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Chitin deacetylases: new, versatile tools in biotechnology

Iason Tsigos, Aggeliki Martinou, Dimitris Kafetzopoulos and Vassilis Bouriotis

Chitin deacetylases have been identified in several fungi and insects. They catalyse the hydrolysis of *N*-acetamido bonds of chitin, converting it to chitosan. Chitosans, which are produced by a harsh thermochemical procedure, have several applications in areas such as biomedicine, food ingredients, cosmetics and pharmaceuticals. The use of chitin deacetylases for the conversion of chitin to chitosan, in contrast to the presently used chemical procedure, offers the possibility of a controlled, non-degradable process, resulting in the production of novel, well-defined chitosan oligomers and polymers.

Chitin, a homopolymer comprising β-(1-4)-linked *N*-acetyl-D-glucosamine residues is one of the most abundant, easily obtained and renewable natural polymers, second only to cellulose. It is commonly found in the exoskeletons or cuticles of many invertebrates and in the cell walls of most fungi[1]. Because of its high crystallinity, chitin is insoluble in aqueous solutions and organic solvents[2].

Chitosan is a polycationic biopolymer that occurs naturally or is obtained by the *N*-deacetylation of chitin; its name does not refer to a uniquely defined compound but rather to a family of copolymers with various fractions of acetylated units. It is biodegradable, non-toxic to animals (in mice, the LD_{50} was $>16\ g\ kg^{-1}$), soluble in acidic solutions, available in various physical forms and much more tractable than chitin[2,3]. Thus, chitosan offers properties with great potential for many industrial applications.

Today, several companies are producing chitin and chitosan products on a commercial scale; the majority are located in Japan, where >100 billion tons of chitosan are manufactured each year from the shells of crabs and shrimps, an amount that accounts for ~90% of the global chitosan market (approximately four trillion yen). The major areas of application include water treatment, biomedical applications (including wound dressing and artificial skin) and personal-care products[2–13].

In addition, oligomers of chitin and chitosan have also attracted considerable attention because they have been reported to exhibit certain interesting physiological

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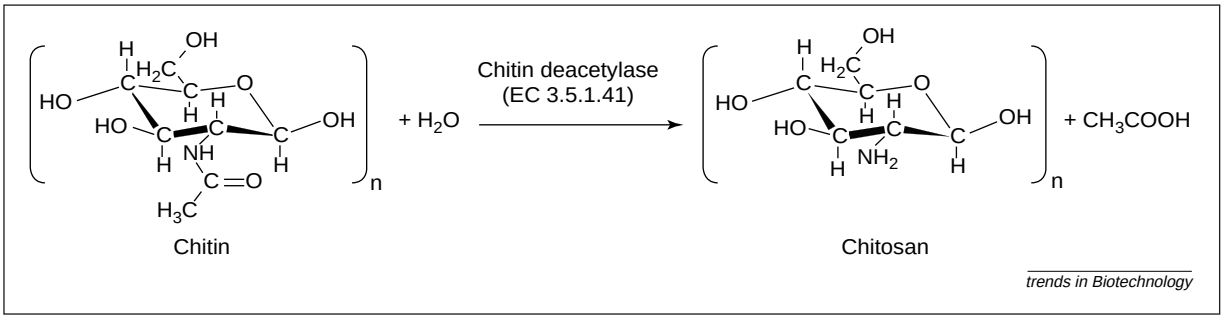

Figure 1

The catalytic action of chitin deacetylases; chitin is deacetylated by the enzyme chitin deacetylase to form chitosan and acetate.

activities, such as antitumour and antimicrobial activity[4,11] and elicitor activity for plants[4]. Furthermore, chitin and chitosan oligomers are soluble in aqueous solutions, and can be easily characterized by a variety of analytical procedures (e.g. nuclear magnetic resonance spectroscopy[14] and mass spectroscopy[15]).

Presently, chitosan is produced from chitin via a harsh thermochemical procedure. This process shares most of the disadvantages of a severe chemical procedure; it is environmentally unsafe and not easily controlled, leading to a broad and heterogeneous range of products[16]. Similarly, chitosan oligomers are prepared by partial acid-hydrolysis of chitosan polymers. A chemo-enzymatic method using lysozyme and tri-*N*-acetylchitotriose derivatives as substrates has also been reported for the preparation of specific chitosan oligomers[17]. However, the resulting products from both methods are mixtures of randomly deacetylated chitosan oligomers with various degrees of polymerization.

The use of chitin deacetylase for the preparation of chitosan polymers and oligomers offers the possibility of the development of an enzymatic process that could potentially overcome most of these drawbacks. Chitin deacetylase (CDA; EC 3.5.1.41) catalyses the hydrolysis of *N*-acetamido bonds in chitin to produce chitosan (Fig. 1). The presence of this enzyme activity has been reported in several fungi[18–24] and insect species[25]. This article reviews the most important characteristics of chitin deacetylases, and highlights some initial studies exploiting their potential use in the deacetylation of chitinous substrates for the production of oligomer and polymer products with novel characteristics.

Chitin deacetylases

Chitin deacetylases have been purified and characterized from several fungi. The most well-studied enzymes are those from the fungi *Mucor rouxii*[18–20], *Absidia coerulea*[21], *Aspergillus nidulans*[22] and two strains of *Colletotrichum lindemuthianum*[23,24]. The most important characteristics of the enzymes are summarized in Table 1. All the enzymes are glycoproteins and are secreted either into the periplasmic region or into the culture medium. Furthermore, all enzymes exhibit a remarkable thermal stability at their optimum temperature (50°C), and exhibit a very stringent specificity for water-soluble β-(1,4)-linked *N*-acetyl-D-glucosamine polymers. Nevertheless, they vary considerably in their molecular weight and carbohydrate content, and display a wide range of pH optima. One interesting property with a potential biotechnological application for the enzymes from *C. lindemuthianum* and *A. nidulans* is that, apart from their thermal stability, they are not inhibited by acetate, a product of the deacetylation reaction[22–24].

The respective genes of chitin deacetylases from the fungi *M. rouxii*[26], *C. lindemuthianum*[27] and *Saccharomyces cerevisiae*[28,29] have been cloned, sequenced and characterized. Furthermore, the expression of chitin deacetylase genes from *C. lindemuthianum* and *M. rouxii* in *Escherichia coli*[30] and *Pichia pastoris*[31] respectively, have been recently reported.

Biological role

Two different biological roles have been suggested for fungal CDAs, namely their involvement in cell-wall formation and plant–pathogen interactions. The

Table 1. Characteristics of chitin deacetylases from different sources

	Source				
	Mucor rouxii	***Absidia coerulea***	***Aspergillus nidulans***	***Colletotrichum lindemuthianum* (ATCC 56676)**	***Colletotrichum lindemuthianum* (DSM 63144)**
Localization	Periplasm	Periplasm	Culture media	Culture media	Culture media
Molecular weight (kDa)	75	75	27	24	150
Isoelectric point	3	NA	2.75	NA	3–5
Optimum pH value	4.5	5.0	7.0	11.5	8.5
Optimum temperature (°C)	50	50	50	50	50
Acetate inhibition	Yes	Yes	No	No	No
Min. DP	3	3	2	2	2
Ref.	19	21	22	24	23

Abbreviations: Min. DP, minimum degree of polymerization of a chitin oligomer required for catalysis; NA, not available.

involvement of CDA in cell-wall chitosan biosynthesis was demonstrated for the first time during studies to investigate chitin and chitosan biosynthesis in fungi. In the case of the fungus *M. rouxii*, it was revealed that chitin synthase operates in tandem with chitin deacetylase; chitin synthase synthesizes chitin by the polymerization of *N*-acetyl-D-glucosamine residues from uridine 5-diphospho-*N*-acetyl-D-glucosamine (UDP-GlcNAc), and chitin deacetylase hydrolyses the *N*-acetamido bonds in the chitin chains, acting more efficiently on nascent rather than on microfibrillar chitin[32]. Similar results were also obtained for the fungus *Absidia coerulea*, where it was also found that CDA was localized near the inner face of the cell wall (periplasmic region)[21]. This spatial arrangement of CDA is in agreement with previous studies, which reported the presence of chitin synthase activity and chitin biosynthesis in the plasma membrane fraction of fungi[33]. A similar biological role has been reported for the two CDAs (Cda1p and Cda2p) from *S. cerevisiae*. It was shown that these enzymes are required for correct ascospore wall formation[28]. Furthermore, it was found that Cda2p was the most active enzyme and performed most of the deacetylation tasks in the ascospore cell wall[29].

An alternative biological role, namely the involvement of the enzyme in plant–pathogen interactions, has been suggested for the CDA from *Colletotrichum lindemuthianum*, considering that the fungus is a plant pathogen, and the enzyme is extracellular and active on chitin oligomers. Taking into account that chitin oligomers (tetramer to hexamer) elicit plant-defence mechanisms (callose formation, lignification and synthesis of coumarin derivatives)[34,35], whereas their deacetylated forms do not[36,37], it has been proposed that CDA might deacetylate chitin oligomers that arise from the fungus cell wall subsequent to the activity of plant chitinases, and thereby diminish their elicitor activity[23].

Finally, a role for CDA during the penetration process of the fungus hypha (which contain a considerable amount of chitin) in plant tissues has been proposed. Because decreasing levels of acetylation result in less-efficient hydrolysis of chitin by plant endochitinases, it was suggested that the penetrated hypha might be protected by enzymatic deacetylation[38].

Mode of action

The mode of action of CDA has been studied in both chitosan polymers[39] and chitin oligomers[40,41]. The mode of action of CDA from *M. rouxii* has been investigated on an ~32% randomly deacetylated water-soluble chitosan substrate, with an average degree of polymerization of 30. Using ^{1}H- and ^{13}C-NMR spectroscopy, it was found that the enzyme hydrolysed the acetyl groups of the substrate according to a multiple-attack mechanism (see Glossary) with a degree of multiple attack of three. This is the maximum number of successive deacetylations that could be achieved by the enzyme because the maximum number of the consecutive *N*-acetyl-D-glucosamine residues that were found in this substrate polymer was three[39].

The mode of action of CDA from *M. rouxii* on chitin oligosaccharides (DP 2–7) has also been studied[40]. The sequence of chitin oligomers following enzymatic deacetylation was identified by the alternative use of two specific exoglycosidases in conjunction with high-pressure liquid chromatography (HPLC) and the results were further verified using ^{1}H-NMR spectroscopy. It was observed that the length of the oligomer was important for enzyme action (Fig. 2). The enzyme could not effectively deacetylate chitin oligomers with a degree of polymerization lower than three. Tetra-*N*-acetylchitotetraose and penta-*N*-acetylchitopentaose were fully deacetylated by the enzyme, however, in tri-*N*-acetylchitotriose, hexa-*N*-acetylchitohexaose and hepta-*N*-acetylchitoheptaose, the reducing end-residue remained intact. Furthermore, the enzyme initially removed an acetyl group from the non-reducing end-residue of all chitin oligomers with a degree of polymerization greater than two, and further catalysed the hydrolysis of the next acetamido groups in a progressive fashion. This mechanism resembles the mode of action that the enzyme exhibits on polymeric substrates[39].

Chitin oligosaccharides (DP2–4) have also been used as model substrates to study the mode of action of CDA from *C. lindemuthianum*[41]. Using HPLC and fast-atom-bombardment mass spectrometry (FAB-MS), it was found that in a similar manner to the *M. rouxii* enzyme, CDA from *C. lindemuthianum* could fully deacetylate tetra-*N*-acetylchitotetraose. However, it could also fully deacetylate tri-*N*-acetylchitotriose and the non-reducing end-residue of di-*N*-acetylchitobiose. Furthermore, it was shown that under specific conditions (in the presence of 3 M sodium acetate), this enzyme could also catalyse the reverse deacetylation reaction[42]. Using FAB-MS and NMR it was demonstrated that CDA from *C. lindemuthianum* could acetylate chitobiose (GlcNGlcN) and convert it into 2-acetamido-2-deoxy-D-glucopyranosyl-(1-4)-2-amino-2-deoxy-D-glucose (GlcNAcGlcN).

In summary, enzymatic deacetylation of both chitin oligomers and chitosan polymers is a well-defined reaction, in contrast to chemical deacetylation, in which the hydrolysis of the *N*-acetamido bonds of *N*-acetyl-D-glucosamine residues is performed in a random fashion. Therefore, enzymatic deacetylation involving chitin deacetylases offers a possibility for the preparation of specific novel chitosan oligomers and polymers.

Genes

The CDA genes from *M. rouxii*, *C. lindemuthianum* and *S. cerevisiae* have been cloned and characterized[26–28].

Glossary

Single chain The enzyme forms an active enzyme–polymer complex and catalyses the reaction in a 'zipper' fashion towards one end of the chitin chain; it does not form an active complex with another substrate until it reaches the end of the first chain

Multiple chain or random type The enzyme forms an active enzyme–polymer complex and catalyses the hydrolysis of only one acetyl group before it dissociates and forms a new active complex

Multiple attack The enzyme forms an enzyme–polymer complex and further catalyses the hydrolysis of several acetyl groups before it dissociates and forms a new active complex with another polymer chain

Degree of multiple attack The maximum number of acetyl groups that can be hydrolysed by the enzyme during the multiple-attack process

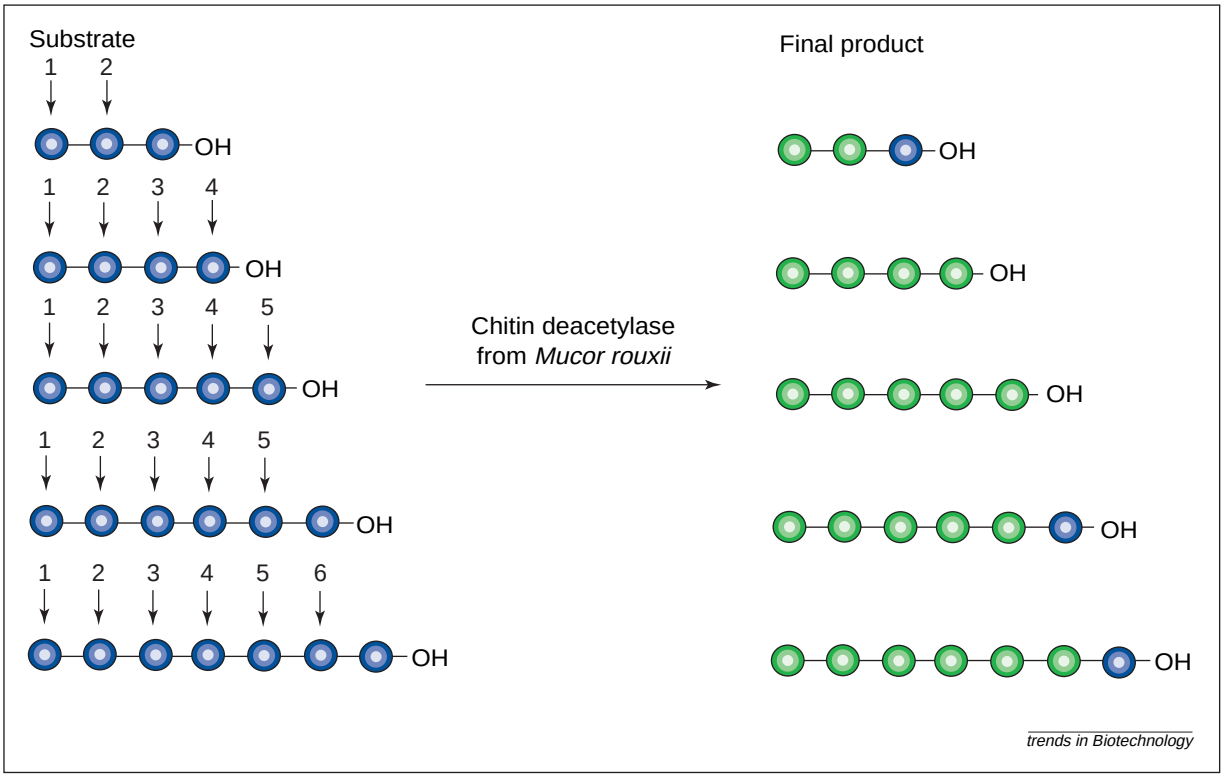

Figure 2

Mode of action of chitin deacetylase from *Mucor rouxii* on chitin oligomers. Blue spheres correspond to *N*-acetyl-D-glucosamine residues; green spheres to their deacetylated counterparts (D-glucosamine). Numbered arrows indicate the order and the site of deacetylation. The enzyme initially deacetylates the non-reducing end-residue of the oligomers, and further catalyses the hydrolysis of the next acetamido groups in a progressive fashion. Tetra-*N*-acetylchitotetraose and penta-*N*-acetylchitopentaose are fully deacetylated; in tri-*N*-acetylchitotriose, hexa-*N*-acetylchitohexaose and hepta-*N*-acetylchitoheptaose, the reducing end-residue remains intact.

The enzymes are highly homologous and, furthermore, there is a universal conserved region that exhibits a significant similarity to the rhizobial nodulation proteins[43,44] (NodB proteins), certain regions in microbial acetyl xylan esterases and xylanases[45,46], and several uncharacterized open reading frames (ORFs) in *Bacillus* sp. (Fig. 3). This conserved region has been assigned as the 'nodB homology domain' because of its similarity to NodB proteins. Apart from this region, no other homologies were found between chitin deacetylases and other proteins.

NodB proteins are chitooligosaccharide deacetylases that are essential for the biosynthesis of bacterial nodulation signals, termed Nod factors[43]. Nod factors from different rhizobial species share a common basic structure: they are all *N*-acetylglucosamine oligomers, with an *N*-acyl substitution at the non-reducing end-residue. This *N*-acyl substitution is required for the biological activity of all Nod factors because *N*-acetylglucosamine oligomers fail to elicit any nodulation-specific responses in host plants. NodB proteins specifically remove the *N*-acetyl group from the non-reducing terminal residue of *N*-acetylglucosamine oligomers, thus providing the necessary free amino group for the subsequent *N*-acylation[44].

The nodB homology domain present in *Cellulomonas fimi* and *Clostridium thermocellum* xylanases was found to be functional, exhibiting deacetylase activity against xylan, and thus contributing to the efficient hydrolysis of acetylated xylan by xylanases[45,46]. Finally, the ORFs from *Bacillus* sp., which also exhibit significant similarity with CDAs have been suggested to correspond to peptidoglycan deacetylases, although this function has yet to be verified biochemically[26].

Thus, the similarities between the CDAs highlights the catalytic domain in the CDA sequences and can direct the design of an enzyme with improved efficiency in the deacetylation of chitinous substrates.

Applications of chitosan

The following major characteristics of chitosan make this polymer advantageous for numerous applications: (1) it has a defined chemical structure; (2) it can be chemically and enzymatically modified; (3) it is physically and biologically functional; (4) it is biodegradable and biocompatible with many organs, tissues and cells; and (5) it can be processed into several products including flakes, fine powders, beads, membranes, sponges, cottons, fibres and gels[2–4]. Furthermore, its main source (crab and shrimp shells) is produced as a byproduct of the seafood industry. Consequently, chitosan has found considerable application in various industrial areas (Table 2).

As a result of its high molecular weight, cationic character and gel-forming ability, chitosan has been extensively used in industry, foremost as a flocculent in the clarification of waste-water and the detoxification of hazardous waste[2,4,5]. It was found that the adsorption capacity of chitosan varies with the amino-group content, and that polymers with ~50% deacetylation were the most effective[5]. The United States Environmental Protection Agency has also approved the use of commercially available chitosan in potable-water purification

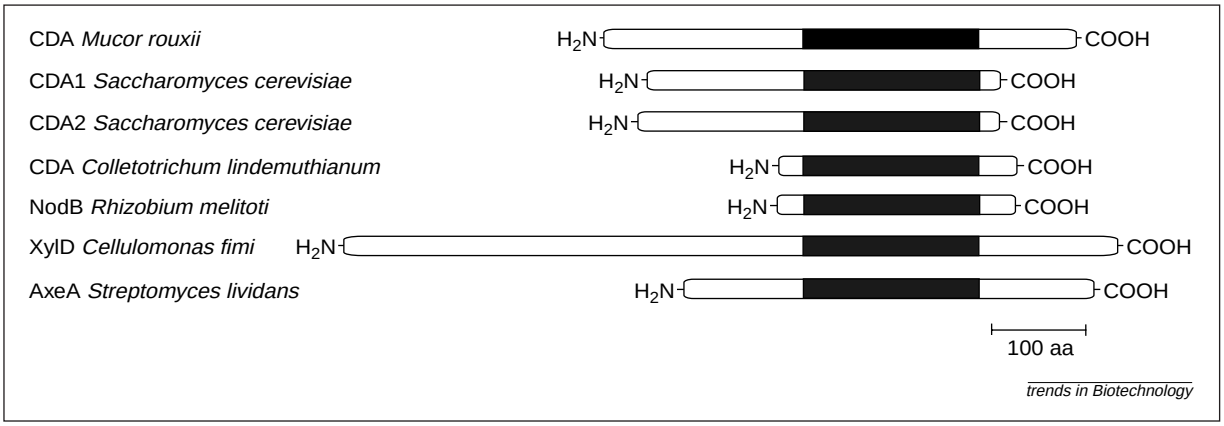

Figure 3

The NodB homology domain. Chitin deacetylases from *Mucor rouxii*, *Saccharomyces cerevisiae* and *Colletotrichum lindemuthianum* are aligned with NodB from *Rhizobium melitoti*, Xylanase D from *Cellulomonas fimi*, and acetyl xylan esterase A from *Streptomyces lividans*. The black segment indicates the NodB homology domain. Abbreviations: CDA, chitin deacetylase; NodB, nodulation protein B; XylD, xylanase D; AxeA, acetyl xylan esterase A; aa, amino acids.

systems[4] up to a maximum level of 10 mg l^{-1}. Furthermore, chitosan has been used as a paper-coating material, increasing the physical strength of cellulose paper and improving the printing quality with anionic inks[2].

Chitosan has also been used for the clarification of beverages (such as fruit juices and beers) and in the agricultural sector[2,4]. Furthermore, chitosans (>70% deacetylated) have recently been introduced to the nutritional-supplement market as a weight-loss aid and a cholesterol-lowering agent[6,47]. They are also used as a constituent of many food products, particularly in Japan[2], and are presently approved as a food additive in Japan, Italy and Finland[6].

Chitosan polymers and oligomers have recently attracted considerable attention in the pharmaceutical and biomedical fields because of their favourable characteristics, such as biocompatibility, biodegradability, antimicrobial action and non-toxicity to animals. Although chitosan has only been used in one registered pharmaceutical product (Tegasorb™, a wound-healing product), several other important applications of chitosan as an excipient in pharmaceutical products are currently being investigated[6]. Interestingly, in all cases the degree of polymerization and *N*-acetylation of the oligomers and polymers proved to be extremely important, because both these parameters influence not only their biochemical characteristics but also their biocompatibility and immunological activity[3]. For example, it was shown that chitin oligomers (DP 4–7) displayed a strong immuno-enhancing effect, inhibiting the growth of various tumour mice cells, whereas chitosan oligomers (DP 2–6) did not exhibit such an effect[4]. Furthermore, high molecular weight chitosans with a high degree of deacetylation were more effective in inhibiting bacterial growth than chitosans with a lower molecular weight and degree of deacetylation[11].

Owing to its cationic character and the presence of reactive functional groups, chitosan has also been used in the development of controlled-release technologies.

Table 2. Applications of chitosan polymers and oligomers

Application	Examples	Refs
Water treatment	Recovery of metal ions and pesticides, removal of phenols, proteins, radioisotopes, PCBs and dyes, recovery of solid materials from food-processing wastes	2,4,5
Agriculture	Seed- and fruit-coating, fertilizer and fungicide	2,6
Food and feed additives	Clarification and de-acidification of fruits and beverages, colour stabilization, reduction of lipid adsorption, natural flavour extender, texture-controlling agent, food preservative and antioxidant, emulsifying, thickening and stabilizing agent, livestock and fish-feed additive, and preparation of dietary fibres	2,3
Biomedical and pharmaceutical materials	Treating major burns, preparation of artificial skin, surgical sutures, contact lenses, blood dialysis membranes and artificial blood vessels, as antitumour, blood anticoagulant, antigastritis, haemostatic, hypocholesterolaemic and anti-thrombogenic agents, in drug- and gene-delivery systems, and in dental therapy	3,7–13
Cosmetics	Skin- and hair-care products	2
Chromatographic media and analytical reagents	Immobilization of enzymes, as a matrix in affinity and gel permeation chromatography and as enzyme substrates	2,3,42,48,49,53
Others	Synthetic fibres, chitosan-coated paper, manufacturing material for fibres, cottons, films and sponges	2

In such systems, the rate of drug administration is controlled while prolonging the duration of the therapeutic effects[6,8]. Chitosans used for these purposes have been fabricated in the form of gels, microspheres and microcapsules, and it was found that the products were more stable and the rate of drug release was more sustained when chitosans with a high molecular weight and degree of deacetylation were used[6].

In addition, chitosan polymers have a moisturizing effect on the skin, offer protection from mechanical hair damage and exhibit an anti-electrostatic effect on hair. Their moisturizing effect (which is proportional to their molecular weight and degree of deacetylation) is comparable to that of an aqueous 20% propyleneglycol or hyaluronic acid solution. They also protect the skin from microbial infections. Consequently, they have been used widely in the cosmetic industry for skin- and hair-care products[2]. Finally, chitosan oligomers and polymers, as well as their derivatives have been used extensively as analytical reagents, for example, in various enzymatic reactions as substrates for chitinases, chitosanases and lysozymes[48,49]. However, a broader use of chitosan has been significantly restricted, primarily as a result of the current methods of preparing these polymers, which lead to products exhibiting non-uniform characteristics.

Enzymatic vs chemical deacetylation

Presently, chitin and chitosan are produced from crab and shrimp shells by a thermochemical procedure. First, shells are deproteinized under alkaline conditions, and are subsequently demineralized to remove $CaCO_3$ under acidic conditions to produce pure chitin. The *N*-deacetylation of chitin is either performed heterogeneously[16] or homogeneously[50]. In the heterogeneous method, chitin is treated with a hot, concentrated solution of NaOH and chitosan is produced as an insoluble precipitate. Chitosan prepared by this method is ~85–93% deacetylated. According to the homogeneous method, chitin is also treated with NaOH, but under milder conditions. This method results in a water-soluble chitosan with an average degree of deacetylation of 48–55%.

However, both methods have three critical disadvantages: (1) they consume considerable amounts of energy; (2) they waste a large amount of concentrated alkaline solution, resulting in an increase in the level of environmental pollution; and (3) they lead to products with a broad range of molecular weights and a heterogeneous extent of deacetylation. However, a uniform material with specific physical and chemical properties is required for many high-value biomedical applications. Furthermore, the degree and distribution of deacetylation has been found to influence the physical and chemical properties, as well as the biological activities of this polymer and subsequently the properties of the pharmaceutical or other industrial formulations that are based on chitosan. In order to overcome these drawbacks in the preparation of chitosan, an alternative enzymatic method exploiting chitin deacetylases has been explored.

The effectiveness of chitin deacetylase for the preparation of chitosan has been tested using an enzyme isolated from the fungus *M. rouxii*[51]. Chitin, both in its crystalline and its chemically modified form, amorphous chitin, has been used as a substrate. The degrees of deacetylation obtained were 0.5% and 9.5%. These relatively low degrees of deacetylation indicate that the enzyme is not very effective in deacetylating insoluble chitin substrates. Similar results were also obtained using chitin deacetylases isolated from different sources[21,23,24]. Pretreatment of crystalline chitin substrates before enzyme addition seems to be necessary in order to improve the accessibility of the acetyl groups to the enzyme and therefore to enhance the yield and the rate of the deacetylation reaction. Experiments have also been performed using chitin deacetylase from *M. rouxii* and partially deacetylated water-soluble chitosans as substrates[39,52]. It was shown that the enzyme was effective in deacetylating polymers, with up to 97% deacetylation[52], although this procedure has not yet been optimized. Moreover, because enzymatic deacetylation is not a random process like chemical deacetylation, new polymers with potentially different physical and chemical characteristics can be produced.

The advantages of an enzymatic process are more evident in the deacetylation of chitin oligomers. These compounds, in contrast to their corresponding polymers, are soluble in aqueous solution and are therefore more accessible for enzyme action. Furthermore, enzymatically deacetylated oligomers, compared with chemically deacetylated oligomers, can easily be produced and have a different distribution of *N*-deacetylated residues. A variety of well-defined chitosan oligomers can be produced from a single enzymatic deacetylation step (Fig. 2). Another example indicating the specificity of the enzymatic method and the facile preparation of products is the selective *N*-deacetylation of *p*-nitrophenyl *N*,*N*′-diacetyl-β-chitobioside [$(GlcNAc)_2$-*p*NP] by CDA from *Colletotrichum lindemuthianum*[48]. The enzyme specifically deacetylates the non-reducing end of the glycoside, converting it to *p*-nitrophenyl-2-acetamido-4-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranoside (GlcNGlcNAc-*p*NP). This disaccharide derivative can be used to further classify chitinases degrading [$(GlcNAc)_2$-*p*NP]. This reaction can also be performed chemically, but a number of synthetic and purification steps would be required.

Finally, the reverse deacetylation reaction catalysed by CDA from *C. lindemuthianum* also has several interesting applications. One example is in the synthesis of a triacetylated chitosan tetramer [$(GlcNAc)_3GlcN$], which can be used to explore the physiological activity of partially deacetylated chitin oligomers[53]. The enzymatic reaction exhibits a regioselectivity that is hard to achieve by chemical methods.

In conclusion, the development of a controllable process using the enzymatic deacetylation of chitinous substrates presents an attractive alternative process that can, in principle, result in the preparation of novel chitosan polymers and oligomers.

Conclusions

There is an increasing interest in chitin deacetylases and as new primary structures become available, the application of molecular biological techniques are anticipated to provide the tools to tailor and manipulate chitin deacetylases, resulting in enzymes with novel properties that can be used for the preparation of chitosan polymers and oligomers. Future developments

in both basic research and biotechnological applications are awaited with great interest.

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Particle-based biofilm reactor technology

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Particle-based biofilm reactors provide the potential to develop compact and high-rate processes. In these reactors, a large biomass content can be maintained (up to 30 g l^{-1}), and the large specific surface area (up to 3000 m^2 m^{-3}) ensures that the conversions are not strongly limited by the biofilm liquid mass-transfer rate. Engineered design and control of particle-based biofilm reactors are established, and reliable correlations exist for the estimation of the design parameters. As a result, a new generation of high-load, efficient biofilm reactors are operating throughout the world with several full-scale applications for industrial and municipal waste-water treatment.

Biofilm reactors are used in situations wherein the reactor capacity obtained by using freely suspended organisms is limited by the biomass concentration and hydraulic residence time. This can be the case either for slow-growing organisms (e.g. nitrifiers, methanogens), whose growth in suspension requires long residence times, or for diluted feed streams (often present in waste-water treatment processes), in which only a very low biomass concentration can be achieved without biomass retention. In these cases, biofilms are an effective solution to successfully retain biomass in the reactors and to improve the volumetric conversion capacity. Biofilm reactors are not particularly useful when fast-growing organisms (i.e. with a maximum specific growth rate >0.1 h^{-1}) or concentrated feed streams are used[1] (e.g. in industrial fermentation processes). In these situations, sufficient biomass will be formed to metabolize the substrate with relatively short residence times without the need for any form of retention; it is the oxygen supply to the liquid phase, not the biomass concentration, which is often the limiting factor. For this reason, in the majority of industrial fermentation processes where high substrate concentrations are used, biofilm formation is either unnecessary or even disadvantageous, and the range of applications of immobilized-cell systems in industry is mainly limited to waste-water treatment processes[2,3]. Biofilms are extensively used in environmental biotechnology because biofilm reactors can be operated at high biomass concentrations to treat the large volumes of dilute aqueous solutions that are typical of industrial and municipal waste-waters without the need for separating the biomass and the treated effluent.

Although the use of biofilms overcomes limitations caused by a low reactor-biomass concentration, for high reactor capacities, a new bottleneck has to be considered because the delivery of poorly soluble substrates (e.g. oxygen) to the biofilm surface might become limiting. Systems with static biofilms (e.g. trickling filters) have small specific biofilm surface areas (typically less than 300 m^2 biofilm m^{-3} reactor) available for substrate transport and reaction, and thus a limited reactor capacity (the oxygen-transfer rate is typically less than 3 kg m^{-3} d^{-1} for trickling filters). Therefore, static biofilm reactors can be useful if the biomass retention and not the mass transfer is the main requirement, for example, when large volumes of liquid with very low substrate concentrations have to be treated (e.g. the removal of xenobiotics from ground water). For more-concentrated streams, the enlargement of the biofilm specific surface area can lead to a substantial reduction in the reactor volume and the area requirements of the process. A dramatic increase in biofilm surface area can be obtained by growing biofilms as small particles. The choice of the optimal particle size is a compromise between the conversion rate and the particle sedimentation rate. If the particles become too small (i.e. their settling velocity is too small), the process might again be limited by the biomass concentration that is achievable in the reactor, as for cell suspensions.

Gravity separation can be enhanced by growing the biomass in the form of dense spherical aggregates. These aggregates can either form spontaneously as large, dense granules[4,5], or attached to suspended carriers[6]

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