enzyme-acyl intermediate. The catalytic cycle ends with the hydrolysis of this intermediate. To insure biologic functionality, the enzymes structure must be robust enough but flexible to allow sufficient substrate binding, chemical reaction and product release. Shan and Herschlag (1996) demonstrated that in a model system, the presence of DMSO led to a strengthening in H-bond between enzyme and substrate as the charge was rearranged during the conversion from the enzyme's ground state to its transition state, which leads to a rate enhancement. Such a rate enhancement in the presence of low concentrations of DMSO was observed in this study, but the reaction begins to show inhibition at higher concentrations (>15%) of the solvent as the solvent competes for the substrate in the binding site.

If feruloyl esterases are to be used in applications involving organic co-solvent concentrations greater than 20–30%, they will either require engineering to make them more resistant, which will also require further structure–function studies on this class of enzyme, or further micro-organisms screened for improved stability esterases.

3.5. Effect of DMSO exposure time on esterase activity

In a dry aprotic solvent, enzymes are kinetically trapped in their conformation due to the high energy barrier required to unfold, and thus they should remain catalytically active for long periods under such conditions (Castillo et al., 2006). The previous experiments involved a short exposure time of the esterases to the co-solvent before the reaction was initiated by the addition of substrate. Exposure of TsFaeC to 20% DMSO for up to 5 h before assaying against MFA showed no effect on the activity of the esterase (results not shown). The enzymes were also exposed to a DMSO concentration corresponding to their IC_{50} value (Table 1) for 96 h

and then assayed for residual activity against MFA and MpCA. Table 3 shows a comparison of activity against the two substrates when solvent was added and after 1 min substrate added, or when the esterase was in contact with the solvent for 96 h before assaying. AnFaeA activity against MFA and MpCA improved upon organic solvent exposure for 96 h while the activities of the other enzymes decreased or remained unchanged. This long-term exposure to the organic solvent before reaction should insure a complete exchange between the enzyme water and the co-solvent containing medium. Therefore, the changes in AnFaeA catalytic properties would be related to the entrance of DMSO into the active site, establishing direct relationships with catalytically-relevant amino-acid residues or modifying the location of water molecules by forming DMSO–water bonds.

Only the crystal structure of the "lipase-type" AnFaeA has been solved to date (Hermoso et al., 2004) of the enzymes examined in this study, and so structure–function aspects between the esterases cannot be compared. Further structural information on this class of enzymes is required to explain their inhibition by organic co-solvents. However, the "acquisition" of MpCA-hydrolyzing activity by AnFaeA after 96 h incubation in DMSO suggests a rearrangement at the active site after partial substitution of water by DMSO, thus conferring to AnFaeA the ability to act on a substrate with a different substitution pattern on the phenolic ring. A similar broadening of specificity was observed upon replacing Tyr80 in the active site, albeit with a decrease in k_{cat} (Faulds et al., 2005). Of the other feruloyl esterases with resolved structures, little biochemical characterization has been performed.

4. Conclusions

Although reactions of lipases and other hydrolases in organic solvents are mainly investigated for synthetic purposes, this study demonstrates for the first time that feruloyl esterases have the potential to hydrolyze water-insoluble substrates, such as acylated lignins, in the presence of co-solvents without strong impairment of the catalytic action of the enzyme. This facilitates further studies into the effect of enzymes on isolated cell-wall polymers dissolved in DMSO, as well as whole cell-walls swelled in DMSO mixtures and that the effects of enzymatic or other pretreatments can be then analyzed "*in situ*" by powerful techniques such as 2D-NMR (Kim et al., 2008; Rencoret et al., 2009).

Acknowledgements

This research was supported by the Project LIGNODECO (KBBE-3-244362) and a Marie Curie Intra-European Fellowship to CBF within the 7th Framework Programme of the EU.

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