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Production of functionalised chitins assisted by fungal lytic polysaccharide monooxygenase†

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The gene *CCT67099* from *Fusarium fujikuroi* was shown to encode a novel enzyme from the Lytic Polysaccharide Monooxygenase (LPMO) Family AA11. The gene was expressed and a truncated version of the enzyme, designated as *Ff*AA11, was purified from the periplasmic space of *Escherichia coli* cells at high yield. *Ff*AA11 exhibited oxidative activity against α- and β-chitins, as well as lobster shells. Under optimised conditions, *Ff*AA11 introduced 35 nmol of carboxylate (COO^-) moieties per milligram of α-chitin. These carboxylate groups were introduced onto the chitin surface under mild enzymatic oxidation conditions in an aqueous solution without changes to the crystallinity of the chitin fibres. *Ff*AA11 was also combined with a simple and environmentally friendly chemical method that transforms recalcitrant chitins into desirable functionalised (nano)materials. The use of ethyl(hydroxyimino)cyanoacetate (Oxyma)-assisted click chemistry allowed the rapid modification of the surface of *Ff*AA11-oxidized chitins, with a fluorescent probe, a peptide, and gold nanoparticles. The chemical steps performed, including the *Ff*AA11 oxidase treatment and surface chemical modification, were achieved without the production of any toxic by-products or waste organic solvents. This approach represents a novel method for the greener production of chitin-based biomaterials.

Introduction

Chitin is a polysaccharide that occurs in the exoskeletons of crustaceans and insects, and in the cell walls of fungal species and mushrooms. An estimated 100 billion tonnes of chitin are produced in biomass each year.[1] Due to the abundance of chitin and other polysaccharides of industrial relevance in marine organisms, such as alginates, a biorefinery approach based on marine biomass has great potential for the production of large amounts of carbohydrates for many applications.[2] Chitosan, the deacetylated form of chitin, is already used in many applications, especially in the biomedical and pharmaceutical sectors, but many more potential commercial products can be derived from this polymer and unmodified chitin.[3] Chitin, however, is not currently exploited at the same large scale as its derivative chitosan or the structurally similar cellulose from plant biomass. A major limitation to the direct exploitation of chitin is its lack of solubility in most aqueous and organic solvents and poor reactivity combined with a compact, resilient crystalline structure.[4]

Chitin is converted into more soluble chitosan using strong alkali, which leads to partial deacetylation of the C-2 acetamide, providing a highly reactive amine handle that can be used for standard coupling chemistries.[4] Indeed, due to the aforementioned solubility issues and low reactivity of chitin, most of the chitin-derived functional materials reported to date have been chitosan-based, and very few chemical modifications have been reported directly on chitin. However, compared to chitosan, native chitin possesses greater mechanical properties, especially strength, modulus, toughness and strain-to-failure.[5] For example, an increase in chitin content provides a greater strength to chitin/chitosan nanocomposites.[5,6] An increase in chitin content also enhances degradability of chitin/chitosan-based materials *in vivo*.[7]

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Despite a low workability, the modification of chitin still attracts a lot of interest due to its relevance to many sectors such as the biomedical, cosmetics, food, biocomposite and agrochemical industries.[8] The primary and secondary alcohols of chitin have a slow reaction rate, and thus require a strong base to be converted into the more reactive alkoxide groups.[9] More often, strong oxidising agents are used to oxidise the C-6 primary alcohols into aldehyde and/or carboxylate groups, and subsequently, desired functionalities can be chemically introduced onto chitin materials.

A typical route to chitin modification is *via* the introduction of a carboxylate group at the C-6 position of the pyranose ring using the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)/NaBr/NaClO radical-mediated oxidation reaction.[10] TEMPO oxidation has been one of the most popular methods in biopolymer modification, but the method itself suffers from a lack of selectivity. Indeed, the TEMPO reagent provokes oxidation of primary alcohols of all polysaccharides that may be present in a reaction mixture, and does not distinguish between chitin and cellulose. TEMPO-mediated oxidation is also of limited use due to its toxicity, and this is a particular concern if functional materials are subsequently to be used in food or biomedical applications.[11] Separation of the TEMPO and halide oxidiser from the reaction products is also problematic, especially for large-scale reactions. Another major drawback of TEMPO-mediated oxidation is that the method is expensive and uses environmentally harsh halide-based oxidising reagents. Therefore, there are several concerns regarding the TEMPO approach, especially when other hazardous chemicals are used for secondary modifications to produce chitin-based biomaterials for medical applications.[12] For these reasons, we have been inspired to develop an alternative avenue to functionalise the resilient native chitin polymer.

Lytic Polysaccharide Monooxygenases (LPMOs) are a recently discovered class of Auxiliary Activity (AA) enzymes that assist the degradation of recalcitrant polysaccharides by glycoside hydrolases (GHs) through an oxidising activity.[13–18] Fungal family AA11 is the most recently discovered class of LPMOs, and has oxidising activity toward crystalline chitins.[19] A similar activity had previously been reported for a bacterial enzyme, chitin-binding protein 21 (CBP21).[13,20–22] Most LPMOs from the AA9, AA10 and AA11 families introduce molecular breaks onto the surface of the target biopolymers, providing an entry point for the action of GHs and considerably enhancing their efficiency.[23–26] LPMOs have therefore attracted considerable attention owing to their potential applications in biomass deconstruction.[27,28] In addition, the oxidative activity of LPMOs has potential in the modification of polysaccharides like chitin for functionalisation, and can be exploited as a sustainable, non-chemical alternative to the TEMPO method.

Here we describe a novel fungal chitin-active LPMO from the AA11 family. So far only one AA11 has been reported,[19] and its potential for the preparation of functionalised materials has not been investigated. Using our newly discovered LPMO, we have introduced carboxylate moieties on chitin surface in a highly specific manner, which is a clear improvement to current non-selective chemical methods. Furthermore, we employed a green and safe type of conjugation chemistry for the grafting of a fluorescent probe, a peptide, and gold nanoparticles onto the chitin surface. We also demonstrate the possibility to use this new AA11 LPMO in combination with a commercially available chitinase for the one-pot production of gold-conjugated chitin nanofibres.

Experimental

Cloning of CCT67099 and *Ff*AA11

The full-length gene *CCT67099* c.1–c.1215 was codon optimized and synthesised by GeneArt (Thermofisher Scientific, MA, USA). The truncated sequence c.52–c.651 of CCT67099.1 encoding the *FfAA11* domain (amino acids 18–217) was amplified by PCR (Table S1†). To generate recombinant enzymes with an N-terminal catalytic amino acid His-1, the *CCT67099* and *FfAA11* genes were each fused to a *pelB* leader sequence using overlap PCR. The chimeric cDNA sequences (*pelB-CCT67099* and *pelB-FfAA11*) were amplified by PCR using a Q5 HF polymerase master mix (New England Biolabs, MA, USA) and subsequently cloned into the pET-26b(+) vector (ThermoFisher Scientific) between the *NdeI* and *XhoI* restriction sites. The sequences were verified at the EMBL sequencing facility (Heidelberg, Germany). Primers and gene sequences (codon optimized) are presented in Table S1.† Final constructs were transformed into *Escherichia coli* BL21 star (DE3, ThermoFisher Scientific) cells by heat shock treatment at 42 °C for 45 seconds, before cells were grown and selected on LB plates supplemented with kanamycin (50 mg L^{-1}).

Expression and purification of recombinant LPMOs

Transformed *E. coli* cells harbouring plasmids containing either *pelB-CCT67099* or *pelB-FfAA11* were grown in LB broth (BD Bioscience, CA, USA) containing 50 mg L^{-1} kanamycin, at 37 °C on an orbital shaker (200 rpm) until the absorbance at 600 nm reached 0.6–0.8. Protein expression was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Amresco, OH, USA) using optimised conditions at 16 °C and 180 rpm over 18 hours, before cells were collected by centrifugation (4000*g*, 15 min). Cell lysis was performed using the following procedure: cell pellets were resuspended in a 30 mM Tris-HCl (pH 8) buffer containing 1 mM EDTA and 20% w/v sucrose, at a ratio of 1 : 50 (wet cell weight : volume in mL), and were gently swirled at room temperature for 10 min. The cells were centrifuged at 16 000*g* for 30 min at 4 °C and rapidly subjected to osmic shock *via* resuspension in ice-cold water with gentle shaking for 10 min. The suspension was centrifuged at 16 000*g* for 30 min and the supernatant containing the periplasmic proteins was harvested and passed through an affinity HisTrap column (GE Healthcare, Uppsala, Sweden). The target proteins were eluted using 20 mM sodium phosphate (pH 7.4) elution buffers containing 0.5 M NaCl and 50–200 mM imidazole. The purified recombinant proteins were concentrated using an Amicon ultracentrifuge filter unit (molecular weight

cut-off value of 10 000 Da, Millipore, Cork, Ireland), and protein concentration was determined by using the dye-binding Bradford assay according to the manufacturer's instructions (Bio-Rad, CA, USA).

Enzyme specificity assay

Purified *Ff*AA11 was saturated with copper by incubation with a 3-fold molar excess of $CuCl_2$ for 1 h at 30 °C. Excess copper was removed by desalting the protein solution using a PD MidiTrap G-25 desalting column (GE Healthcare). The reactions were set up with 1% (w/v) chitin substrate. Substrates utilised were shrimp shell α-chitin from Sigma (MO, USA), squid pen β-chitin from Mahtani Chitosan Ltd (Veraval, India), lobster shells prepared in-house using 1 M hydrochloric acid (HCl) and ethanol,[5] and commercial substrates including Avicel (Sigma), chitosan (Mahtani Chitosan), arabinoxylan, xyloglucan, glucomannan, and mixed-linked glucan (all from Megazyme, Wicklow, Ireland). The substrates were incubated with 10 μM of Cu^{2+}-saturated *Ff*AA11 in 50 mM ammonium acetate, pH 5.0, and 1 mM ascorbic acid.[19] After 16 h or 72 h incubation at 30 °C, the mixture was centrifuged and the supernatant was collected for further analysis.

Analyses of enzymatic reaction products were performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS, Applied Biosystems, CA, USA). The reaction product (10 μL) was mixed with 10 mM NaCl (6 μL) and 2,5-dihydroxybenzoic acid (DHB) (10 mg mL^{-1}, 10 μL) in 50% (v/v) acetonitrile. Then 1 μL of the mixture was spotted onto a stainless steel plate and rapidly dried under vacuum for homogeneous crystallization. MALDI-ToF MS analysis was performed using an accelerating voltage of 20 000 V with a delay time of 200 ns, and operating the instrument under the reflectron mode.

Substrates binding assay

α-Chitin, β-chitin, lobster shell preparations, chitosan, and Avicel, were used as substrates for the *Ff*AA11 binding assay. The assay was performed essentially as described elsewhere.[21] In brief, reaction mixtures contained 1% (w/v) of substrate and 10 μM enzyme in 50 mM ammonium acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 3 h, and the chitin substrates were subsequently sedimented by centrifugation for 2 min at 13 300*g*. The supernatant contained unbound protein and was collected into a new tube. To collect the chitin-bound protein, the chitin pellet was washed with water 5 times, before a swab was used to absorb the remaining liquid over the substrate. The residual dry pellet should only contain bound *Ff*AA11. Both supernatants and pellets were mixed with 5 × SDS-PAGE reagents and incubated at 95 °C for 10 min to denature and desorb the bound enzyme for subsequent SDS-PAGE analysis.

Microscopy of chitin reaction products after *Ff*AA11 treatment

The action of *Ff*AA11 on crystalline α-chitins was monitored using a Leica DMIL LED microscope (IL, USA) and the images were processed with the Leica Application Suite (LAS). The samples were prepared as follows: (1) α-chitins (10 mg) were mixed in enzyme buffer without *Ff*AA11, or (2) the α-chitins (10 mg) were mixed in enzyme buffer with *Ff*AA11 (10 μM). Both samples were incubated in Eppendorf tubes for 48 h at 30 °C in Eppendorf tubes. The insoluble pellet was collected after a brief centrifugation and washed with water before being deposited onto a microscope slide and observed.

Enzymatic breakdown of chitins using *Ff*AA11 and chitinase

Chitin nanofibres were prepared using α-chitin pre-treated for 48 h with *Ff*AA11 (15 μM) followed by 16 h treatment with a chitinase mixture from *Trichoderma viride* (*Tv*-chi, Sigma-Aldrich) (0.4 U mL^{-1}). The products from the reaction mixture were subsequently analysed by scanning transmission electron microscopy (STEM).

α-Chitin, β-chitin and lobster shell powder were used as the substrates to test for synergistic activity between *Ff*AA11 and *Tv*-chi. The reactions were performed in three different ways: (1) *Tv*-chi only + chitins, (2) *Ff*AA11 + *Tv*-chi + chitins, (3) chitin substrates with *Ff*AA11 pre-treatment, followed by addition of *Tv*-chi. The reaction systems contained 0.4 U ml^{-1} of *Tv*-chi (one U liberates 1.0 mg of *N*-acetyl-D-glucosamine (GlcNAc) from chitin per h at pH 6.0 at 25 °C in a 2 h assay) and/or 10 μM of Cu^{2+}-saturated *Ff*AA11, as well as 1 mM ascorbic acid in 50 mM ammonium acetate, pH 5.0. The reactions were quenched by adding an equal volume of 50 mM sulfuric acid, and stored at −20 °C prior to further analysis. GlcNAc produced from the hydrolysis of chitin substrates was quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), using a Dionex ICS-3000 system (Dionex, CA, USA) equipped with a CarboPac PA1 analytical column (4 mm × 250 mm) at 30 °C, and operated at a flow rate of 1 mL min^{-1}.[29] A standard curve for the monosaccharide GlcNAc was produced to allow for quantification.

X-ray diffraction (XRD) analysis

XRD analysis was performed on a Philips X'Pert Pro Diffractometer (Model PW 3040/60; PANalytical, Almelo, Netherlands) with Cu Kα radiation (λ = 1.5418 Å), at 40 kV and 35 mA, monochromatized using a 20 μm Ni filter that primarily absorbs Cu Kβ radiation (λ = 1.392 Å). Diffractograms were recorded at room temperature in reflection mode in the range from 5° to 50° 2θ. The crystallinity index[30] was calculated as $1 - I_{am}/I_{cryst}$ where I_{am} is taken as the amorphous baseline at 16.0° and I_{cryst} is the intensity of the (020) diffraction peak at 9.5°.[31]

X-ray photoelectron spectroscopy (XPS)

Two samples were analysed by XPS: an α-chitin reference sample and α-chitin pre-treated for 48 h with *Ff*AA11. XPS spectra were recorded using a Kratos AXIS Ultra^{DLD} X-ray photoelectron spectrometer (Kratos Analytical, Manchester, UK). The samples were analysed using a monochromatic Al X-ray source. The analysis area was below 1 mm^2, with most signals from an area of *ca.* 700 × 300 μm^2. The relative elemental composition of the surface expressed in atomic % was then

obtained from quantification of detailed spectra performed for each individual element. All elements except hydrogen and helium were detected. In addition, the high-resolution carbon spectra were curve-fitted, showing chemical shifts in the carbon signals due to different functional groups (*e.g.* carbonyl and hydroxyl groups).

Radioactive labelling for the quantification of carboxyl groups on α-chitin

A modified radioactive labelling approach[32] was used to determine the content of carboxylic acid groups in chitins treated with *Ff*AA11. Briefly, carboxymethyl cellulose (CM-cellulose, 50% CM substitution, Megazyme) and different chitins were suspended in 250 μL water and mixed with 100 μL MES buffer (200 mM, pH 4.75). *N*-Cyclohexyl-*N*′-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (11.8 mM, 100 μL) was added to the polymers, followed by 500 μL of 2 M Tris-HCl buffer (pH 7.0) to raise the pH before the addition of 0.4 μmol of tritiated sodium borohydride (100 mCi/mM; PerkinElmer, MA, USA) to the reaction mixture. The reaction was incubated at 30 °C for 2 h.

The [^{3}H]-labelled CM-cellulose and [^{3}H]-labelled chitins in the buffer were precipitated by adding 2 volumes of absolute ethanol, and dried onto a 2.2 cm GFA glass fibre disk (Whatman, Maidstone, England). The CM-cellulose on the disk was exhaustively washed with 100 mM HCl and, in a step-wise manner, with 90% (v/v) ethanol, 80% (v/v) ethanol, and absolute methanol to remove the excess of tritiated sodium borohydride. The radioactivity of the [^{3}H] labelled CM-cellulose and α-chitin was measured using a MicroBeta$^{2®}$ Microplate Counter (PerkinElmer). The carboxyl groups of the modified α-chitin were quantified using a standard curve of CM-cellulose (Fig. S1†).

Labelling of α-chitins with fluorescein

The α-chitins (with or without *Ff*AA11 pre-treatment) were conjugated with fluorescein-5-thiosemicarbazide (FTSC) (Sigma) using the phosphonium coupling reagent benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (NovaBiochem), or the Oxyma-derived uranium salt ((1-cyano-2-ethoxy-2-oxoethylidenaminooxy)-dimethyl-aminomorpholino-carbenium hexafluoro-phosphate (COMU)) (NovaBiochem). The reaction was first carried out in a time-course. Labelled chitins were pelleted by centrifugation, and the fluorescence in the supernatant containing uncoupled FTSC was measured under UV light (492 nm) in a plate reader (BMG CLARIOstar, Offenburg, Germany). Next, 100 mg (with 3.47 μmol COOH, measured using the abovementioned assay) of labelled chitin was incubated with 2 equivalents (eq.) of PyBOP, 4 eq. of *N*,*N*-diisopropylethylamine (DIPEA), and 2 eq. of FTSC in dimethylformamide (DMF) (2 mL). The mixture was gently tapped for 1 min. The pellet was washed thoroughly in DMF and then water until FTSC was completely removed. The final washing solutions were monitored under UV light, until no further FTSC was detected in the wash liquid, before the α-chitin-FTSC was examined under a confocal microscope (Zeiss LSM 510 Pascal, Oberkochen, Germany) using an argon laser (488 nm).

The lobster shell was cut into multiple small sections (5 mm × 10 mm). Sections of equal weight were incubated with an excess of propylamine, PyBOP, and DIPEA for 30 min, to completely block the carboxylate moieties derived from any residual proteins in the shell section. The sections were washed thoroughly then dialysed and dried, before a 16 h treatment with *Ff*AA11 or denatured *Ff*AA11 (control, boiling for 2 min). The lobster shell section was successively washed in water, 6 M guanidine hydrochloride and water, then dialysed to ensure that all propylamine and reactants were removed. Subsequent modification of the shell section (1.94 μmol COOH/section) was achieved using (1) PyBOP, DIPEA, FTSC in DMF (2 mL), or (2) COMU, 2,6-lutidine, and FTSC in H_2O. Fluorescence was measured under UV light (320 nm) for quantification.

Solid-phase peptide synthesis

A model peptide NH_2-Gly-Ala-Leu-NH_2 (GAL) was synthesised by solid-phase synthesis using rink amide resin LL (200 μmol, NovaBiochem, Darmstadt, Germany). For N-terminal Fmoc deprotection, a solution of piperidine/DMF (1 : 5 v/v, 2 × 5 mL) was added to the resin and agitated at room temperature for 5 min. The resin was subsequently filtered and washed with DMF (5 × 3 mL), dichloromethane (DCM) (5 × 3 mL), and DMF (5 × 3 mL). The resulting resin-bound amine was used immediately in the next peptide coupling step. The amino acid coupling solution consisted of Fmoc-protected amino acid (4 eq.), PyBOP (4 eq.) and DIPEA (8 eq.) in DMF (4 mL).[33] The solution was added to the resin and the resulting suspension gently agitated at room temperature for 1 h. The resin was filtered and washed with DMF (5 × 3 mL), DCM (5 × 3 mL), and DMF (5 × 3 mL). After coupling, a mixture of acetic anhydride/pyridine (1 : 9 v/v, 2 × 5 mL) was added to the resin and agitated for 5 min. At this time the resin was filtered and washed with DMF (5 × 3 mL), DCM (5 × 3 mL), and DMF (5 × 3 mL). Steps were repeated until the target peptide was assembled. The peptide was cleaved from the resin using a mixture of trifluoroacetic acid (TFA)/iPr_3SiH/thioanisole/H_2O (5 mL, 85 : 5 : 5 : 5 v/v/v/v) and the filtrate was concentrated under a stream of nitrogen gas. The peptide was recovered from cold ether before being purified by preparative high-performance liquid chromatography (HPLC) (Agilent, CA, USA) using a Sunfire C18 column.

Conjugation of synthetic peptides and gold nanoparticles onto the α-chitin or chito-aldonic acid oligosaccharide

LPMO-treated α-chitin (10 mg) or aldonic acid (DP3) (0.5 mg) purified on a Bond Elut carbon cartridge column (Agilent, CA, USA) were conjugated with the GAL peptide (2 mg, synthesised as described above) or gold nanoparticles (20 nm, amine functionalised, PEG5000, Sigma). The coupling reaction was performed using COMU, as described above. After incubation at 30 °C and 200 rpm for 1 h, the GAL-DP3 glycopeptide was analysed directly by MALDI-ToF MS without further purification,

whereas visible particles of GAL-α-chitin were washed with 6 M guanidine hydrochloride, DMF, and water 5 times, before being hydrolysed using 4 M TFA at 121 °C for 1 h. The molecular weight of the monomeric hydrolysate (GAL-GlcN) was confirmed by MALDI-ToF MS. The chitin labelled with the gold nanoparticles was subjected to a chitinase (*Tv*-chi) treatment (16 h) as described above.

Scanning transmission electron microscopy (STEM)

STEM was performed using a cold field emission scanning electron microscope (JEM-7401 Joel Japan) equipped with transmission detector. Chitin nanofibres bearing gold nanoparticles (produced as described above) were deposited on a carbon-coated copper grid. The STEM images were captured at 30 kV. Images were processed using ImageJ.

Results and discussion

Cloning, expression and characterization of *Ff*AA11 (CCT67099)

After Pfam and MUSCLE sequence alignment analysis using Geneious 8.1.3 (Biomatters Ltd, Auckland, New Zealand) to mine for potentially undiscovered AA11 enzymes, a gene with accession number CCT67099 encoding a putative AA11 enzyme was identified by its X278 binding domain from a *Fusarium fujikuroi* genome database.[27] To investigate the function of this candidate gene, its sequence was codon optimised and synthesised with the 5′ end fused to a pelB coding sequence. The final construct, *pelb-CCT67099*, was cloned into the pET26b vector and heterologously expressed *via* periplasmic secretion in *E. coli* BL21 cells. The CCT67099-His_6 recombinant protein was purified and assayed against α-chitin, β-chitin, chitosan, and Avicel (microcrystalline cellulose), but no activity was detected from the soluble CCT67099 protein. We investigated whether the auxiliary activity may be obstructed when the CCT67099 protein is produced in full length by performing a predictive secondary structure analysis using PSIPRED.[34] This simulation study indicated that the C-terminal of CCT67099 largely possesses a random coil structure, whereas the N-terminal domain (39–188 segment) is composed of a compact β-sheet structure, suggesting that this region could be an important structural core of the enzyme. This is consistent with all previously characterised LPMOs, which have a central four-stranded β-sandwich structure. It is feasible that the C-terminal random coil polypeptide interferes with activity in the recombinant protein.

We therefore generated a smaller truncated version of the CCT67099 protein lacking the C-terminal stretch. The truncated *CCT67099* sequence (606 bp) was ligated onto *pelb*, and re-cloned into pET26b (Fig. S2†). The soluble protein (His1–His207) without the C-terminal peptide was successfully expressed in excellent yield. This truncated protein is hereafter referred to as *Ff*AA11. Sequence alignment of *Ff*AA11 shows that the protein shares 83% sequence similarity with *Aspergillus oryzae* AA11 domain (*Ao*AA11), the only AA11 so far reported in the literature.[19] The optimised yield of purified *Ff*AA11 was 56 mg L^{-1} of LB. The protein has a predicted molecular mass of approximately 22 kDa (Fig. 1). MS techniques were utilised to confirm the molecular mass of the purified protein (Fig. 1), and to confirm its identity. A 78% coverage of the protein sequence was obtained from the trypsin-liberated peptide fragments. The *Ff*AA11 domain without the C-terminal peptide was able to facilitate an oxidative catalysis on crystalline α- and β-chitins, as well as lobster shells. No activity was found against cellulose, non-cellulosic polysaccharides, including chitosan, even after an extended 72 h treatment. This confirms that the C-terminal coil of CCT67099 is redundant and hinders activity in the recombinant protein, and also confirms that the active form of the enzyme is specific for chitin.

Oxidised chitooligosaccharides (aldonic acids) with degrees of polymerization (DP) of 2, 3 and 4, and their corresponding 1,5-lactone intermediates released from chitin, were detected by MALDI-ToF MS (Fig. S3†), suggesting that the *Ff*AA11 is a Type-1 LPMO, oxidising substrates at the C1 position.[35] The lack of activity against chitosan could be the result of weak substrate interactions. Indeed, a substrate-binding assay revealed that while *Ff*AA11 binds favourably to both α- and β-chitins, it adsorbs to the surfaces of chitosan and cellulose only very weakly (Fig. S4†).

Enzymatic hydrolysis of chitin into monomers assisted by *Ff*AA11

Much research into LPMOs has so far focussed on assisting the breakdown of recalcitrant polysaccharides for saccharification purposes. We have investigated the potential of using *Ff*AA11 to convert chitin into monomeric GlcNAc. The traditional approach to produce GlcNAc from chitin involves the use of 15–36% HCl at 40–80 °C, often resulting in low yields because of acid-catalysed hydrolytic *N*-deacetylation of the GlcNAc residues.[13,14] The replacement of harsh acid by chitinolytic enzymes has been proposed, which typically makes use of synergistic chitinases, *N*-acetylglucosaminidases, and chito-

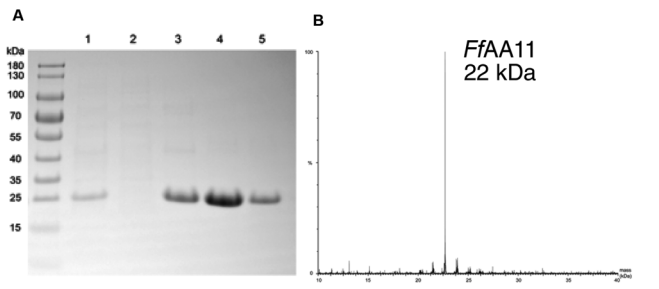


Fig. 1 Heterologous protein expression of *Ff*AA11. (A) SDS-PAGE analysis of the purified recombinant proteins: Lane 1, total protein extract from *E. coli* periplasm; Lane 2, Ni-NTA column flow through; Lanes 3–5, recombinant protein eluted with 50 mM, 100 mM, and 200 mM imidazole, respectively. (B) Mass spectrometric analysis of *Ff*AA11 confirming the predicted molecular weight.

biases.[36] While the *N*-deacetylation of GlcNAc is avoided, yields of GlcNAc from the enzymatic process tend to be low. Indeed, although chitinolytic enzymes are excellent catalysts, as in most proposed biorefinery processes the challenge remains to identify a new enzyme or new combination of enzymes capable of giving a truly competitive overall yield. This is particularly true when highly crystalline recalcitrant α-chitins are used as the starting material.

We have investigated the potential of using a combination of *Ff*AA11 with a highly efficient commercial chitinolytic enzyme mixture (*Tv*-chi) isolated from *Trichoderma virde* for GlcNAc production from α-chitin, β-chitin and lobster shell. An advantage of a one-pot reaction is that it allows several catalytic reaction steps to occur at the same time. Over a 24 h reaction, the *Ff*AA11/*Tv*-chi one-pot reaction released more soluble GlcNAc, up to 54–77%, compared to *Tv*-chi treatment alone, for all three chitin substrates tested (Fig. S5†). In a two-step hydrolysis, where the substrates were treated separately with *Ff*AA11 and then *Tv*-chi, the release of GlcNAc was even more efficient, with up to 67%, 75%, and 64% yield improvement for the α-chitin, β-chitin and lobster shells, respectively (Fig. S5†). This improved yield over the one-pot reaction may have arisen because *Ff*AA11 tends to compete for substrate binding sites with *Tv*-chi when all enzymes are present at the same time. For all three substrates tested, pre-treatment with *Ff*AA11 clearly facilitated a greater conversion to GlcNAc. Altogether these results show that *Ff*AA11 represents an effective biocatalyst to improve GlcNAc yield from chitin when used in combination with a chitinase mixture. Furthermore, given that mild reaction conditions were used, the *Ff*AA11-assisted degradation of chitin could be a useful enzyme-based platform for marine biomass refinery alleviating the need for harsh chemicals or extreme conditions.

Structure of α-chitins after *Ff*AA11 pre-treatment

Next, we investigated the effect of *Ff*AA11 pre-treatment on the structure of chitin. We used commercial α-chitins derived from shrimp shells as a model to examine the structural changes accompanying the enzymatic treatment. After 48 h incubation with the enzyme, we observed that the crystallite size of α-chitin had reduced up to 32.5 times compared to untreated chitin (Fig. 2A–C). Our results are in agreement with evidence showing that the bacterial AA10 LPMO CPB21 can breakdown large crystalline β-chitin without the additional activity of any other enzymes.[13] We then investigated the impact of *Ff*AA11 treatment on the crystallinity of these α-chitin particles. Interestingly, even with prolonged *Ff*AA11 treatment, the X-ray diffraction pattern and crystallinity index (C.I.) of the original chitin and *Ff*AA11-treated chitin do not show any clear differences; both chitins have a C.I. of approximately 0.8 (Fig. S6, Table S3†). Therefore, *Ff*AA11 appears to reduce the polycrystalline particle size but it has no impact on the C.I. of chitin. This result correlates with the proposed models of LPMO activity reported in the literature, which suggest that the oxidative action of LPMOs only results in surface puncturing of recalcitrant polymers.[24] A resulting decrease in particle size can be rationalised by the disruption of the polycrystalline aggregate nature of the chitin.

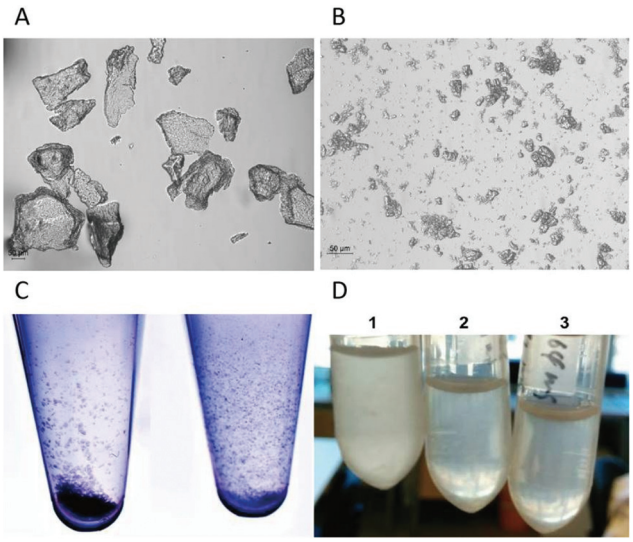
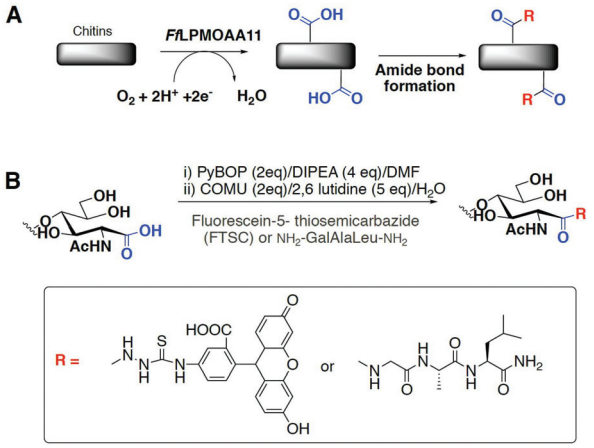


Fig. 2 Morphological studies of α-chitin before and after LPMO and chitinase treatment. (A) α-Chitin. (B) α-Chitin treated by *Ff*AA11 (scale bar is 50 μm). (C) Comparison of α-chitin suspension before (left) and after (right) enzyme treatment. (D) Change in α-chitin dispersibility after 48 h treatment with 1, *Tv*-chi only; 2, *Ff*AA11 and *Tv*-chi co-incubation; and 3, *Ff*AA11 pretreatment combined with action of *Tv*-chi.

We also analysed the surface carboxylate groups of insoluble chitins (after removal of enzymes and solubilised sugars) using XPS. The enzymatic formation of a C-1 carboxylate on GlcNAc can be evaluated by an increased abundance of the corresponding C4-carbon (peak at 289.1 eV) in *Ff*AA11-treated chitins. However, we observe very similar carbon spectra for both the reference and enzyme-treated chitins (Table S2, Fig. S7†). It could be that the carboxylate moieties on the chitin surface are not sufficiently abundant to be detected by XPS analysis. Therefore we optimised a highly sensitive radiochemical approach for quantification, using carbodiimide-activated carboxylate followed by sodium borohydride ($NaBH_4$-[^{3}H]) reduction. The optimised protocol is described in the Experimental section. After 16 h of *Ff*AA11 treatment, the carboxylate content was calculated to be of 3.47 ± 0.53 μmol per 100 mg of chitin.

Chitin surface functionalisation with Oxyma-assisted conjugation chemistry

Having successfully introduced C-1 carboxylates onto the chitin surface with the newly discovered *Ff*AA11 (Scheme 1A), we were interested in further testing the capacity of the enzyme-based approach to functionalise the chitin surface with a fluorescent probe or peptide, using covalent amide bond conjugation chemistry. First, the C-1 carboxylate was activated using a phosphonium-based coupling reagent, benzotriazole-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP). A coupling reaction of fluorescein-5-thiosemicarbazide (FTSC) (2 eq.) to chitin was carried out in the pres-



Scheme 1 (A) *Ff*AA11 facilitates oxidation on crystalline chitin fibre surface. (B) Conjugation chemistries investigated.

ence of PyBOP (2 eq.), Hünig's base (DIPEA; 4 eq.) and DMF (Scheme 1B). The reaction was completed within 1 min (Fig. S8†), after which time the excess FTSC, the carcinogenic by-product hexamethylphosphoramide (HMPA), and the organic solvent DMF were washed away with water. The modified chitin surface displayed intact FTSC, and emitted green fluorescent light at 480 nm, whereas no emission was observed on the α-chitin that was treated with non-activated *Ff*AA11 (Fig. 3A).

To improve the environmental impact of current chemical methods, and to increase the scope for biomedical applications, we must first consider the risk of autocatalytic decomposition of coupling reagents (EDC, PyBOP, HATU) that would produce toxic benzotriazole scaffolds including HMPA. Therefore we additionally trialled a safer approach based on the use of modified ethyl(hydroxyimino)cyanoacetate (Oxyma)-assisted click chemistry. Specifically, 2 eq. of the Oxyma-derived uranium salt COMU was used as the coupling reagent,[32,33] with the pyridyl base 2,6-lutidine (5 eq.) (Scheme 1B). The reaction was performed in water without pH adjustment. The reaction is conceivably as efficient as that using PyBOP (Fig. S8†), and there were no toxic by-products or organic solvent waste produced in this reaction.[34]

The combination of *Ff*AA11 catalysis and Oxyma-assisted green chemistry was further demonstrated on a small section of lobster shell. First, this required us to block the COOH moieties derived from residual lobster shell proteins with propylamine, before the surface of chitin on the lobster shell was oxidized chemoselectively by *Ff*AA11. Approximately 1.94 ± 0.4 μmol of COOH was introduced per section (5 mm × 10 mm). Amide bond formation was performed under two conditions, using either PyBOP or COMU. Under UV light exposure (320 nm), the LPMO-treated shell sections emitted bright yellow fluorescence whereas the control shell sections without activated *Ff*AA11 treatment displayed no labelling (Fig. 3B).

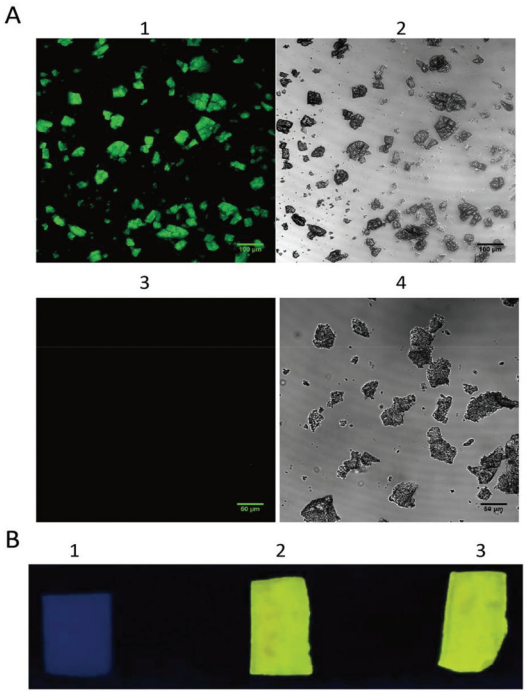


Fig. 3 Fluorescent capture images of *Ff*AA11-treated chitins (A), and lobster shells (B). (A) *Ff*AA11-treated (16 h) chitin (A1) compared to non-activated *Ff*AA11 treated chitin (A3). A2 and A4 are corresponding bright-field images of A1 and A3, respectively. (B) UV light detection of FTSC on the surface of lobster shell: B1, Section of non-activated *Ff*AA11 treatment combined with COMU coupling reagent; B2, LPMO-treated section combined with COMU coupling reagent; B3, LPMO-treated section using PyBOP as the coupling reagent. Additional negative controls are shown in Fig. S9.†

Having successfully modified the recalcitrant chitins using COMU as the coupling reagent, we tested the possibility to synthesise a C-1 specific chitin glycopeptide conjugate. The model peptide Gly-Ala-Leu (GAL) was assembled using iterative Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS), commenced from Rink amide resin on a 200 μmol scale. GAL was purified in excellent yield (96%). With this in hand, we purified its conjugation partner, the aldonic acid trisaccharide (DP3) derived from chitin with *Ff*AA11 treatment. Conjugation between GAL and the DP3 aldonic acid was performed using Oxyma-assisted click chemistry as described earlier, this time performed in the presence of a micellar surfactant (2 wt% TPGS-750 M/H_2O). The aqueous surfactant is recyclable by using a small quantity of isopropyl acetate, which is sufficient for COMU removal. Without further purification, the formation of the DP3-GAL glycopeptide product (907.2 Da) was confirmed by MALDI-ToF MS (Fig. 4A). The same strategy was also employed on a different substrate, an insoluble chitin polymer bearing surface carboxylate moieties. The product derived from the coupling reaction (chitinGAL) was acid hydrolysed in 4 M TFA to produce the low molecular weight glycopeptide GlcN-GAL (459.0 Da) (Fig. 4B). Compared to traditional methods, which involve hazardous and unsustainable chemical processes,[37]

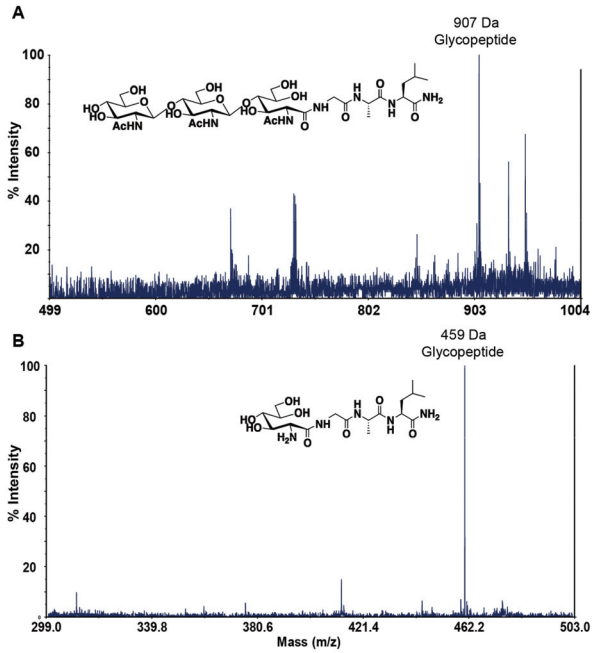


Fig. 4 MALDI-ToF MS analysis of glycopeptides. (A) GAL conjugated with $DP3_{+16}$, *m/z* 907.2 Da; (B) GAL conjugated to chitin and hydrolysed into GlcN-GAL, *m/z* 459.0 Da.

the approach described here for the preparation of chitin-peptide conjugates is based on a greener click technology. Our approach can potentially be used to synthesise inhibitors of tumor metastasis.[37]

Enzymatic hydrolysis of chitin into chitin nanofibres

In another example of efficient labelling of the chitin surface we conjugated gold nanoparticles onto the surface of chitin using an *Ff*AA11-assisted reaction. In the time-course experiments for GlcNAc production discussed earlier, we observed in both the one-pot and two-step reactions that chitins treated with *Ff*AA11 and *Tv*-chi chitinase became fully transparent after 48 h whereas chitinase treatment without *Ff*AA11 formed a product that remained turbid in solution (Fig. 2D). As we had verified that *Ff*AA11, in combination with chitinolytic enzymes, was highly effective in trimming crystalline chitins, we suspected that chitin nanofibres were also being produced, although these were not visible by the naked eye. To verify this, we designed a new multi-step reaction procedure. After treatment with *Ff*AA11, gold nanoparticles were coupled onto the chitin using the sustainable and non-hazardous COMU protocol; this was followed by chitin hydrolysis with *Tv*-chi for 16 h (Fig. 5A). Subsequent STEM showed that nanofibres were indeed produced (Fig. 5B) and they were efficiently labelled with gold nanoparticles (20 nm), showing that our novel LPMO-based method for chitin modification allows the near-simultaneous production and functionalization of nano-scale chitin structures with relatively large groups.

Conclusions

A novel fungal LPMO enzyme, *Ff*AA11, has been characterised in this study. To the best of our knowledge this is the second AA11 reported in the literature. Oxidative enzymatic modification of the chitin surface is shown to be effective, chemoselective and sustainable. Despite the positives, the surface modification assisted by *Ff*AA11 is limited to small scale application because the production of the pure enzyme is expensive, thus far we are limited to perform a chitin modification on a small scale, with a rather dilute concentration. Indeed, coupled with Oxyma-click chemistry, the enzymatic surface

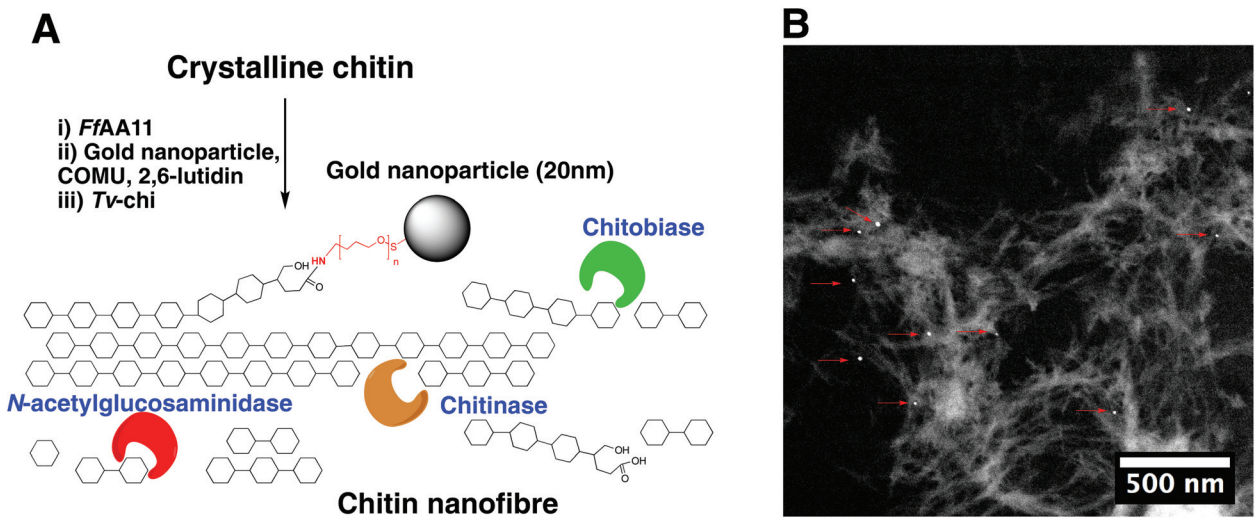


Fig. 5 (A) Preparation of gold nanoparticle labelled chitin nanofibres using *Ff*AA11-facilitated chitin surface oxidation and Oxyma-assisted conjugation to conjugate gold nanoparticles onto chitin; the labelled chitin underwent 16 h chitinolytic enzyme-assisted breakdown prior to STEM imaging of the chitin nanofibres (B). In (B), the red arrows points to gold nanoparticles. Negative control is shown in Fig. S10.†

modification process offers an interesting avenue for the further preparation of functional chitin-based biomaterials with various biomedical applications. Supporting this statement is our demonstration of the effective coupling of three different types of partner molecules and nanoparticles. When used with a commercial chitinase mixture with already potent degrading activity against recalcitrant chitins, the addition of *Ff*AA11 further promotes the production yields of GlcNAc, indicating a promising application for this novel enzyme in marine biorefinery processes. The preliminary production of chitin nanofibres using *Ff*AA11 and chitinases has also been demonstrated in this study.

Conflicts of interest

There are no conflicts to declare.

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