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# 3. Cellulose biomodification with cutinase fusion proteins

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**Abstract.** The textile industry presents well succeed examples of enzyme applications on the processing of natural fibres. The design of hybrid enzymes mimics the strategies that Nature uses to evolve and it is a powerful tool in biotechnology. The production and application of the cutinase fused to carbohydrate-binding modules (CBMs) has provided strong evidences of being an interesting strategy to pursuit. The CBMs act synergistically with the catalytic domains by increasing the effective enzyme concentration at the substrate surface and, for some CBMs, by physical disruption of the solid substrate. Future work is needed to improve the recombinant production of modular cutinases and to study in detail their affinities toward the cellulose substrates.

## 1. Introduction

Sustainable solutions for industrial processes are the major goal and driving force of applied biotechnology research. The global warming, the limited water resources, the increasing energy costs, the waste disposal problems, the increasing personal health and life quality concerns are

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inevitable issues and requirements that modern industry faces. Looking to chemical transformation processes used extensively across many industries important drawbacks, either from a commercial or an environmental point of view, can be pointed out. High temperatures and/or high pressures needed to drive reactions lead to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving high temperatures, pressures, acidity, or alkalinity need specially designed equipment and control systems. Nonspecific reactions may result in poor product yields and unwanted by-products that may prove difficult or costly to dispose[1].

In a number of cases, some or all of these drawbacks can be virtually eliminated by using enzymes[2]. The chiral nature of enzymes results in an outstanding chemical precision. Most enzymes operate at room temperature, under neutral aqueous conditions and in the absence of functional group-protection. Enzymes are environmental friendly catalysts not just because they are biodegradable themselves but also because of their mild operating conditions. They can result in processes that generate fewer waste disposal problems and that require lower energy input, leading to lower costs and lower emissions of greenhouse gases to the environment. Besides, industrial enzymes originate from microorganisms which are fermented using primarily renewable resources[1].

Nowadays, it is widely recognized that enzyme-catalysed chemical transformations are convenient alternatives to traditional (non-biological) transformations and suitable solutions to difficult synthetic problems[2]. The enzyme can be used as the sole catalyst in a reaction, in combination with other enzymes, or with inorganic reagents. Besides, many enzymes accept unnatural substrates, and genetic, pathway and medium engineering can improve further their stability and specific activity as well as modulate their substrate specificity[2].

The Nature's biodiversity provides us with a large collection of enzymes well suited for supporting life, however, they may not always be well suited for our technological interests[2,3]. Some important issues that often need to be addressed are limited substrate range, limited stability to temperature, pH and solvent, limited enantioselectivity and limited turnover number[4]. Although screening the biodiversity continues to be an important approach to find better biocatalysts, the manipulation of the protein molecule itself and/or the reaction media are gaining increasing importance[2].

A revolution in the biocatalysts design at the molecular level was provided by the establishment of the recombinant DNA technology. It allowed the manipulation of DNA sequences in a highly specific manner and the expression of their protein products in a variety of organisms, from

animal cells to bacteria[3]. If *a priori* there is the knowledge of the protein structure, function and catalytic mechanism, it is possible to rational redesign enzymes through molecular site-specific changes[2]. These changes can be a single point mutation, several point mutations or they can consist in the exchange/addition of a whole structural domain. It is also common to fuse two enzymes or an enzyme with a non-catalytic domain[5].

This chapter is devoted to cutinases genetically fused to carbohydrate-binding modules. These enzymes were engineered to be tailored for the modification of cellulose and cellulose derivatives, either in an aqueous environment where cutinase behaves as a hydrolase or in an organic environment where cutinase behaves as ester synthase.

## 2. Cutinase - A versatile enzyme

### 2.1. General description, structure and function

Cutinases are carboxylic ester hydrolases that degrade cutin which is the structural component of the outer envelope (the cuticle) of higher plants[2]. Cutin is a natural polyester composed mainly of C16 and C18 hydroxyl and epoxy fatty acids as monomers. The cuticle constitutes an efficient barrier against desiccation and entry of pathogens in plants[6].

Cutinases have been purified from different sources, especially fungi but also from pollen and bacteria[7]. The first and most studied cutinase is from the fungal pathogen of peas, *Fusarium solani pisi*[8,9]. This is a small ellipsoid protein (22 KDa, 45x30x30 Å) that bridges functional properties of lipases and esterases, because it hydrolyses both soluble esters and emulsified triacylglycerols[10,11]. Studies with triglyceride analogues revealed that cutinase activity is very sensitive to the length and distribution of the acyl chains, with highest activities observed for short chain lengths of three to five carbons[10,11]. The optimum pH for the hydrolysis is around 8.5, depending on the particular substrate used, and the maximum thermal stability is obtained for the pH range 6-9[12,13].

Cutinase is a serine esterase that shares the basic catalytic features with serine proteases[14]. The essential feature is the catalytic triad involving the hydroxyl group of a serine, the imidazole side chain of a histidine and a carboxylic side chain of an acidic residue which, in cutinase, is an aspartic residue (Figure 1)[15]. The *F. solani pisi* cutinase belongs to the superfamily of *α/β*-hydrolases, to which lipases also belong. Cutinases have a central slightly twisted β-sheet consisting of five parallel strands covered by two α-helices on either side of the sheet[11,16]. The nucleophilic serine is located in an extremely sharp turn between a β-chain and an α-helice, named nucleophilic elbow. The

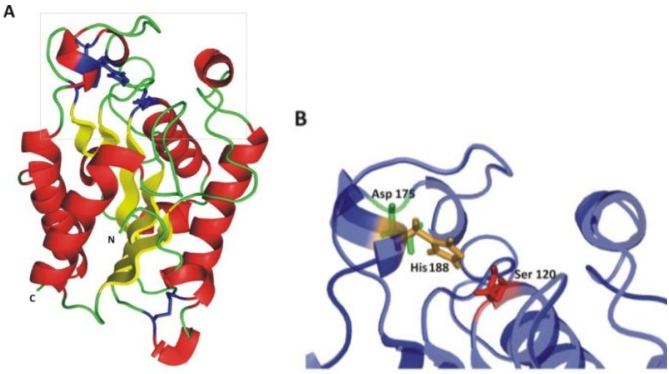


**Figure 1. A)** Schematic ribbon diagrams depicting the overall structure of cutinase from *Fusarium solani pisi* (Protein Data Bank ID: 1cex); **B)** Closed view of the catalytic triad composed by serine, histidine and aspartic acid, shown by the stick model; the two possible locations of the Ser120 side chain are both represented[2].

amino acid sequence around the catalytic serine (Gly-Tyr-Ser-Gln-Gly) matches the consensus sequence commonly present in lipases (Gly-His/Tyr-Ser-X-Gly). The catalytic triad is located at one edge of the ellipsoid protein, it is rather accessible to the solvent, in contrast with lipases, and it is surrounded by two loops with high mobility[17,18]. These loops delimit the catalytic site and the substrate binding cleft which is hydrophobic, reflecting the lipolytic nature of cutinases[11].

The *F. solani pisi* cutinase is also classified as a member of the family 5 of carbohydrate esterases[19] sharing a very similar 3D-structure with other two members with known structure: the acetyl xylan esterase (E.C. 3.1.1.72) from *Trichoderma reesei* and the acetyl xylan esterase II (AXE II) from *Penicillium purpurogenum*[20,21]. Although they present very similar overall structures, the conformation of the active site is different. These esterases have more exposed catalytic residues than cutinase, reflecting their specificity towards nonlipidic polar substrates[21]. There is also an additional loop, delimiting the active site, in the AXE II from *P. purpurogenum*, which is responsible for the preference for short substrates. When this loop is deleted, AXE II is also able to hydrolyse long chain fatty acidic esters, up to at least 14 carbons, resembling cutinase specificity[22].

## 2.2. Applications

Cutinase is a very versatile enzyme: it can utilise a broad range of substrates, it is rather stable and at low water activities cutinase catalyses the

reverse reaction – transesterification of fats and selective esterification of alcohols[2,7]. Both, hydrolytic and synthesis reactions (Figure 2) have potential use in food processing, beverages, perfume industries, pharmaceutical industries, agriculture, chemical industries and others like pulp/paper, textile and leather industries[23]. Some of its potentialities are already applied in industry, but many others are still at a research level. Some cutinase preparations have been produced by Genencor and Unilever for detergent and surfactant formulations[7]. A lot of research is devoted to the study and optimization of cutinase activity in non conventional media (organic media, supercritical fluids and gas/solid systems) and in the stabilization of the enzyme through immobilization, micro-encapsulation and lyophilized preparations[24].

Another important application of cutinase is the degradation of plastics. The synthetic polyester, polycaprolactone, was hydrolysed to soluble products that were used by *F. solani* wild type strains as source of carbon and energy[25]. In cotton industry, cutinases are used to reduce backstaining during stonewash processing of cotton denim fabrics[26]. Finishing effects such as biopolishing can be obtained using this enzyme[27]. The potential use of cutinase in bioscouring of cotton has been proposed as a good ally of pectinase[28-30]. Cutinases have also been extensively studied for the modification of polyester and polyamide fibres in order to increase their hydrophilicity and dyeability[31-37], and even for the functionalization of acrylic fibres[38]. Recently, cross-linked aggregates of cutinase from *F. solani pisi* have been used to synthesize polyamide[39].

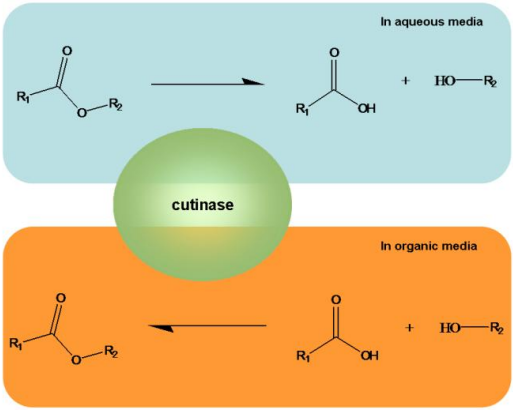


**Figure 2.** Schematic diagram depicting the reactions that cutinases in general are able to catalyse *in vitro* depending on the reaction medium.

## 2.3. Carbohydrate-binding modules

In 1950, it was proposed that the initial stage of enzymatic degradation of cellulose involved the action of two components: C1 was the non-hydrolytic component and Cx was the catalytic component[2]. Decades later, the C1 component, named cellulose-binding domain, was isolated from the fungus *Trichoderma reesei* and from the bacterium *Cellulomonas fimi* by proteolytic cleavage of the linker that connected this domain to the catalytic domain of cellulose hydrolytic enzymes[40-43]. Meanwhile, the name cellulose-binding domain evolved to carbohydrate-binding module (CBM) to reflect the diverse ligand specificity of these non-catalytic polysaccharide-recognizing domains. Many putative sequences from bacteria, fungi, plants and animals have been identified and the CBMs have been classified into more than 60 different families based on the amino acid sequence, binding specificity and structure[19].

CBMs functions are to increase the concentration of the enzyme on the surface of the substrate - the proximity effect, especially on solid polysaccharides and the substrate binding and selectivity - the targeting effect[2]. The most controversial role attributed to CBMs is the non-hydrolytic disruptive effect of the polysaccharide structure. This effect was described only for a few CBMs. The first evidence was found for the CBM from the endoglucanase A (CenA) from *C. fimi*, which alone or attached to the linker region led to a rough surface on cellulose fibres and to the release of small particles[44].

The CBMs can be grouped in two three general classes according to structural and functional similarities[2]. The type A CBMs bind to surfaces of crystalline polysaccharides and they have flat or platform-like binding sites. The type B CBMs interact with single chains, thus with the amorphous phases of the polysaccharides, and they have binding sites in the shape of a groove or cleft of varying depth. The type C CBMs bind to small sugars having one to three units.

The application of CBMs in several areas of biotechnology has been growing in the recent years due to the fact that CBMs are independently folded domains and *a priori* they can function perfectly when fused to other proteins[2]. Moreover, their ligands are abundant and renewable materials with good properties and normally low-priced. CBMs can be expressed as an affinity tag for protein immobilization, processing and purification using cellulose as a matrix in many formats, from affinity chromatography to two phase liquid separations[45]. Targeting of compounds to polysaccharides that are present in many daily products, especially cellulose, is also an important application of CBMs. This area includes oral care products, denim

stonewashing, the targeting of enzymes that do not possess natural affinity to cotton, in laundry detergents, and other chemicals, like fragrances[46-50].

## 2.4. Gain of function through fusion of cutinase with carbohydrate-binding modules

Teresa Matamá, 2010, published the first report on the cutinase engineering through fusion of a carbohydrate-binding module (CBM) to improve the surface hydrolysis of acetyl groups on cellulose acetate fibres (cellulose diacetate and cellulose triacetate). Using molecular genetic tools, four constructions were obtained using two distinct CBMs, fused independently to the C-terminal of fungal cutinase from *F. solani pisi*, and varying the linker DNA sequence. CBMs were selected on the basis of ligand affinity - one type A and the other type B, as the two cellulose acetate fibres used were structurally distinct from cellulose (the native ligand) and between themselves, with different overall crystallinities. The results presented demonstrated that hydrolysis of surface acetyl groups from cellulose diacetate and triacetate with a chimeric cutinase-CBM constitutes a promising approach to increase reactivity and hydrophilicity in these fibres, exhibiting an enhanced reactive dye uptake of treated fabrics compared with the native cutinase. By mimicking nature strategies they have designed and produced chimeric cutinases fused with a CBM which increased the performance of native cutinase[51].

Zhang, 2010, fused independently two CBMs, one from *T. fusca* cellulase Cel6A (CBMCel6A) and the other from *Cellulomonas fimi* cellulase CenA (CBMCenA), to the bacterial cutinase from *Thermobifida fusca*. The authors verified that the fusion proteins bound and catalyzed the release of fatty acids from cotton fibres significantly better in the presence of pectinase which suggested that most of the cellulose on the surface of cotton is not well exposed to the solvent. When pectinase was added to the reaction mixture, removal of pectin must have exposed the cellulose increasing the adsorption of cutinase-CBMs, which eventually led to a higher scouring efficiency of the cotton fibre[52,53].

Zhang, 2013, went further and modulated the affinity of the CBM of *T. fusca* cellulase Cel6A. They genetically modified the CBM domain of the cutinase-CBMCel6A, so that it could accommodate the structure of PET. Mutants W86L and W86Y exhibited an improvement in polyester binding and catalytic efficiency compared with the native enzyme[54].

Another innovative concept was the application of CBMs to confer a proximity effect to the chimeric cutinase for cellulose based substrates in a non-aqueous environment. Cutinase is rather stable and at low water

activities where it catalyses the reverse reaction—transesterification of fats and selective esterification of alcohols[55-58]. A commercial immobilized cutinase from *F. solani* was used to acylate cellulose with long fatty acids in non conventional media with the ultimate purpose of conferring a thermoplastic behaviour to the polymer[59-61], reported a new approach for the simple and direct biomodification of hydroxyl groups on the surface of cellulose, either as Avicel or as plain woven cotton fabric, using cutinase from *F. solani pisi* and its chimeric partner fused with the CBM N1 of the Endoglucanase C (CenC) from *C. fimi*[51]. Cutinase treatment resulted in relative increases in the carbonyl stretching peak area of 31 and 9% when octanoic acid and vegetable oil were used as substrates, respectively. Cutinase-N1 treatment resulted in relative increases of 11 and 29 % in the peak area when octanoic acid and vegetable oil were used as substrates, respectively. A major outcome of the present study was the insight that the fusion with CBM N1 also presented particular advantages in organic media to the reverse esterase activity of cutinase on cellulosic substrates.

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