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REVIEW

Methods for discovery and characterization of cellulolytic enzymes from insects

Jonathan D. Willis, Cris Oppert and Juan L. Jurat-Fuentes

Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, Tennessee, USA

Abstract Cellulosic ethanol has been identified as a crucial biofuel resource due to its sustainability and abundance of cellulose feedstocks. However, current methods to obtain glucose from lignocellulosic biomass are ineffective due to recalcitrance of plant biomass. Insects have evolved endogenous and symbiotic enzymes to efficiently use lignocellulosic material as a source of metabolic glucose. Even though traditional biochemical methods have been used to identify and characterize these enzymes, the advancement of genomic and proteomic research tools are expected to allow new insights into insect digestion of cellulose. This information is highly relevant to the design of improved industrial processes of biofuel production and to identify potential new targets for development of insecticides. This review describes the diverse methodologies used to detect, quantify, purify, clone and express cellulolytic enzymes from insects, as well as their advantages and limitations.

Key words biofuels, cellulases, cellulase discovery methods, cellulase substrate, insect digestive fluids, lignocellulosic biomass

Insect relevance to lignocellulosic biofuels

Current world energy needs demand the development of industrial-scale processes for the sustainable production of fuel from renewable biological resources as economic and environmentally sound alternatives to finite fossil fuels. In the US, lignocellulosic ethanol has been suggested as a desirable biofuel, mostly due to its sustainability, reduced competition as a food resource, net energy production, and reduced input costs related to production of ethanol from corn-derived starch (Lynd *et al.*, 1991; McLaughlin *et al.*, 2002; Schmer *et al.*, 2008). Cost-efficient production of ethanol from lignocellulosic biomass is mostly dependent on development of efficient hydrolysis technologies (Sun & Cheng, 2002; Wyman, 2007). Enzymatic degradation of cellulose is considered the hydrolysis method with the greatest potential for improvement and cost reduction (Wyman, 1999, 2007). Current estimates suggest that reducing the cellulase enzyme amounts by half through biotechnology could decrease processing costs by up to 13% (Lynd *et al.*, 2008).

Correspondence: Juan Luis Jurat-Fuentes, Department of Entomology and Plant Pathology, University of Tennessee, Knoxville, TN 37996-4560, USA. Tel: (865) 974 5931; email: jurat@utk.edu

Lignocellulosic recalcitrance, which prevents enzymatic access to fermentable sugars, is derived from the tight association of cellulose with hemicellulose and lignin to form the plant cell wall (Delmer & Amor, 1995). Pre-treatment steps are currently necessary to achieve efficient glucose yields from lignocellulosic feedstocks (Chandra *et al.*, 2007). Even though novel pretreatment technologies based on ionic liquids (Zhao *et al.*, 2009) or expression of hydrolases in plants (Taylor *et al.*, 2008) are being developed, there is still a need to also identify and develop more efficient cellulolytic enzymes that can be applied into both pretreatment and/or cellulolytic technologies (Mosier *et al.*, 2005).

Cellulose degradation requires the synergistic action of three types of glycoside hydrolases (GH): endo-β-1,4-glucanases (EG; EC. 3.2.1.4), exo-β-1,4-cellobiohydrolases (CBH; EC. 3.2.1.91), and β-glucosidases (EC. 3.2.1.21) (Clarke, 1997). EG enzymes work by random cleavage of β-1,4 glycosidic bonds in the internal portions of cellulose strands to reduce the degree

of polymerization of the cellulose chain into smaller subunits. CBH enzymes remove subunits at both reducing and non-reducing ends of the cellulose chain, releasing either cellobiose or glucose. Due to the inhibition of EG enzymes by accumulation of cellobiose, the presence of β-glucosidases to hydrolyze cellobiose to glucose is important for complete degradation of cellulose (Holtzapple *et al.*, 1990; Gruno *et al.*, 2004).

Enzymes currently used for production of cellulosic ethanol were identified in fungal and bacterial systems (Lynd, 1996). Current limitations of enzymatic degradation of lignocellulosic biomass are mostly related to enzymatic stability and susceptibility to inhibitory agents or byproducts (Mousdale, 2008; Kristensen *et al.*, 2009). Continuous prospecting and bioengineering efforts should provide novel enzymes with higher specific activity and with lower susceptibility to inhibitors (Lynd *et al.*, 2008).

Some insects have evolved very effective strategies to use lignocellulosic substrates as sources of energy (Martin, 1983), which makes them an optimal resource to prospect for novel cellulolytic enzymes. Evidence for the prospecting potential in insects is most notably found in species of termites, which are known to live almost exclusively on substrates with high lignocellulosic content. In lower termites cellulolytic activity is mostly dependent on enzymes produced by symbiotic protozoa (Ohkuma, 2008), while higher termites combine cellulases secreted by the gut cells with bacterial enzymes (Tokuda *et al.*, 1997; Warnecke *et al.*, 2007). The importance of insect-produced cellulases for survival has also been suggested as a potential target for development of termite control technologies (Zhu *et al.*, 2005; Zhou *et al.*, 2008). Increased thermostability and specific activity of termite cellulases achieved through random mutagenesis of non-conserved residues highlights the potential development of technologies for biofuel production from these insect enzymes (Ni *et al.*, 2007). Recent evidence also suggests that lignin degradation, which is one of the main issues of plant biomass use for biofuels, may be common in insects feeding on lignocellulosic substrates (Geib *et al.*, 2008). In comparison to extensive studies in termite cellulolytic systems, detailed information on alternative insect cellulolytic systems is limited. This review focuses on the methods reported to detect and characterize cellulolytic systems in insects, with emphasis on the insect-produced enzymes.

Discovery of insect cellulases

Based on data from termites and wood-feeding roaches, cellulolytic activity in insects has been attributed to symbiotic gut flora (Cleveland, 1924, 1934). The role of symbiotic microbes in production of cellulolytic enzymes has been widely recognized for insects feeding on lignocellulosic biomass (Martin, 1983; Morrison *et al.*, 2009). However, examples of insect-produced cellulolytic activity have been described for species belonging to five taxonomic orders: Isoptera (Martin & Martin, 1978; Slaytor, 1992; Bignell *et al.*, 1994), Thysanura (Treves & Martin, 1994), Coleoptera (Genta *et al.*, 2006), Blattodea (Scrivener *et al.*, 1989; Genta *et al.*, 2003) and Hemiptera (Adams & Drew 1965). Low numbers of bacteria in gut regions that display cellulolytic activity has been suggested as evidence for endogenous cellulase degradation in some species of Orthoptera and Phasmatodea (Cazemier *et al.*, 1997).

The first insect cellulase gene, encoding an endo-β-1,4-glucanase, was cloned from *Reticulitermes speratus* (Watanabe *et al.*, 1998). While no endogenous CBHs have been reported in insects, EG genes have been identified in other isopteran species (Tokuda *et al.*, 1999; Scharf *et al.*, 2005; Zhang *et al.*, 2009a), as well as species in the orders Coleoptera (Ferreira *et al.*, 2001; Sugimura *et al.*, 2003; Lee *et al.*, 2004, 2005; Wei *et al.*, 2006b) and Orthoptera (Kim *et al.*, 2008). Availability of sequenced genomes is allowing a more complete identification and characterization of insect cellulolytic systems (Kunieda *et al.*, 2006). Sequenced insect EGs have been included in diverse GH families based on sequence homology, including GHF1, GHF5, GHF7, GHF9, GHF10 and GHF45. The only three-dimensional structure for an insect cellulase, the NtEgl endo-glucanase from *Nasuritermes takasagoensis*, revealed the common alpha helical barrel folding and catalytic domain observed for GHF9 members (Khademi *et al.*, 2002).

Quantitative and qualitative detection of cellulolytic activity in insects

Detection of enzymatic activity is directly dependent on the sample preparation as well as the specific substrate used. Due to their involvement in digestion, salivary glands, gut tissues and gut digestive fluids are usually used for the detection of cellulolytic activity in insects (Martin, 1983; Cazemier *et al.*, 1997), and due to localized expression, levels of activity or cellulases detected are greatly dependent on the tissue of origin or sample preparation method (Martin, 1983; Ferreira *et al.*, 2002). Another important consideration for the quantification of cellulolytic activity is the product inhibition reported for both EG and CBH activities (Zhang *et al.*, 2009b). This limitation is generally overcome by addition of β-glucosidases to the reaction to degrade inhibiting cellobiose to glucose.

In most cases, degradation of cellulase substrates is measured using modifications of the 3,5-dinitrosalicylic acid (DNSA) (Miller, 1959) or tetrazolium blue (Jue & Lipke, 1985) assays, which detect reducing sugars generated during cellulose degradation. Initially, the DNSA assay was found susceptible to interferences when using native or pretreated lignocellulosic materials, which limited its use depending on the substrates (Rivers *et al.*, 1984). More recently, optimized microplate assays based on the DNSA method to quantify degradation of modified cellulose (Xiao *et al.*, 2005), filter paper (FP) (Xiao *et al.*, 2004), or pretreated lignocellulosic material (King *et al.*, 2009), have been developed to overcome these limitations. Other techniques to quantify production of glucose in solution include the alkaline copper (Somogyi, 1952) and the glucose oxidase/peroxidase (Dahlqvist, 1968) methods. However, the oxidase method has been shown to be affected by the presence of lignin (Breuil & Saddler, 1985), and although it is rarely used for lignocellulosic substrates, it has been used with more processed substrates to determine β-glucosidase activity in insect samples (Ferreira & Terra, 1983; Chipoulet & Chararas, 1985a; Zinkler & Gotze, 1987; Marana *et al.*, 1995, 2000; Yapi *et al.*, 2009). Methods based on HPLC or thin layer chromatography separations to estimate produced sugars have also been described (Adams & Drew, 1965; Berlin *et al.*, 2006; Chundawat *et al.*, 2008). To further characterize the activity of specific cellulolytic enzymes, activity is measured by spectrophotometry using substrates containing *p*-nitrophenol (Terra *et al.*, 1979; Chipoulet & Chararas, 1985a; Cazemier *et al.*, 1997; Marana *et al.*, 2000; Ferreira *et al.*, 2001; Marana *et al.*, 2004; Yapi *et al.*, 2009) or methyl-umbelliferyl (MU) groups (Jacobson & Schlein, 1997; Marana *et al.*, 2001).

Use of diverse cellulase substrates can help differentiate the specific contribution of specific insect gut areas to the digestion of plant material (Tokuda *et al.*, 2005), which can contribute to a more complete characterization of the cellulolytic process. Due to their low levels of chemical modification, FP, microcrystalline cellulose (MCC) and cotton, have been used as preferred cellulase substrates to determine the existence of complete cellulolytic systems (EG, CBH and β-glucosidases). However, there is evidence for the degradation of MCC in insects lacking CBH activity (Scrivener & Slaytor, 1994). As shown in Table 1, reports of CBH activity in insects are uncommon (Cazemier *et al.*, 1997), and this activity is related to enzymes produced by symbionts or parasites (Martin & Martin, 1978; Martin, 1983). The main limitation in the use of these CBH substrates is their insolubility, which limits their use to in-solution assays with continuous mixing and complicates the removal of particulates. A high throughput microplate assay for

Table 1 Insects with reported cellobiohydrolases and the methods used for the quantitative detection of this activity.

Order (family)	Species	Substrate	Detection method	Reference
Coleoptera (Cerambycidae)	*Anoplophora glabripennis*	MCC	DNSA	Li *et al.*, 2008
	Rhagium inquisitor	MCC	AC	Chipoulet & Chararas, 1985b
Diptera (Psychodidae)	*Phlebotomus papatasi*	MeUMB	Flu	Jacobson & Schlein, 1997
Isoptera (Heterotermitidae)	*Coptotermes lacteus*	MCC	TB	Hogan *et al.*, 1988
(Kalotermitidae)	*Neotermes koshunensis*	MCC	TB	Tokuda *et al.*, 2005
(Rhinotermitidae)	*Reticulitermes flavipes*	pNPC	Abs.	Zhou *et al.*, 2007
	R. speratus, Coptotermes formosanus	MCC	TB	Tokuda *et al.*, 2005
(Termitidae)	*Odontotermes formosanus*, *Nasutitermes takasagoensis*	MCC	TB	Tokuda *et al.*, 2005; Tokuda & Watanabe, 2007
(Termopsidae)	*Hodotermopsis sjoestedti*	MCC	TB	Tokuda *et al.*, 2005
Thysanura (Lepismatidae)	*Thermobia domestica*	MCC	GOP	Zinkler & Gotze, 1987

AC, alkaline copper; Abs, absorbance; DNSA, dinitrosalicylic acid; Flu, fluorescence; GOP, glucose oxidase-peroxidase; MCC, microcrystalline cellulose; MeUMB, 4-methylumbelliferyl-β-cellobiopyranoside; pNPC, *p*-nitrophenyl-β-D-cellobioside, TB, tetrazolium blue.

cellulolytic activity was developed using MCC as substrate (Chundawat *et al.*, 2008), although this approach has not been used with insect samples. An alternative approach is the detection by absorbance or fluorescence of *p*-nitrophenol or methyl-umbelliferyl (MU) cleavage after hydrolysis of glycosides such as *p*-nitrophenyl-β-D-cellobioside (pNPC) (Zhou *et al.*, 2007), or 4-methylumbelliferyl-β-cellobiopyranoside (MeUMB) (Jacobson & Schlein, 1997). In termites it has been suggested that localization of activity against MCC correlates with the presence of hindgut symbionts (Tokuda *et al.*, 2005). The same cellulase substrate was also used to qualitatively determine the existence of cellulolytic activity in gut symbionts isolated from three coleopteran species (Delalibera *et al.*, 2005) or to establish the role of fungal cells for cellulolytic activity in fungus-growing termites (Abo-Khatwa, 1978). A similar direct correlation between activity against FP and numbers of hindgut symbionts was reported in *Periplaneta americana* (Gijzen *et al.*, 1994). Survival and assimilation of glucose from FP digestion by *Panesthia cribrata* in the presence of antibiotics was used as evidence for the presence of endogenous complete cellulolytic systems in this insect (Scrivener *et al.*, 1989).

An alternative to insoluble cellulase substrates are modified celluloses, such as carboxymethylcellulose (CMC), which are derived to improve water solubility. In CMC the hydroxyl groups are methylated, resulting in high water solubility compared to crystalline or amorphous cellulose. Due to its ease of use and easy degradation by EG activity, CMC is the most documented cellulase substrate used for solution assays or incorporation into agar or acrylamide gel matrices (Table 2). As indicated in Table 2, degradation of CMC quantified by the DNSA assay is the most common technique used to demonstrate cellulolytic activity in insect samples. CMC has been used as substrate in agarose plates for qualitative determinations of EG activity localization in gut regions of *Rhagium inquisitor* (Zverlov *et al.*, 2003), as early screening for cellulolytic symbionts (Delalibera *et al.*, 2005), and to screen modified (Ni *et al.*, 2007; Zhang *et al.*, 2009a) or heterologously produced insect cellulases (Lee *et al.*, 2004; Ni *et al.*, 2005; Wei *et al.*, 2005, 2006b). In this method, plates are incubated with digestive fluids and activity revealed as clear zones when staining undigested CMC with Congo red dye. The diameter of this activity area has been utilized as a relative measurement to compare activity of heterologously expressed enzymes from *Coptotermes formosanus* (Zhang *et al.*, 2009a). In a similar strategy, cellulolytic zymograms with CMC as substrate can be used to detect proteins with cellulolytic activity after electrophoretic separation of proteins (Schwarz *et al.*, 1987). The advantage of this method over activity assays in agar plates is that specific protein bands with cellulolytic activity can be visualized and their molecular weight estimated. To prevent CMC degradation during electrophoresis, proteins are only partially heat denatured and gels run at low temperature so that discrete activity bands, rather than smears, can be detected. Using this technique, specific cellulases have been detected from digestive fluids of diverse insects, including *R. inquisitor* (Zverlov *et al.*, 2003), *T. molitor* (Genta *et al.*, 2006), *Psacothea hilaris* (Sugimura *et al.*, 2003), *Anoplophora glabripennis* (Li *et al.*, 2008), and *Phaedon cochleariae* (Girard & Jouanin, 1999). These zymograms have also been used to characterize cellulolytic activity of insect cellulases overproduced in heterologous systems (Ni *et al.*, 2005; Zhang *et al.*, 2009a).

Due to the diverse range of specificities of β-glucosidases, a variety of substrates are used for detecting and quantifying this enzymatic activity. Cleavage of β-D-glucopyranosides (such as salicin, octyl β-glucoside or helicin), or disaccharides (such as cellobiose or amygdalin) by β-glucosidases is generally quantified by detection of the generated glucose using the glucose oxidase/peroxidase method (Ferreira & Terra, 1983; Chipoulet & Chararas, 1985a; Zinkler & Gotze, 1987; Marana *et al.*, 1995; Marana *et al.*, 2000; Yapi *et al.*, 2009). Alternatively, β-glucosidase activity is also quantified by measuring the hydrolysis of *p*-nitrophenol from glycoside derivatives (NP β-glycosides) such as glucoside (NP βGlu) (Terra *et al.*, 1979; Chipoulet & Chararas, 1985a; Cazemier *et al.*, 1997; Marana *et al.*, 2000; Ferreira *et al.*, 2001; Marana *et al.*, 2004; Yapi *et al.*, 2009), or methyl-umbelliferyl (MU) fluorescence after hydrolysis of MU glycosides such as 4-methyl-umbelliferyl β-D-glucoside (MUG) (Marana *et al.*, 2001). Activity properties and specificity of purified β-glucosidases from digestive systems of lepidopteran (Marana *et al.*, 2000, 2001), coleopteran (Chipoulet & Chararas, 1985a, 1985b; Ferreira & Terra, 1989; Genta *et al.*, 2006), orthopteran (Marana *et al.*, 1995), and dipteran (Terra *et al.*, 1979; Ferreira & Terra, 1983) insects have been reported using these methods. Table 3 provides a comprehensive list of insects prospected for β-glucosidases and the specific substrate and methodology used. Combined use of diverse substrates allowed determination of enzymatic specificities in insect midgut β-glucosidases from diverse taxonomic groups (Ferreira *et al.*, 1998). Protein bands displaying β-glucosidase activity after electrophoretic separation can be detected using MUG as substrate (Genta *et al.*, 2006).

Table 2 Insects with documented β-1,4-endoglucanase activity and the methods used for the quantitative detection and characterization of this activity. When available, information on the enzyme purification and molecular size are also presented. NP = not provided by the authors.

Order (family)	Species	Substrate	Detection method	Purification	Size (kDa)	Reference
Blattodea (Blaberidae)	*Panesthia cribrata*	CMC	DNSA	SEC, AEC, HIC	53.6, 48.8	Scrivener & Slaytor, 1994
	Pycnoscelus surinamensi	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997
(Blattidae)	*Blaberus fuscus*, *Periplaneta americana*, *P. australasia*	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997; Gijzen *et al.*, 1994
Coleoptera (Buprestidae)	*Agrilus planipennis*	CMC	CMC-AP	NP	NP	Vasanthakumar *et al.*, 2008
(Cerambycidae)	*Anoplophora glabripennis*	CMC	DNSA, Zymogram	NP	NP	Li *et al.*, 2008
	Apriona germari	CMC	DNSA, CMC-AP	SEC, AEC	29, 36, 47	Lee *et al.*, 2004; Lee *et al.*, 2005; Wei *et al.*, 2006b
	Hylotrypus bajules	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997
	Psacothera hilaries	CMC	TB	Native PAGE	47	Sugimura *et al.*, 2003
	Rhagium inquisitor	CMC	AC	NP	NP	Chipoulet & Chararas, 1985b
	Saperda vestita	CMC	CMC-AP	NP	NP	Delalibera *et al.*, 2005
(Chrysomelidae)	*Aulacophora foveicollis*	CMC	Zymogram, DNSA	Native PAGE	NR	Sami & Shakoori, 2008
(Curculionidae)	*Dendroctonus frontalis*	CMC	CMC-AP	NP	NP	Delalibera *et al.*, 2005
	Ips pini	CMC	CMC-AP	NP	NP	(Delalibera *et al.*, 2005)
(Scarabeidae)	*Pachnoda marginata*	CMC	DNSA	NP	NP	(Cazemier *et al.*, 1997)
Diptera (Psychodidae)	*Phlebotomus papatasi*	OBRH, CA	Abs.	NP	NP	(Jacobson & Schlein 1997)
(Sciaridae)	*Rhynchosciara americana*	CMC	Colorimetry	NP	NP	(Terra *et al.*, 1979)
(Tipulidae)	*Tipula abdominalis*	CMC	DNSA	NP	NP	(Walters & Smock 1991)
Isoptera (Heterotermitidae)	*Coptotermes lacteus*	CMC	TB	NP	NP	(Hogan *et al.*, 1988)
(Kalotermitidae)	*Neotermes koshunensis*	CMC	TB	NP	NP	(Tokuda *et al.*, 2005)
	Cryptotermes pingyangensis	CMC	AC	NP	NP	(Mo *et al.*, 2004)
(Mastotermitidae)	*Mastotermes darwiniensi*	CMC	Zymogram, DNSA	AEC	36	(Cazemier *et al.*, 1997; Li *et al.*, 2003)
(Rhinotermitidae)	*Reticulitermes flaviceps*	CMC	DNSA, AC	NP	NP	(Mo *et al.*, 2004; Zhou *et al.*, 2007)
	R. speratus	CMC	TB	NP	NP	(Watanabe *et al.*, 1998)
	Coptotermes formosanus, *R. leptomandibularis*	CMC	AC	NP	NP	(Mo *et al.*, 2004)

Continued

Table 2 Continued

Order (family)	Species	Substrate	Detection method	Purification	Size (kDa)	Reference
(Termitidae)	*Nasutitermes takasagoensis*	CMC	TB, Zymogram	SEC	47	Tokuda & Watanabe, 2007; Tokuda *et al.*, 1997
	Odontotermes formosanus	CMC	AC	AEC	80	Mo *et al.*, 2004; Yang *et al.*, 2004
Lepidoptera (Papilionidae)	*Parnassius apollo* ssp. *frankenbergeri*	CMC	DNSA	NP	NP	Nakonieczny *et al.*, 2006
(Saturniidae)	*Philosamia ricini*	CMC	AC	NP	NP	Pant & Ramana, 1989
Orthoptera (Acrididae)	*Schistocerca gregaria*	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997
(Gryllidae)	*Acheata domesticus*	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997
	Teleogryllus emma	CMC	DNSA	TAC	47	Kim *et al.*, 2008
Plecoptera (Peltoperlidae)	*Peltoperla arcuata*	CMC	AC	NP	NP	Walters & Smock, 1991
(Pternoarcidae)	*Allonarcys proteus*	CMC	AC	NP	NP	Walters & Smock, 1991
Phasmatodea (Phasmatidae)	*Eurycantha calcarata*	CMC	DNSA	NP	NP	Cazemier *et al.*, 1997
Trichoptera (Limnephilidae)	*Pycnopsyche* spp.	CMC	AC	NP	NP	Walters & Smock, 1991
Thysanura (Lepismatidae)	*Thermobia domestica*	CMC	GOP	NP	NP	Zinkler & Gotze, 1987
	Ctenolepisma lineata	RC	AC	NP	NP	Lasker & Giese, 1956

Abs, Absorbance; AEC, anion exchange chromatography; AC, alkaline copper; AP, agarose plates; CA, cellulose azure; CMC, carboxymethylcellulose; DNSA, dinitrosalicylic acid; HIC, hydrophobic interaction chromatography; NP, not provided; OBRH, ostazin brilliant red hydroxyethyl-cellulose; RC, regenerated cellulose; SEC, size exclusion chromatography; TAC, tag affinity chromatography; TB, Tetrazolium blue.

Identification, cloning and expression of insect cellulases

In order to characterize specificity, substrate affinity and activity, detected insect cellulases need to be either purified or cloned and expressed heterologously. As in the case of fungal and bacterial cellulases, insect cellulases are usually not expressed at high levels, hindering their characterization through purification efforts. Probably reflecting their abundance over other glycosidases, most purified insect cellulases are β-glucosidases. Purification procedures usually consist of multiple steps, including size exclusion, anion exchange and/or hydrophobic interaction chromatography (Marana *et al.*, 1995, 2000; Ferreira *et al.*, 2001; Yapi *et al.*, 2009). Even though proteins displaying CBH activity have not been purified from insect systems, EG enzymes have been purified and characterized from cockroach species (Scrivener & Slaytor, 1994; Genta *et al.*, 2003) or termite symbiotic flagellates (Li *et al.*, 2003) using also liquid chromatographic procedures. Alternative reported purification methods for glycosidases include isoelectric focusing (Ferreira & Terra, 1983) and preparative electrophoresis (Chipoulet & Chararas, 1985a; Sugimura *et al.*, 2003; Sami & Shakoori, 2008).

Once glycosidases are purified, protein sequencing may facilitate primer design for polymerase chain reaction (PCR) cloning (Marana *et al.*, 2001; Sugimura *et al.*, 2003). A disadvantage to this cloning method is primer degeneracy, which may result in lack of specific amplicons from PCR reactions. Additionally, selection of template material may be difficult if no information on the origin (insect *vs.* symbiont) of the enzyme is available. Probably due to these limitations of PCR cloning, the most reported method to clone and sequence insect cellulases is the generation and screening of cDNA libraries. In the case of

Table 3 Insects with documented β-D-glucosidase and the methods used for the quantitative detection and characterization of this activity. When available, information on the enzyme purification and molecular size are also presented. NP = not provided by the authors.

Order (family)	Species	Substrate	Detection method	Purification	Size (kDa)	Reference
Blattodea (Blaberidae)	*Panesthia cribrata*	pNPG	Abs.	NP	NP	Scrivener & Slaytor, 1994
	Gromphadorrhinna portentosa	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997
(Blattidae)	*Blaberus fuscus Periplaneta americana P. australasia*	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997
(Blattodea)	*Pycnoscelus surinamensi*	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997
Coleoptera (Carabidae)	*Pheropsophus aequinoctialis*	CB, pNPG, amygdalin, salicin	GOP	DC	27	Ferreira & Terra, 1989; Ferreira *et al.*, 1998
(Cerambycidae)	*Anoplophora glabripennis*	Salicin	DNSA	NP	NP	Li *et al.*, 2008
	Hylotrypus bajules	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997
	Rhagium inquisitor	CB, lactose, gentiobiose, maltose, pNPG, salicin	GOP, Abs.	DC, PE	NR	Chipoulet & Chararas, 1985a, 1985b
(Curcolinidae)	*Rhynchophorus palmarum*	pNPG	Abs.	SEC, AEC, HIC	58	Yapi *et al.*, 2009
(Elateridae)	*Pyrearinus termitilluminans*	pNPG, CB, salicin, amygdalin	Abs., GOP	NP	NP	Ferreira *et al.*, 1998
(Scarabeidae)	*Pachnoda marginata*	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997
(Tenebrionidae)	*Tenebrio molitor*	pNPG, MUG, CMC, CB, amygdalin, salicin	Abs., zymograms, GOP	PE	59	Ferreira *et al.*, 1998, 2001
Diptera (Nematocera)	*Ptychoptera paludosa*	MUG	Flu	NP	NP	Wolf *et al.*, 1997
(Sciaridae)	*Rhynchosciara americana*	pNPG, CB, maltose, salicin	Flu, GOP	DGC	106 and 65	Ferreira & Terra, 1983; Ferreira *et al.*, 1998; Terra *et al.*, 1979
Isoptera (Heterotermitidae)	*Coptotermes lacteus*	CB	TB	NP	NP	Hogan *et al.*, 1988

Continued

Table 3 Continued

Order (family)	Species	Substrate	Detection method	Purification	Size (kDa)	Reference
(Kalotermitidae)	*Cryptotermes pingyangensis*	Salicin	AC	NP	NP	Mo *et al.*, 2004
	Neotermes koshunensis	CB	GOM	Recombinant	60	Ni *et al.*, 2007
(Macrotermitdidae)	*Odontotermes formosanus*	Salicin	AC	NP	NP	Mo *et al.*, 2004
(Mastotermitidae)	*Mastotermes darwiniensis*	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997
(Rhinotermitidae)	*Coptotermes formosanus* *Reticulitermes flaviceps*, *R. leptomandibularis*	Salicin	AC	NP	NP	Mo *et al.*, 2004
(Termitidae)	*Nasutitermes takasagoensis*	CB	GOM	NP	NP	Tokuda *et al.*, 1997
	N. exitiosus, *N. walkeri*	CB	GOP	NP	NP	McEwen *et al.*, 1980
Hymenoptera (Apidae)	*Scaptotrigona bipunctata*	CB, pNPG, salicin, amygdalin	GOP	NP	NP	Ferreira *et al.*, 1998
Lepidoptera (Noctuidae)	*Anticarsia gemmatalis* *Heliothis zea* *Spodoptera frugiperda* *Trichoplusia ni*	pNPG, helicin, salicin, MUG, CB	Abs., GOP, Flu	NP	NP	Yu, 1989
	S. frugiperda	pNPG, MUG, CB, amygdalin, gentiobiose, cellotriose	Abs., Flu, GOP	SEC, AEC, HIC	47 and 50	Ferreira *et al.*, 1998; Marana *et al.*, 2000
(Pyralidae)	*Diatraea saccharalis*	pNPG, CB, salicin, amygdalin	GOP	NP	NP	Ferreira *et al.*, 1998
(Sphingidae)	*Erinnyis ello*	pNPG, CB, salicin, amygdalin	GOP	NP	NP	Ferreira *et al.*, 1998
(Papilionidae)	*Parnassius apollo* ssp. *frankenbergeri*	pNPG, CB	DNSA, Abs.	NP	NP	Nakonieczny *et al.*, 2006
(Saturniidae)	*Philosamia ricini*	CB	AC	NP	NP	Pant & Ramana, 1989
Orthoptera (Acrididae)	*Abracis flavolineata*	pNPG, CB, salicin, ABG, lactose, LB	Abs., GOP	SEC, AEC	82	Ferreira *et al.*, 1998; Marana *et al.*, 1995
	Locusta migratoria	pNPG, CB	Abs., GOP	SEC	65	Morgan, 1975
	Schistocerca gregaria	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997

Table 3 Continued

Order (family)	Species	Substrate	Detection method	Purification	Size (kDa)	Reference
(Gryllidae)	*Acheata domesticus*	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997
Phasmatodea (Phasmatidae)	*Eurycantha calcarata*	pNPG	Abs.	NP	NP	Cazemier *et al.*, 1997
Thysanura (Lepismatidae)	*Thermobia domestica*	CB, sucrose, trehalose, lactose	GOP	NP	NP	Zinkler & Gotze, 1987
	Ctenolepisma lineata	CB	AC	NP	NP	Lasker & Giese, 1956

Abbreviations: ABG, alkyl-β-glucosidase; Abs, absorbance; AEC, anion exchange chromatography; AC, alkaline copper; CB, cellobiose; DGC, density gradient centrifugation; DC, differential centrifugation/precipitation; DNSA, dinitrosalicylic acid; Flu, fluorescence; GOM, glucose-oxidase-mutarotase; GOP, glucose oxidase/peroxidase; HIC, hydrophobic interaction chromatography; LB, laminaribiose; MUG, -methyl-umbelliferyl β-D-glucoside; NR, not reported; pNPG, *p*-nitrophenyl-β-D-glycosides; PE, preparative electrophoresis; SEC, size exclusion chromatography; TB, tetrazolium blue.

termites, this approach has proved useful to sequence endogenous and symbiont-produced EG, CBH and β-glucosidases (Scharf *et al.*, 2003; Todaka *et al.*, 2007). Both whole body (Lee *et al.*, 2004; Wei *et al.*, 2006b; Kim *et al.*, 2008) and midgut (Girard & Jouanin, 1999; Marana *et al.*, 2001) or salivary gland (Watanabe *et al.*, 1998; Yuki *et al.*, 2008) cDNA libraries have been reported as useful to identify endogenous insect cellulases. Due to limited sequence information in some cases, these libraries are usually used as sources to sequence expression sequence tags (ESTs) that are then used for database searching to identify putative cellulases (Girard & Jouanin, 1999; Lee *et al.*, 2004, 2005; Wei *et al.*, 2006b; Kim *et al.*, 2008; Yuki *et al.*, 2008). The advantage of this approach is that multiple enzymes can be identified simultaneously, yet their levels of activity cannot be assigned and may result in identification of enzymes with low activity or with a secondary role for cellulose digestion in the insect. Alternatively, partial sequence can be obtained from specific cellulolytic proteins of interest and used to design probes to screen cDNA libraries (Watanabe *et al.*, 1998; Ferreira *et al.*, 2001; Marana *et al.*, 2001) or primers for PCR amplification of full-length cellulase cDNAs (Li *et al.*, 2003; Sugimura *et al.*, 2003). Availability of insect genomes has facilitated the identification of endogenous cellulases in *Apis mellifera* (Kunieda *et al.*, 2006) and *Tribolium castaneum* (Morris *et al.*, 2009). Metagenomic projects aimed at identifying cellulase genes from whole genome shotgun libraries have proved successful in identifying putative symbiont-derived cellulolytic enzymes in insects (Todaka *et al.*, 2007; Warnecke *et al.*, 2007). Similarly, high throughput pyrosequencing projects have allowed detection of multiple cellulase enzymes in *Chrysomela tremulae* (Pauchet *et al.*, 2009) and *Melitaea cinxia* (Vera *et al.*, 2008). The obvious advantage of these genomic methods is the characterization of the complete insect cellulolytic system, even though further research is necessary to demonstrate functionality and specificity of the identified cellulases. With the increased availability of whole insect genomes and next generation sequencing projects, the number of identified endogenous and symbiont-derived insect cellulases is expected to increase in the near future (Matsui *et al.*, 2009; Morrison *et al.*, 2009).

A number of insect cellulolytic enzymes have been expressed and purified in heterologous systems to characterize their activity, specificity and stability (Table 4). EG enzymes from *Apriona germari* (Lee *et al.*, 2004, 2005; Wei *et al.*, 2006b) and *Teleogryllus emma* (Kim *et al.*, 2008) have been expressed as soluble proteins and purified from *Spodoptera* SF9 cell cultures. Even though the purified enzymes displayed the expected β-1,4-endoglucanase activity, N-glycosylation was reported to be necessary for activity of *A. germari* cellulases (Wei *et al.*, 2005, 2006a). In comparison, cellulases from *Spodoptera frugiperda* and *Coptotermes formosanus* have been expressed and purified as active enzymes in *Escherichia coli* (Marana *et al.*, 2004; Zhang *et al.*, 2009a), suggesting that in this case glycosylation may not be necessary for enzymatic activity. However, in the case of *C. formosanus* cellulase, it was reported that C-terminal tagging, a process which greatly facilitates recombinant enzyme purification,

Table 4 Insect-derived cellulases that have been cloned and heterologously expressed for their characterization. NP = not provided by the authors.

Order (family)	Species	Expression system	Activity	pH optima	Thermo-stability	Reference
Coleoptera (Cerambycidae)	*Apriona germari*	Sf9 cells	β-1,4 endoglucanase	6.0	50–60°C	Lee *et al.*, 2005; Wei *et al.*, 2006b
Isoptera (Kalotermitidae)	*Neotermes koshunensis*	*E. coli*	β-glucosidase	5.0	45°C	Ni *et al.*, 2007b
(Rhinotermitidae)	*Coptotermes formosanus*	*E. coli*	β-1,4 endoglucanase	5.0	42°C	Zhou *et al.*, 2007
	Reticulitermes speratus	*E. coli*	β-1,4 endoglucanase	6.9	50°C	Ni *et al.*, 2007b
(Termitidae)	*Nasutitermes takasagoensis*	*E. coli*	β-1,4 endoglucanase	7.2	45°C	Ni *et al.*, 2007b
Lepidoptera (Noctuidae)	*Spodoptera frugiperda*	*E. coli*	β-glucosidase	NP	NP	Marana *et al.*, 2004
Orthoptera (Gryllidae)	*Teleogryllus emmaa*	Sf9 cells	β-1,4 endoglucanase	5.0	45°C	Kim *et al.*, 2008

affects activity and stability of the enzyme (Zhang *et al.*, 2009a). Random DNA shuffling between termite cellulases has been used to increase expression levels as well as thermostability in the mutated genes (Ni *et al.*, 2005, 2007). Similarly, expression of cellulase genes from termite symbionts in *Aspergillus oryzae* has been suggested to be optimized by codon optimization (Sasaguri *et al.*, 2008). An alternative heterologous expression system based on infection of *Bombyx mori* larvae with transgenic nucleopolyhedrovirus containing an insect cellulase gene has also been reported to be efficient for insect cellulase production (Lee *et al.*, 2006). In this system, active cellulase was recovered as a soluble protein in the hemolymph of infected larvae.

Optimization of heterologous expression of insect cellulases would be necessary to produce the high enzyme amounts needed for lignocellulose degradation during biofuel production. Alternative approaches to lignocellulose degradation and preprocessing of feedstock, such as inducible expression of cellulases in plants (Taylor *et al.*, 2008), have not been tested with insect enzymes.

Conclusions/insights from insect cellulases and their applications to biofuels

Since diverse lignocellulosic feedstocks are being considered for biofuel production, optimized cellulase mixtures will be needed for each feedstock and pre-treatment method used. Optimally, cellulolytic enzymes being used in bioreactors for ethanol production would be stable under high heat and acidic conditions used to make cellulose in the lignocellulosic biomass available. High production costs and activity limitations of currently available enzymatic mixtures highlight the need for cellulase prospecting and improvement through genetic engineering (Lee, 1997; Wyman, 2007). Traditional biochemical and genetic methods have demonstrated the presence of effective cellulolytic systems in insects, and have provided insight on some of these enzymes. Broader understandings of cellulose digestion in insects will continue to grow under the advent of novel technologies. High throughput metagenomic, transcriptomic and proteomic projects should vastly increase our knowledge on the components of these insect cellulolytic systems as well as their regulation. Expression of insect-derived cellulases in insect cell cultures has proven successful to characterize specificity and activity of these enzymes, as well as to identify key residues for activity (Marana *et al.*, 2004). However, and since glycosylation seems important for activity in some cases (Wei *et al.*, 2006a), further research would be necessary to optimize expression of these enzymes in bacterial or fungal systems to be used in bioreactors. Similarly, expression of insect cellulases in plants (Oraby *et al.*, 2007) needs to be investigated as a possibility for early pretreatment of lignocellulosic feedstocks.

Despite the low number of characterized insect cellulases compared to fungal or bacterial counterparts, some insect enzymes have been reported to display novel features that may be of interest for biofuel production. For example, an EG from *Aulocophora foveicollis* was reported to display optimal activity at pH 7.8 (Sami & Shakoori, 2008), which is unusual among animal (Watanabe & Tokuda, 2001) or even fungal (Lee, 1997) cellulases. Genetic manipulation has also shown that insect-derived cellulases are amenable to improvement for levels of expression (Sasaguri *et al.*, 2008), activity (Ni *et al.*, 2005), as well as thermostability (Ni *et al.*, 2007). This information highlights the promise of insect cellulolytic systems to provide improvements to the production of ethanol from plant biomass. Additionally, and considering the importance of these cellulases for insect survival (Sami & Shakoori, 2008; Zhou *et al.*, 2008), insect cellulases may also be used as targets for the design of novel insecticidal technologies.

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