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Physiology of microbial degradation of chitin and chitosan

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Abstract

Chitin is produced in enormous quantities in the biosphere, chiefly as the major structural component of most fungi and invertebrates. Its degradation is chiefly by bacteria and fungi, by chitinolysis via chitinases, but also via deacetylation to chitosan, which is hydrolysed by chitosanases. Chitinases and chitosanases have a range of roles in the organisms producing them: autolytic, morphogenetic or nutritional. There are increasing examples of their roles in pathogenesis and symbiosis. A range of chitinase genes have been cloned, and the potential use for genetically manipulated organisms over-producing chitinases is being investigated. Chitinases also have a range of uses in processing chitinous material and producing defined oligosaccharides.

Introduction: chitin and chitosan

Chitin, the (1–4)-β-linked homopolymer of *N*-acetyl-D-glucosamine (Fig. 1), is produced in enormous amounts in the biosphere. A recent working estimate for both annual production and steady-state amount is of the order of 10^{10} to 10^{11} tons (Gooday 1990a). Chitin is utilized as a structural component by most species alive today. Its phylogenetic distribution is clearly defined:

Prokaryotes. Despite its chemical similarity to the polysaccharide backbone of peptidoglycan, chitin has only been reported as a possible component of streptomycete spores and the stalks of some prosthecate bacteria.

Protista. Chitin provides the tough structural material for many protists: in cyst walls of some ciliates and amoebae; in the lorica walls of some ciliates and chrysophyte algae: in the flotation spines of centric diatoms; and in the walls of some chlorophyte algae and oomycete fungi (Gooday 1990a).

Fungi. Chitin appears to be ubiquitous in the fungi (Bartnick-Garcia & Lippman 1982). Reported exceptions, such as *Schizosaccharomyces*, prove to have small but essential amounts of chitin (H. Sietsma, pers. comm.). *Pneumocystis carinii*, of uncertain affinity, has chitin the walls of its cysts and trophozoites (Walker et al. 1990).

Animals. Chitin is the characteristic tough material playing a range of structural roles among most invertebrates (Jeuniaux 1963, 1982). It is absent from vertebrates.

Plants. Chitin sensu stricto is probably absent from plants, but polymers rich in (1–4)-β-linked *N*-acetylglucosamine have been reported (Benhamou & Asselin 1989).

Chitin occurs in a wide variety of types. Three hydrogen-bonded crystalline forms have been characterized: α-chitin with antiparallel chains, β-chitin with parallel chains and γ-chitin with a three-chain unit cell, two 'up' – one 'down' (Blackwell

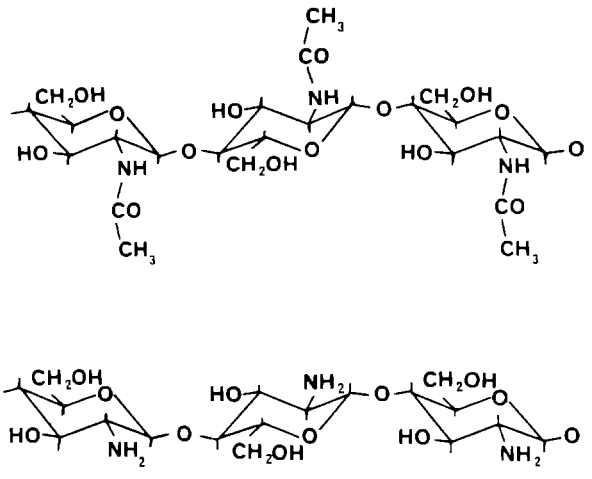

Fig. 1. Structures of chitin (top) and chitosan (bottom).

1988). α-Chitin is by far the most common, being the form found in fungi and most protistan and invertebrate exoskeletons. The importance of physical form to biological function is indicated by squid, *Loligo,* having α-chitin in its tough beak, β-chitin in its rigid pen, and γ-chitin in its flexible stomach lining.

With one exception, the chitin of diatom spines, chitin is always found cross-linked to other structural components. In fungal walls it is cross-linked covalently to other wall components notably β-glucans (Sietsma et al. 1986; Surarit et al. 1988). In insects and other invertebrates, the chitin is always associated with specific proteins, with both covalent and noncovalent bonding, to produce ordered structures (Blackwell 1988). There are often also varying degrees of mineralization, in particular calcification, and sclerotization, involving interactions with phenolic and lipid molecules (Poulicek et al. 1986; Peter et al. 1986).

Another modification of chitin is its deacetylation to chitosan, the (1–4)-β-linked polymer of D-glucosamine (Fig. 1). This is mediated by the enzyme chitin deacetylase. In the fungi this occurs in the Mucorales, where chitosan is a major component of the cell wall (Datema et al. 1977; Davis & Bartnicki-Garcia 1984) and in *Saccharomyces cerevisiae,* where it is a major component of ascospore walls. The biological significance of this deacetylation in fungi may be to give them added resistance to lysis by chitinolytic organisms. Deacetylation also occurs in arthropods, where its occurrence seems to be related to chitinous structures that undergo subsequent expansion, such as the abdominal cuticle of physogastric queen termites, and eye-lens cuticles (Aruchami et al. 1986).

Pathways of chitin degradation

The vast annual production of chitin is balanced by an equal rate of recycling. The bulk of this chitin degradation is microbial; in the sea chiefly by bacteria-free-living and in association with animal guts; in the soil chiefly by fungi and bacteria. Their biochemical pathways have been reviewed by Davis & Eveleigh (1984). Organisms that degrade chitin solely by hydrolysis of glycosidic bonds are known as chitinolytic; a more general term, not specifying the mechanism, is 'chitinoclastic'.

The best-studied pathway is the action of the chitinolytic system, of hydrolysis of the glycosidic bonds of chitin. Exochitinase cleaves diacetylchitobiose units from the non-reducing end of the polysaccharide chain. Endochitinase cleaves glycosidic linkages randomly along the chain, eventually giving diacetylchitibiose as the major product, together with some triacetylchitotriose. There may not always be a clear distribution between these two activities (see also Davis & Eveleigh 1984), as the action of these enzymes is dependant on the nature of the substrate. Thus the pure crystalline β-chitin of diatom spines is degraded only from the ends of the spines by *Streptomyces* chitinase complex, to yield only diacetylchitobiose, whereas colloidal (reprecipitated) chitin is degraded to a mixture of oligomers and diacetylchitobiose (Lindsay & Gooday 1985a). Lysozyme has a low endochitinolytic activity, but can readily be distinguished from chitinases as it readily hydrolyses *Micrococcus* peptidoglycan whereas they do not. Diacetylchitobiose (often called chitobiose, but beware confusion with the product of chitosanase) is hydrolysed to *N*-acetylglucosamine by β-*N*-acetylglucosaminidase. Some β-*N*-acetylglucosaminidases can also act weakly as exochitinases, cleaving monosaccharide units from the non-reducing ends of chitin chains.

Together, the chitinases and β-*N*-acetylglucosaminidases are known as 'the chitinolytic system'.

An alternative system for degrading chitin is via deacetylation to chitosan which is hydrolysed by chitosanase to give chitobiose, glucosaminyl-(1–4)-β-glucosaminide, which in turn is hydrolysed by glucosaminidase to glucosamine. This pathway appears to be important in some environments, for example in estuarine sediments, where chitosan is a major organic constituent (Hillman et al. 1989a, b; Gooday et al. 1991). As yet, there are no reports of a third possible pathway, involving deamination of the aminosugars (Davis & Eveleigh 1984).

Autolytic and morphogenetic chitinolysis

Where investigated in detail, all chitin-containing organisms also produce chitinolytic enzymes. In some cases, such as arthropod moulting, a role is obvious. Microbial examples include the basidiomycete fungi, the inkcaps *Coprinus* species and the puff-balls, *Lycoperdon* species, where massive autolysis follows basidiospore maturation (Iten & Matile 1970; Tracey 1955). In the case of *Coprinus*, the basidiospore discharge starts at the outermost, i.e. bottom, edges of the gills, and the gills then progressively autolyse upwards so that the spores are always released with only a fraction of a millimetre to fall into the open air for dispersal. Thus, unlike most agarics, precise vertical orientation of the gills is not required, and they are not geotropic. In the case of *Lycoperdon*, the spore-producing gleba autolyses to give a capillitium of long dry springy hyphae packed with dry spores. Raindrops cause the puff-ball to act like bellows, expelling puffs of spores into the open air. Autolytic chitinases must also act in consort with other lytic enzymes to allow plasmogamy during sexual reproduction in fungi, for example to break down the gametangial walls in the Mucorales (Sassen 1965), and to break down septa to allow nuclear migration during dikaryotization in basidiomycetes (Janszen & Wessels 1970). The accumulation of autolytic enzymes in culture filtrates of senescent fungal cultures in well-documented (Reyes et al. 1984; Isaac & Gokhale 1982) but it is unclear to what extent the chitin is recycled by these mycelia.

Chitinous fungi also produce chitinases during exponential growth. Examples include *Mucor* (Humphreys & Gooday 1984a,b,c; Gooday et al. 1986), *Neurospora crassa* (Zarain-Herzberg & Arroyo-Begovich 1983) and *Candida albicans* (Barrett-Bee & Hamilton 1984).

Possible roles for these chitinases are discussed by Gooday et al. (1986) and Gooday (1990b). They include:

Maturation of chitin microfibrils. The form of microfibrils in the wall differs in different fungi and between different life stages in the same fungus (Gow & Gooday 1983). The formation of antiparallel α-chitin microfibrils of particular orientation, length and thickness may require modelling of the chitin chains by chitinases, both by their lytic activities and their transglycosylase activities (Gooday & Gow 1991). Their transglycosylase activities may also have a role in covalently linking chains with other wall polysaccharides.

Apical growth. The 'unitary model' of hyphal growth (Bartnicki-Garcia 1973) envisages a delicate balance between wall synthesis and wall lysis, allowing new chitin chains to be continually inserted into the wall, with concomitant lysis of pre-existing chains to allow this. There is much circumstantial evidence for the role of chitinases and other lytic enzymes in this process (Gooday & Gow 1991), but as yet there is no direct evidence. The membrane-bound *Mucor* chitinase studied by Humphreys & Gooday (1984a, b, c) shared with chitin synthase the property of being activatable by trypsin, i.e. being zymogenic, suggesting that the two enzymes could be co-ordinately regulated, as would be required for orderly chitin deposition.

Branching. It is generally accepted that chitinase action will be required to form a branch. The cylindrical wall of a hypha, unlike the apex, is a rigid structure. Its chitin microfibrils are wider, more crystalline, and are cross-linked with other wall components (Wessels 1988). The site of the new branch must be weakened to allow a new apex to be

formed, and lytic enzymes are obvious contenders for this process.

Spore germination. Germination of fungal spores, and indeed hatching of protozoal cysts, requires the breaching of the wall. It seems likely that chitinases have a role in this process in at least some cases, for example in sporangiospore germination of *Mucor mucedo,* where the initial spherical growth is accompanied by a co-ordinated activation of chitinase and chitin synthase (Gooday et al. 1986).

In the budding yeast *Saccharomyces cerevisiae*, chitin is mostly confined to the septum separating the bud from the mother cell, where it is a major component. Elango et al. (1982) showed that chitinase is a periplasmic enzyme in these yeast cells, and suggested that it plays a role in cell separation. More direct evidence for this is provided by the findings that the chitinase inhibitors, allosamidin and demethylallosamidin, inhibit cell separation and lytic damage during budding (Cabib et al. 1990; Sakuda et al. 1990).

Nutritional chitinolysis

Bacteria

Chitinolytic bacteria are widespread in all productive habitats. Chitinases are produced by many genera of Gram negative and Gram positive bacteria, but not by Archaebacteria (Gooday 1979; Berkeley 1979; Monreal & Reese 1969).

The sea produces vast amounts of chitin, chiefly as carapaces of zooplankton, which are regularly moulted as the animals grow. Most of this chitin is produced near to the surface, and studies have shown that its recycling occurs both in the water column and in sediments (reviewed by Gooday 1990a). The rate of degradation will be enhanced by phenomena of adherence of chitinolytic microflora and by passage through animals guts. The importance of these processes is highlighted by the repeated finding of chitinolytic bacteria, principally of the genera *Vibrio* and *Photobacterium*, associated with zooplankton and particulate matter (e.g. Hood & Meyers 1977). Estimations of population densities of chitinolytic bacteria, both as total counts and as percentages of total heterotrophs, have shown considerable variation, but consistently higher counts have been reported from marine sediments than from the overlying seawater (Gooday 1990). Studies such as that by Helmke & Weyland (1986) conclude that indigenous bacteria are capable of decomposing chitin particles throughout the depth of the Antarctic Ocean, as are chitinases produced in surface waters and transported down by sinking particles.

Estuaries are particularly productive: Reichardt et al. (1983) isolated 103 strains of chitinolytic bacteria from the estuarine upper Chesapeake Bay. Of these, 44 were yellow-orange pigmented *Cytophaga*-like bacteria , with a range of salt requirements. Others were vibrios, pseudomonads and *Chromobacterium* strains. Chan (1970) presented studies of chitinolytic bacteria from Puget Sound: genera identified, in decreasing order of abundance, were *Vibro, Photobacterium, Aeromonas, Cytophaga, Streptomyces, Photobacterium, Bacillus* and *Chromobacterium*.Pel & Gottschal (1986a, b; 1989) and Pel et al (1989, 1990) have investigated chitinolysis by *Clostridium* strains isolated from sediments and the anoxic intestine of plaice from the Eems-Dollard estuary. They found that in pure culture chitin was degraded slowly; diacetylchitobiose accumulated, but soon disappeared as *N*-acetylglucosamine accumulated. They suggested that the *Clostridium* strains are specialised utilizers of diacetylchitobiose, and accumulation of *N*-acetylglucosamine represents non-utilizable monomers appearing during random hydrolysis of chitin oligomers. Chitin degradation was greatly enhanced by coculture with other bacteria from the sediments. One aspect of this enhancement they suggest is the release of stimulatory growth factors, such as a thioredoxin-type compound that maintained the reduced state of essential sulphydryl groups in the chitinolytic system. Interspecies interactions may also play a role for this bacterium if it is exposed to oxygen in the upper layers of sediments, as accumulating mono- and disaccharides could provide substrates

for facultative aerobic bacteria, which would consume oxygen to render the microenvironment anaerobic again.

Chitinolytic bacteria are also abundant in freshwaters, characteristic genera in the water column being *Serratia, Chromobacterium, Pseudomonas, Flavobacterium,* and *Bacillus*, with *Cytophaga johnsonae* and actinomycetes in sediments (Gooday 1990a).

The soil contains many chitinous animals and fungi as its normal living components, and chitinolytic bacteria can be isolated readily. The numbers and types reported vary greatly with different soils and methods of isolation, but major genera are *Pseudomonas, Aeromonas, Cytophaga johnsonae, Lysobacter, Arthrobacter, Bacillus* and actinomycetes (Gooday 1990a).

When grown in liquid culture, most of the chitinolytic bacteria secrete chitinases into the medium. *Cytophaga johnsonae*, a ubiquitous soil organism, characteristically binds to chitin as it degrades it. Wolkin & Pate (1985) describe a class on non-motile mutants with an interesting pleiotropy. They are all unable to digest and utilize chitin, as well as being resistant to phages that infect the parental strain and having relatively non-adherent and non-hydrophobic surfaces compared with wild-type strains. The authors conclude that all characteristics associated with this pleiotropy require moving cell surfaces, and that chitin digestion requires some feature of this, presumably involving enzymatic contact between bacterium and substrate. Pel & Gottschal (1986a) illustrate direct contact between cells of the chitinolytic *Clostridium str.* 9.1 and chitin fibrils, and as for cellulolytic *Clostridium* species, this may involve specific enzymatic structures on the cell surface. Particular attention has been paid to adsorption of the pathogenic but also chitinolytic *Vibrio* species. Kaneko & Colwell (1978) describe strong adsorption to chitin of *Vibrio parahaemolyticus* from the estuarine Chesapeake Bay. They suggest that this has an ecological as well as digestive significance to the bacteria, as the adsorption was reduced by increasing values of salinity and pH from those of the estuary to those of sea-water. This phenomenon would favour retention of the bacteria within the estuary. Bassler et al. (1989) have found that not only does *Vibrio furnissii* adhere to chitin, but also it shows specific chemotaxis to chitin oligosaccharides (monosaccharide to hexasaccharide), with at least two or three distinct chemoreceptors. In contrast it shows slight to no chemotaxis to a range of other nutrient sources, such as glycerol, lactate and amino acids, with the exception of L-glutamic acid.

Where investigated, chitinase production by bacteria has been shown to be inducible by chitin oligomers and low levels of *N*-acetylglucosamine (Jeaniaux 1963; Monreal & Reese 1969; Kole & Altosaar 1985).

Fungi

Chitinolytic fungi are readily isolated from soils, where they rival or even exceed the chitinolytic activities of bacteria. Most common are Mucorales, especially *Mortierella* spp. and Deuteromycetes and Ascomycetes, especially the genera *Aspergillus, Trichoderma, Verticillium, Thielavia, Penicillium* and *Humicola* (Gooday 1990a). These fungi characteristically have inducible chitinolytic systems (Sivan & Chet 1989). Baiting of freshwater sites with chitin yields a range of chitinolytic fungi, interesting members of which are the chytrids, such as *Chytriomyces* species (Reisert & Fuller 1962), and *Karlingia astereocysta*, which has a nutritional requirement for chitin that can only be relieved by *N*-acetylglucosamine; i.e. it is an 'obligate chitinophile' (Murray & Lovett 1966). Fungi are rare in the sea, but the sea is rich in chitin, and Kohlmeyer (1972) described a range of fungi degrading the chitinous exoskeletons of hydrozoa. Only one could be identified, the ascomycete *Abyssomyces hydrozoicus*.

Slime moulds, protozoa and algae

The Myxomycetes, 'true slime moulds', are a rich source of lytic enzymes, and, for example, *Physarum polycephalum* produces a complex of extracel-

lular chitinases (Pope & Davies 1979). Soil amoebae, *Hartmanella* and *Schizopyrenus* produce chitinases. These enzymes must participate in the digestion of chitinous food particles engulfed by the slime mould plasmodium and by the amoebae. Phagocytotic ciliates probably also have the capacity to digest chitin, and chitinase activities have been implicated in the unusual feeding strategies of *Ascophrys*, a chitinvorous ectosymbiont of shrimps (Bradbery et al. 1987), and *Grossglockneria*, which feeds by digesting a tiny hole through a fungal hypha and sucking out cytoplasm (Petz et al. 1986). The colourless heterotrophic diatom, *Nitzschia alba*, is also reported to digest chitin (A.E. Linkens, quoted by Hellebust & Lewin 1977).

Chitinolysis in pathogenesis and symbiosis

Pathogens of chitinous organisms characteristically produce chitinases. These can have two roles; they can aid in the penetration of the host; and they can provide nutrients directly in the form of amino sugars and indirectly by exposing other host material to enzymatic digestion. Examples include the oomycete *Aphanomyces astaci*, a pathogen of crayfish (Soderhall & Unestam 1975); the fungus *Paecilomyces lilacius*, a pathogen of nematode eggs (Dackman et al 1989); the entomopathogenic fungi, *Beauveria bassiana, Metarhizium anisopliae* and *Verticillium lecanii* (Smith & Grula 1983; St Leger et al. 1986); mycophilic fungi, *Cladobotryum* species and *Aphanocladium album* (G.W.Gooday unpubl.; Zhloba et al. 1980; Srivastava et al. 1985); the bacterial *Serratia* species, insect pathogens (Lysenko 1976; Flyg & Boman 1988); and a *Photobacterium* species causing exoskeleton lesions of the tanner crab (Baross et al. 1978). Other examples where chitinases may be implicated but have yet to be characterised include the ciliate protozoa feeding on fungi and shrimps, discussed in the previous section, and the baculoviruses infecting insect larvae. Infection by these viruses is mediated by a disruption of the chitinous peritrophic membrane by a viral factor of unknown action (Derksen & Granados 1988).

That the chitin of the gut peritrophic membrane is a site of attack by insect pathogenic bacteria is suggested by experiments with *Drosphila melanogaster* (Flyg & Boman 1988). Flies with mutations in two genes *cut* and *miniature* are more susceptible than the wild type to infection by *Serratia marcescens*. That the *cut* and *miniature* mutations lead to deficiencies in chitin content was demonstrated by showing that pupal shells from the mutant strains were more readily digested by *Serratia* chitinase, and especially by synergistic action of chitinase and protease, than those of other strains. Also a mutant bacterial strain, deficient in chitinase and protease, was much less pathogenic to the flies. Daust & Gunner (1979), studying bacterial pathogenesis of larvae of the gypsy moth, showed that the virulence of the chitinolytic bacterium strain 501B was synergistically enhanced by co-feeding the larvae with fermentative nonpathogenic bacteria. They explained this by the acid production by the fermentative bacteria having the effect of lowering the alkaline pH of the larval gut to a value that gave greater activity of the chitinase from 501B, leading to disruption of the peritrophic membrane. The sugar beet root maggot, however, has turned the chitinolysis by *Serratia* to its advantage by developing a symbiotic relationship with *S. liquefaciens* and *S. marcescens* (Iverson et al. 1984). These bacteria become embedded in the inner puparial surface, and aid the emergence of the adult fly by their digestion of the chitin of the puparium. The symbiotic bacteria are present in all developmental stages, including the eggs. Maternally inherited chitinolytic bacteria are also implicated in susceptibility of tsetse flies to infection with trypanosomes (Maudlin & Welburn 1988). The susceptible flies have infections of 'rickettsia-like organisms', which produce chitinase when in culture in insect cells. The resistance of refractory tsetse flies (lacking the bacterial infection) is ascribed to killing of the trypanosomes in the gut mediated by a lectin. Maudlin & Welburn suggest that the bacterial chitinolysis releases amino sugars that inhibit the lectin-trypanosome binding, and thus results in survival of the trypanosomes.

Chitinase production by the entomopathogenic fungi is inducible by chitin oligomers, *N*-acetylglucosamine and glucosamine (Smith & Gruler 1983;

St. Leger et al. 1986). St Leger et al. also report that chitosanase is co-induced with chitinase in *M. anisopliae*. In insect pathogenesis, chitinase has its importance in acting in synergism with proteases, and Bibochka & Khachatourians (1988) suggest that both activities are coordinately regulated. They show that low levels of *N*-acetylglucosamine will induce a serine protease in *Beauveria bassiana*, and suggest that an initial constitutive chitinase attack on the insect cuticle would yield *N*-acetylglucosamine, leading to the coordinate induction of chitinases and proteases.

Chitin in fungi and invertebrates composes a considerable part of the diet of many herbivorous and carnivorous animals. There can be three sources of chitinolytic enzymes in the animal's digestive system; from the animal itself, from the endogenous gut microflora, or from the ingested food (Gooday 1990a). Most work has been done with fish, where a typical marine fish gut microflora is dominated by chitinolytic strains of *Vibrio, Photobacterium* and enterobacteria. However, it is clear that the fish produce their own chitinases, which they use as food processing enzymes rather than directly nutritional enzymes. Thus the gut bacteria cannot be regarded as mutualistic symbionts with respect to chitin as the rumen symbionts are with respect to cellulose (Lindsay et al. 1984; Lindsay & Gooday 1985b; Gooday 1990a). With mammals the situation is less clear: whales have chitinolytic microflora in their stomachs, which may contribute to a rumen-type fermentation (Seki & Taga 1965; Herwig et al. 1984); Patton & Chandler (1975) describe digestion of chitin by calves and steers, implying a chitinolytic rumen flora; and Kuhl et al. (1978) found elevated caecal weights in chitin-fed rats, suggesting participation of intestinal bacteria in chitin digestion.

Among invertebrates, chitin digestion is widespread with or without participation of a microbial chitinolytic flora (Jeuniaux 1963). Borkott & Insam (1990), working with the soil springtail, *Folsomia candida*, conclude that at least in this arthropod there is a mutualistic symbiosis with its gut chitinolytic bacteria, *Xanthomonas* and *Curtobacterium* species. Thus the steady increase in biomass in animals fed every four days with chitin plus yeast extract was prevented by treatment with the antibiotic tetracycline. In a food preference experiment, the animals chose to feed on chitin agar strips that had been pre-inoculated with the chitinolytic bacteria or the animal's faeces, suggesting that some pre-digestion of the chitin was aiding its utilization by the animal.

Degradation of chitosan

As described earlier, chitosan is a major component of the walls of the common soil fungi, the zygomycetes, and is produced by deacetylation of chitin to form a major organic component of estuarine sediments. Chitosanase was discovered and shown to be widespread among microbes by Monaghan et al.(1973) and Monaghan (1975). It is produced by bacteria such as species by *Myxobacter, Sporocytophaga, Arthrobacter, Bacillus* and *Streptomyces*, and by fungi such as species of *Rhizopus, Aspergillus, Penicillium, Chaetomium* and the basidiomycete that is a very rich source of glucanase, 'Basidiomycete sp. QM 806'. Davis & Eveleigh (1984) screened soils from barnyard, forest and salt marsh for chitosan-degrading bacteria, and found them at 5.9, 1.5 and 7.4%, respectively of the total heterotrophic isolates, compared with 1.7, 1.2 and 7.4% chitin-degraders. They investigated chitosanase production by a soil isolate of *Bacillus circulans* in more detail, and showed that it was inducible by chitosan but not by chitin or carboxymethylcellulose, and was only active on chitosan. In contrast, the chitosanase from a soil isolate of *Myxobacter* species was active against both chitosan and carboxymethylcellulose (Hedges & Wolfe 1974).

Biotechnology of chitinases and chitosanases

With chitin and chitosan being an enormous renewable resource, much currently going to waste from the shellfish and fungal fermentation industries, and with their essential roles in fungi and invertebrates, it is not surprising that there is much current interest in these polysaccharides and in

their degradative enzymes (Muzzarelli & Pariser 1978; Hirano & Tokura 1982; Zikakis 1984; Muzzarelli et al. 1986; Skjak-Braek et al. 1989). Nevertheless the number of successful applications has been disappointedly low. Some of those involving lytic enzymes are dealt with here.

Cloning of chitinase genes

Chitinases from bacteria, fungi and plants have been cloned. Of many bacterial isolates, Monreal & Reese (1969) found *Serratia marcescens* and *Serratia liquefaciens* (*Enterobacter liquefaciens*) to be the most active producers of chitinases. Roberts & Cabib (1982) describe purification of the chitinases, and mutant strains with increased production of chitinase have been produced (Kole & Altosaar 1985; Reid & Ogrydziak 1981). Two chitinases genes *chiA* and *chiB* from random cosmid clones of *S. marcescens* have been inserted into *Escherichia coli*, and then into *Pseudomonas fluorescens* and *Pseudomonas putida*, resulting in four strains of genetically manipulated *Pseudomonas* that have considerable chitinase activities (Suslow & Jones 1988). The rationale to this work is to produce chitinolytic rhizosphere bacteria potentially of value for the biocontrol of soil-borne fungal and nematode diseases of crop plants, as chitin is an essential component of fungal walls and nematode egg cases (Gooday 1990d). In another approach using the same genes, Jones et al. (1986, 1988, Taylor et al. (1987) and Dunsmuir & Suslow (1989) have obtained expression of *chiA* in transgenic tobacco plants, using a range of promoters. These transgenic plants showed increased resistance to the tobacco brown-spot pathogen *Alternaria longipes*. Lund et al. (1989) showed that the *chiA* gene product was secreted by the plant cells in a modified form, and suggest that the bacterial signal sequence is functioning in the plant cells and that the chitinase is *N*-glycosylated through the secretory pathway. Fuchs et al. (1986) have characterized five chitinases in *S. marcescens*, and identified clones from a cosmid library encoding for the *chiA* gene. Their aim was biological control via phylloplane and rhizoplane bacteria. Horwitz et al. (1984) describe attempts at cloning the *Serratia* chitinases into *E. coli,* then back into *S. marcescens* on a high copy number plasmid, to produce a bacterium of value for a bioconversion process to treat shellfish waste. They isolated multiple phage clones encoding both *N*-acetylglucosaminidase and chitinase activity, and suggested that these are linked in a chi operon, which was also suggested by Soto-Gil & Zyskind (1984) in their work towards cloning these genes from *Vibrio harveyi* in *E. coli*. Shapira et al. (1989) have cloned a chitinase gene from *S. marcescens* into *E.coli*, and shown that both the *E.coli* containing the appropriate plasmid and enzyme extracts produced by this strain, have potential for biological control of fungal diseases of plants under greenhouse conditions.

Streptomyces species are well-known producers of active chitinases (Jeuniaux 1963). A chitinase from *S.erythraeus* has been purified and sequenced, with 290 amino acid residues, a molecular weight of 30,4000 and two disulphide bridges, but no homology with other chitinases or lysozymes (Hara et al. 1989; Kamei et al. 1989). A chitinase from *S. plicatus* has been cloned from a DNA library and expressed in *Escherichia coli* (Robbins et al. 1988). The *Streptomyces* chitinase was secreted into the periplasmic space of *E.coli* and its signal sequence was removed. A gene for chitinase from *Vibrio vulnificus* has also been cloned into *E. coli*, and was expressed but not secreted into the medium (Wortman et al. 1986). A gene for chitinase in *Saccharomyces cerevisiae* has been cloned by transforming the yeast with vector plasmids containing a genomic library, and screening for over-producing transformants (Kuranda & Robbins 1988).

Plants produce chitinases as major component of their 'pathogenesis-related proteins' induced following attack by potential pathogens or treatment with ethylene (Mauch & Staehlin 1989). These plant chitinases have antifungal activity (Mauch et al. 1988) that can be greater than that of some bacterial chitinases (Roberts & Selitrennikoff 1988). There is now sufficient information to classify the plant chitinases into at least three structural groups: Class I, basic proteins located primarily in the vacuole, sharing amino-terminal sequence homology with wheat germ agglutinin and hevein;

Class II, acidic, extracellular, having sequence homology with the catalytic domain of Class I, but without the hevein domain; Class III, acid, extracellular, with no homologies to Classes I or II (Payne et al, 1990; Shinshi et al. 1990). Several genes for plant chitinases have been cloned (e.g. Broglie et al. 1986; Payne et al. 1990) and expressed in transgenic plants (Linthorst et al. 1990) with the aim of increasing the plants' resistance to fungal pathogens.

Uses of chitinases and chitosanases

Oligomers of chitin and chitosan have value as fine chemicals and as potential pharmaceuticals (Gooday 1990c). As well as direct hydrolysis of chitin by chitinases, a promising development is the characterization of the transglycosylase activities of these enzymes. Thus Usai et al. (1987, 1990) and Nanjo et al. (1989) describe the use of a chitinase from *Nocardia orientalis* for the interconversion of *N*-acetylglucosamine oligomers, especially to produce hexa-*N*-acetylchitohexose, an oligosaccharide with reported antitumour activity (Suzuki et al. 1986). The transglycosylase activity is favoured by a high substrate concentration and a lowered water activity, i.e. in increasing concentrations of ammonium sulphate. The production of the disaccharide, *N,N'*-diacetylchitobiose, from chitin is described by Takiguchi & Shimahara (1988,1989). They isolated two bacteria, *Vibrio anguillarum* strain E-383a and *Bacillus licheniformis* strain X-Fu, the growth of which in chitin-containing medium resulted in the accumulation of 40 and 46%, respectively conversion of chitin to diacetylchitobiose. Pelletier & Sygusch (1990) and Pelletier et al. (1990) describe the characterization of chitosanases from *Bacillus megaterium*, and their use to assay the degree of deacetylation in samples of chitosan. A direct medical use has been suggested for chitinases in the therapy of fungal diseases, in potentiating the activity of antifungal drugs (Pope & Davies 1979; Orunsi & Trinci 1985). Immunological problems however, probably debar this until anti-iodiotypic antibodies for appropriate chitinases are developed.

Chitinases have found extensive use in the preparation of protoplasts from fungi, a technique of increasing importance in biotechnology (Peberdy 1983). Examples include the chitinases from *Aeromonas hydrophila* subsp. *anaerogenes* (Yabuki et al. 1984) and *Streptomyces olivaceoviridis* (Beyer & Diekmann 1985). Chitosanases are required to make protoplasts from species of the Mucorales (Reyes et al. 1985).

Uses of chitinolytic organisms in biocontrol

As most fungal and invertebrate pests and pathogens have chitin as an essential structural component (Gooday 1990d), chitinase activity could have an important place in the repertoire of mechanisms for biological control. Thus the strongly chitinolytic fungus *Trichoderma harzianum* has good potential for the control of a range of soil-borne plant pathogens (Lynch 1987; Sivan & Chet 1989). Dackman et al. (1989) report that chitinase activity is required for soil fungi to infect eggs of cyst nematodes. Sneh (1981) discusses the use of rhizophere chitinolytic bacteria for biological control. Use of genetic manipulation for the development of organisms with enhanced chitinolytic activities for biological control has been discussed earlier. As well as application of the organisms themselves, there have been reports of biological control by addition of chitin to the soil, presumably as this encourages the growth of chitinolytic microbes which then have a better inoculum potential to infect the soil-borne pathogens and pests, but results currently are variable, and the procedures need further investigation (Gooday 1990a).

Allosamidin and demethylallosamidin

These are antibiotics produced by *Streptomyces* strains, discovered independently by Sakuda et al (1987a) and as metabolite A82516 by Somers et al. (1987), in screens for chitinase inhibitors as potential insecticides. Allosamidin is insecticidal to the silkworm by preventing ecdysis. It does not affect egg hatching of the housefly, but prevents devel-

opment from larvae to pupae. It has an interesting spectrum of activity, strongly inhibiting chitinases from nematodes and fish, less strongly those of insects and fungi, weakly those of bacteria, and not inhibiting yam plant chitinase (Gooday 1990a, c). Allosamidin is a pseudotrisaccharide, being a disaccharide of *N*-acetylallosamine (until now unknown in nature) linked to a novel aminocyclitol derivative, named allosamizoline (Sakuda et al. 1987b). Demethylallosamidin, a minor cometabolite, has similar activity to allosamidin in inhibiting the silkworm chitinase, but is more inhibitory to the chitinase from *Saccharomyces cerevisiae* (Isogai et al. 1989; Sakuda et al. 1990).

Conclusions

It is clear that the simple definition of chitinase activity, 'hydrolysis of N-acetyl-D-glucosaminide (1–4)-β-linkages in chitin and chitodextrins', belies the complexity and diversity of this group of enzymes. There is increasing awareness of the biological roles and importance of chitin and related glucosaminylglycans, both in nature and technology, and we can look forward to major advances in the next few years.

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