9. Physiology of microbial degradation of chitin and chitosan

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I. 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:

(a) Prokaryotes. Despite its chemical similarity to the polysaccharide backbone of peptidoglycan, chitin has only been reported as a possible component of streptomycete spores and of the stalks of some prosthecate bacteria.
(b) 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).
(c) Fungi. Chitin appears to be ubiquitous in the fungi (Bartnicki-Garcia and Lippman 1982). Reported exceptions, such as *Schizosaccharomyces*, prove to have small but essential amounts of chitin. *Pneumocystis carinii*, of uncertain affinity, has chitin in the walls of its cysts and trophozoites (Walker et al. 1990).
(d) Animals. Chitin is the characteristic tough material playing a range of structural roles among most invertebrates (Jeuniaux 1963, 1982). It is absent from vertebrates.
(e) Plants. Chitin *sensu stricto* is probably absent from plants, but polymers rich in (1–4)-β-linked *N*-acetylglucosamine have been reported (Benhamou and Asselin 1989).

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

C. Ratledge (ed.), Biochemistry of Microbial Degradation, 279–312.

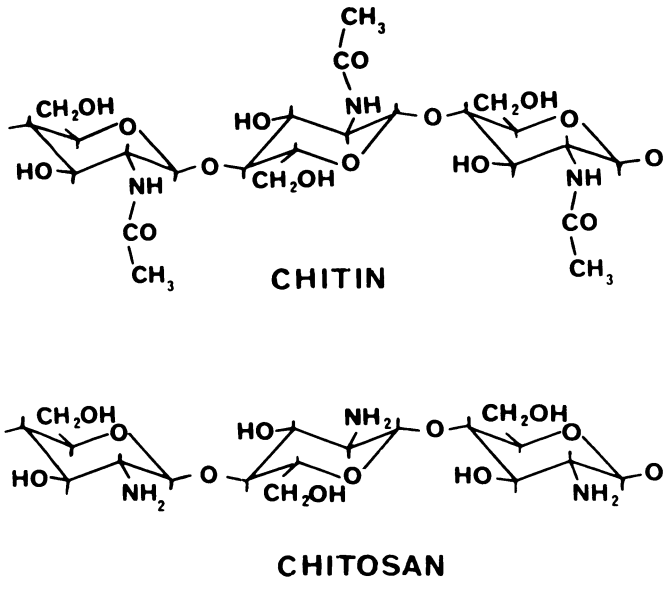

Fig. 1. Structures of chitin and chitosan.

having α-chitin in its tough beak, β-chitin in its rigid pen, and γ-chitin in its flexible stomach lining (Rudall and Kenchington 1973).

With one exception, that 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 and 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).

With this complexity of chemical and physical form in nature, it is not surprising that a wide range of lytic enzymes are produced, each with activities specific for particular forms of chitins, chitosans, related glucosaminoglycans and their oligomers. Typically a chitinolytic microbe will produce several chitinases and *N*-acetylglucosaminidases, distinguished from each other by their substrate specificities and other properties.

II. 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 are reviewed by Davis and 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 (Cabib 1987). 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 distinction between these two activities (see also Davis and 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 and Gooday 1985a). Lysozyme has a low endochitinolytic activity, but can readily be distinguished from chitinases as it 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 (sometimes called chitobiase but beware confusion with glucosaminidase). 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 and Eveleigh 1984).

III. Identification and assay of chitinolytic activities

A ready method for screening for microbial chitinolytic activities is to look for zones of clearing around colonies growing on agar plates containing colloidal or regenerated chitin (e.g. Lindsay and Gooday 1985b; Cody et al. 1990). This, however, only detects production of excreted lytic activities, and not all chitinolytic microbes give such a zone of clearing. Neugebauer et al. (1991), for example, describe the chitinolytic activity of *Streptomyces lividans* when grown in liquid medium that was not readily apparent on solid medium. O'Brien and Colwell (1987) have described a preliminary rapid screen to detect *N*-acetylglucosaminidase as being a good indicator for chitinolysis, but in a survey of *Bacillus* spp., Cody (1989) reported that many strains negative for endochitinase gave a strong positive response for *N*-acetylglucosaminidase. Clearing of chitin or glycolchitin agar overlays can also be used to detect chitinase activity in gels, with sensitivity being enhanced by staining with Congo red or Calcofluor White (e.g. Trudel and Asselin 1989; Cole et al. 1989).

There is a wide range of assays for chitinolytic activities in culture media and cell fractions, differing widely in sensitivity, applicability and cost. They fall into two categories: those using macromolecular chitin or its derivatives in various forms, and those using soluble oligomers or their derivatives. In the former category, examples include measurement of release of reducing sugars or *N*-acetylglucosamine (requiring *N*-acetylglucosaminidase together with chitinase) (Ulhoa and Peberdy 1991; Vasseur et al. 1990); the use of [^{3}H]- or [^{14}C]-chitin (Molano et al. 1977; Cabib 1988; Rast et al. 1991); viscometric measurements of soluble chitin derivatives (Ohtakara 1988; Lindsay and Gooday 1985b); and release of soluble dye-labelled products from dyed chitin derivatives (Wirth and Wolf 1990; Evrall et al. 1990). In the latter category, chromogenic soluble model substrates have provided the basis for useful assays, notably 3,4-dinitrophenyl tetra-*N*-acetyl-β chitotetraose (Aribisala and Gooday 1978; Rast et al. 1991). More versatile, however, are assays following the hydrolysis of glycosides of the fluorophore, 4-methylumbelliferone. By using a range of these, comparative activities of *N*-acetylglucosaminidases, exochitinases and endochitinases can be characterised (Robbins et al. 1988; Watanabe et al. 1990a; Butler et al. 1991; Hood 1991; McCreath and Gooday 1992). The release of the fluorophore can also be used to detect chitinase activity cytochemically in cells (Manson et al. 1992) or in gels after non-denaturing electrophoresis (McNab and Glover 1991).

An important point that should be emphasised is that an enzyme designated as a chitinase by its action in a chitinase assay may not have chitin as its direct natural substrate. Instead, *in vivo* it may act on an as yet unrecognised glucosaminoglycan/mucopolysaccharide/glycoprotein in that tissue. Thus De Jong et al. (1992) described a morphogenetic role for an acidic endochitinase in

the development of carrot somatic embryos, in which neither substrate nor product of the enzyme activity have been identified.

IV. 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 and Matile 1970; Tracey 1955). In the case of *Coprinus*, the basidiospore discharge starts at the outermost edges of the gills which 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. 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 and Wessels 1970). The accumulation of autolytic enzymes in culture filtrates of senescent fungal cultures in well-documented (Reyes et al. 1984, 1989; Isaac and 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 and Gooday 1984a,b,c; Gooday et al. 1986; Pedraza-Reyes and Lopez-Romero 1989; Rast et al. 1991), *Neurospora crassa* (Zarain-Herzberg and Arroyo-Begovich 1983) and *Candida albicans* (Barrett-Bee and Hamilton 1984). Humphreys and Gooday (1984a,b,c) report that as well as soluble chitinase activities, in *Mucor mucedo* there is also membrane-bound chitinase requiring phospholipids for activity and having properties in common with chitin synthase activities. Similar results for related fungi were reported by Manocha and Balasubramanian (1988), but Dickinson et al. (1991) report that in *C. albicans*, the membrane-associated activity was only 0.3% of the total, and was not associated with any particular membrane fraction.

Possible roles for these soluble and membrane-bound chitinases are discussed by Gooday et al. (1986), Gooday (1990b) and Rast et al. (1991) and they include the following.

(a) 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 and 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 and Gow 1991). Their transglycosylase activities may also have a role in covalently linking chains with other wall polysaccharides.

(b) 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 and Gow 1991) but as yet there is no direct evidence. The membrane-bound *Mucor* chitinase studied by Humphreys and Gooday (1984a,b,c) shared with chitin synthase the property of being activatible by trypsin, i.e. being zymogenic, suggesting that the two enzymes could be co-ordinately regulated, as would be required for orderly chitin deposition.

(c) 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. Rast et al. (1991) presented a detailed speculative scheme for the controlled lysis of chitin during branching, and perhaps during apical growth, through the concerted action of chitinase, β-*N*-acetylglucosaminidase and chitin synthase. This scheme is based on their observations of a multiplicity of chitinase activities with a range of properties arising during exponential growth of *Mucor rouxii*. The localised outgrowth of a new tip from the hyphal tube is envisaged as involving successive interrelated stages. Co-operation of chitinase molecules in the densely packed chitin of the wall results in a high incidence of transglycosylation events, leading to a slow onset of wall-loosening. As chitinolysis proceeds, the proportion of transglycoslation events will be decreased and the concentration of oligomers and monomer will increase. These will allosterically activate the chitin synthase (cf. Gooday 1977), allowing insertion of chitin into the stretching wall.

(d) 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). Pedraza-Reyes and Lopez-Romero (1991a,b) presented results of a study of chitinase activities of germinating cells of *M. rouxii*, during spherical growth at four hours, when they found the highest specific activity. This was confirmed by Gooday et al. (1992) who showed that germination was delayed, but not

prevented, by treatment with high concentrations of the inhibitor, allosamidin. In a similar way, hatching of eggs of nematodes is also delayed but not prevented by treatment with allosamidin (K. Arnold et al. 1993).

(e) Cell separation in yeasts. 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 treatment with the chitinase inhibitors, allosamidin and demethylallosamidin, inhibits cell separation during budding (Sakuda et al. 1990). Budding yeast cells of *Candida albicans* show the same response with treatment with allosamidin leading to clumps of cells (Gooday et al. 1992). The chitinase of *S. cerevisiae* is a mannoprotein (Correa et al. 1982; Orlean et al. 1991). Its structural gene *CTS1* has been cloned and sequenced by Kuranda and Robbins (1988, 1991). In cultures growing in rich medium, most of this chitinase was secreted to the medium in parallel with growth but a significant amount was also associated with the cell wall through binding of the carboxyl-terminal domain to chitin. Kuranda and Robbins suggested that it is this wall-bound enzyme fraction that is active in cell separation. SDS-polyacrylamide gel electrophoresis showed the enzyme to be a single polypeptide of about 130 kDa, corresponding to the predicted molecular mass of protein of 60 kDa with about 90 short *O*-linked mannose oligosaccharides on its serine and threonine residues. Its size varied between different strains. Different strains provided two chitinase genomic clones, probably allelic variations of a single chitinase locus. Strains were constructed in which the *CTS1* gene was disrupted. Growth was unaffected but the cells could not separate after budding and formed large aggregates attached by their septal regions. Thus chitinase is required for cell separation. Kuranda and Robbins (1991) also studied the secretion of the chitinase by using temperature-sensitive secretory mutants and showed that these accumulated a form of the enzyme that was clearly different to the one that was normally secreted. During studies of chitin synthesis in *S. cerevisiae*, Cabib et al. (1989) showed that deletion of the chitin synthase 1 gene gave yeast cells that grew normally except in acidic conditions when some of the mother cells lysed with leakage of cytoplasm from their bud scars. This damage, which was prevented by allosamidin, led Cabib et al. (1990) to suggest that it was the result of over-action of chitinase during bud separation. Cabib et al. (1992) showed that this is the case as this cell lysis was prevented by disruption of the chitinase gene. The chitin synthase 1 can thus be seen as a repair enzyme, replenishing chitin during cytokinesis, following the action of chitinase. During investigation of the cell-cycle regulated transcription of *ACE2*, a transcriptional activating gene encoding a zinc-finger DNA-binding protein in *S. cerevisiae*, Dohrmann et al. (1992) observed that an *ace2* mutant strain had

a clumpy phenotype, similar to that of strains with a disrupted *CTS1* gene. They showed that *CTS1* mRNA was absent from *ace2* strains and concluded that *ACE2I* is a major transcriptional activator of *CTS1* in late G_1 phase of cell cycle. Further, from similarity with activation of *HO* mating-type switching gene by the homologous regulator *SW15* they suggested that *ACE2* expression may only activate *CTS1* in the mother cell, which will bear the chitinous bud scar, and not in the daughter cell.

Villagomez-Castro et al. (1992) described a chitinase activity expressed during formation of the chitinous cyst wall by the protozoan, *Entamoeba invadens*. They suggested that it is involved in the orderly deposition of the chitin. Treatment with allosamidin slowed, but did not prevent, the process of encystment.

V. Nutritional chitinolysis

A. 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 and 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 and 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 1990a). Pisano et al. (1992) described the isolation of chitinolytic actinomycetes from marine sediments and comment on the high correlation between chitinolysis and antibiotic production in their isolates. Studies such as that by Helmke and 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 and Reichardt et al. (1983) isolated 103 strains of chitinolytic bacteria from the estuarine upper Chesapeake Bay, Maryland. 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, Washington. Genera identified, in decreasing order of abundance, were *Vibrio*, *Pseudomonas*, *Aeromonas*, *Cytophaga*, *Streptomyces*, *Photobacterium*, *Bacillus* and *Chromobacterium*.

Pel and 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, The Netherlands. 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 that the accumulation of *N*-acetylglucosamine represents non-utilizable monomers appearing during random hydrolysis of chitin oligomers. Chitin degradation was greatly enhanced by co-culture 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 O_2 in the upper layers of sediments, as accumulating mono- and disaccharides could provide substrates for facultative aerobic bacteria which would consume O_2 to render the microenvironment anaerobic again. While investigating the chitinolytic microflora of a solar saltern, Liaw and Mah (1992) isolated a novel, halophilic, anaerobic chitinolytic bacterium, *Haloanaerobacter chitinovorans*. This isolate grew at NaCl concentrations of 0.5 to 5 M and at temperatures between 23 and 50°C. The remarkable ecosystems of the deep-sea thermal vents should be rich areas for the isolation of novel chitinolytic microbes, as their dominant fauna produces chitinous structures such as clam shells, crab carapaces and pogonophoran tubes.

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. Consequently, 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). In a recent survey, Cody (1989) reported that 17 of 52 strains of *Bacillus* were chitinolytic. Recent reports of chitinases from *Streptomyces* species include those by Ueno et al. (1990), Okazaki and Tagawa (1991) and Neugebauer et al. (1991).

When grown in liquid culture, most chitinolytic bacteria secrete chitinases into the medium. *Cytophaga johnsonae*, a ubiquitous soil organism, characteristically binds to chitin as it degrades it. Wolkin and Pate (1985) described a class on non-motile mutants with an interesting pleiotropy: they

were all unable to digest and utilize chitin, as well as being resistant to phages that infect the parental strain, and had relatively non-adherent and non-hydrophobic surfaces compared with wild-type strains. The authors concluded 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 and Gottschal (1986a) illustrated 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 and Colwell (1978) described strong adsorption to chitin of *Vibrio parahaemolyticus* isolated from the estuarine Chesapeake Bay. They suggested that this has an ecological as well as digestive significance to the bacteria as the adsorption was decreased 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 1991a,b) and Yu et al. (1991) presented a detailed study of the utilization of chitin by *Vibrio furnissii*. Adhesion to model substrates was assessed by nixing radio-labelled cells with gel beads that had been covalently coated with carbohydrate residues. Cells of *V. furnissii* adhered to glycosides of *N*-acetylglucosamine and, to a lesser extent, of glucose and mannose. A calcium-requiring lectin was responsible for this binding to the three sugars. Adherent cells continued to divide, and to stay attached, but the population gradually shifted to a large fraction of free swimming cells. Metabolic energy was required for binding but either transient or no adhesion occurred in incomplete growth media. The authors suggested that this active adhesion/de-adhesion process allows the cells to continuously monitor the nutrient status of their environment, enabling them to colonise a suitable chitinous substrate. They suggested that the next step is chemotaxis to chitin hydrolysis products. In a capillary assay, swimming cells of *V. furnissii* showed low level constitutive chemotaxis to *N*-acetylglucosamine (GlcNAc), but induction by prior growth in the presence of GlcNAc greatly increased the effect. No taxis was observed to GlcNAc oligomers by cells grown on lactate, but strong inducible taxis occurred. Bassler et al. (1991a) described the induction of two or more receptors recognizing $(GlcNAc)_n$, n = 2 to 4. Bassler et al. (1991b) described the utilization of chitin oligomers by the cells. They characterized two cell-associated enzymes hydrolysing oligomers that entered the periplasmic space: a membrane-bound chitodextrinase and an *N*-acetylglucosaminidase. Both enzymes were inducible by chitin oligomers, especially *N*,*N*′-diacetylchitobiose $(GlcNAc)_2$.

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

B. 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 and Chet 1989). Induction and characterization of an extracellular chitinase from *Trichoderma harzianum* have been described by Ulhoa and Peberdy (1991, 1992). Chitinase production was induced by chitin but repressed by glucose and *N*-acetylglucosamine. Vasseur et al. (1990) have isolated chitinase over-producing mutants of *Aphanocladium album*, by screening for increased clearing zones around colonies on colloidal chitin agar following mutagenesis. One strain showed a 26-fold increase in maximal extracellular chitinase activity in liquid medium with crystalline chitin as sole carbon source, compared to the wild-type strain. McCormack et al. (1991) described the production of a thermostable chitinolytic activity from *Talaromyces emersonii* which was optimally active at 65°C. Baiting of freshwater sites with chitin can yield a range of chitinolytic fungi, interesting members of which are the chytrids, such as *Chytriomyces* species (Reisert and 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 and 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*.

C. Slime moulds, protozoa and algae

The Myxomycetes, "true slime moulds", are a rich source of lytic enzymes, and *Physarum polycephalum* produces a complex of extracellular chitinases (Pope and 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 chitinivorous ectosymbiont of shrimps (Bradbury et al. 1987) and *Grossglockneria*, which feeds by digesting a tiny hole through a fungal hypha and sucking out the cytoplasm (Petz et al. 1986). The colourless heterotrophic diatom, *Nitzschia alba*, is also reported to digest chitin (A.E. Linkens, quoted by Hellebust and Lewin 1977).

VI. Chitinolysis in pathogenesis and symbiosis

Pathogens of chitinous organisms characteristically produce chitinases. These can have two roles; they can aid the penetration of the host; and they can provide nutrients both 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 and Unestam 1975); the fungus *Paecilomyces lilacius*, a pathogen of nematode eggs (Dackman et al. 1989); the entomopathogenic fungi, *Beauveria bassiana*, *Metarhizium anisopliae*, *Nomuraea rileyi* and *Verticillium lecanii* (Smith and Grula 1983; Coudron et al. 1984; St Leger et al. 1986); mycophilic fungi, *Cladobotryum* species and *Aphanocladium album* (G.W. Gooday unpublished; Zhloba et al. 1980; Srivastava et al. 1985; Kunz et al. 1992); the bacteria *Serratia*, insect pathogens (Lysenko 1976; Flyg and Boman 1988); and a *Photobacterium* species causing exoskeleton lesions of the tanner crab (Baross et al. 1978).

As well as being a component of insect exoskeletons, chitin also has a major structural role in the ephemeral protective lining of insect guts, the peritrophic membrane. Treatment of isolated peritrophic membranes with chitinase leads to their perforation (Brandt et al. 1978; Huber et al. 1991). Addition of exogenous chitinase aids the pathogenesis of insects by *Bacillus thuringiensis* (Smirnoff 1974; Morris 1976), and by a gypsy moth nuclear polyhedrosis baculovirus (Shapiro et al. 1987). There are now several examples where the pathogen's endogenous chitinolytic activities appear to aid penetration of the peritrophic membrane or other chitinous barriers, perhaps aiding eventual release and spread of the pathogen as well as uptake. Gunner et al. (1985) reported a positive correlation between chitinase activity among chitinase-producing strains of *B. thuringiensis* and host mortality. That the chitin of the peritrophic membrane is a site of attack by other insect pathogenic bacteria is suggested by experiments with *Drosophila melanogaster* (Flyg and 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.

Daoust and 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 and 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 and Welburn (1988) suggested that bacterial chitinolysis releases amino sugars that inhibit the lectin-trypanosome binding and thus results in survival of the trypanosomes. An alternative explanation is that the chitinolytic bacteria weaken the insect's peritrophic membrane, aiding the penetration of the trypanosomes. Schlein et al. (1991) reported that cultures of the trypanosomatids, *Leishmania* species, produced their own chitinase activities to aid penetration of the insect gut. This needs re-investigating, however, as their culture medium included bovine serum, a rich source of chitinase. They did, however, find activity associated with the *Leishmania* cells. Arnold et al. (1992) detected no chitinase activity in cells or medium of *Trypanosoma brucei* var. *brucei* when cultured in medium depleted of chitinase by affinity adsorption onto chitin. The invasive form of the malarial parasite, *Plasmodium gallinaceum*, is the ookinete, which penetrates the peritrophic membrane of the host mosquito. Huber et al. (1991) reported the formation of chitinase during the maturation process of *Plasmodium* zygotes to ookinetes and implicated its appearance with the invasion of parasites. The filarial nematode, *Brugia malayi*, also has mosquitoes as its vectors between mammalian hosts. Microfilariae, produced during infection of the mammal, are covered by a chitin-rich coat, formed by stretching of the original eggshell. In model infections in gerbils, Fuhrman et al. (1992) have shown that a major antigen of the microfilariae is a nematode chitinase. This is recognized by the monoclonal antibody, MF1, that they had previously shown to be responsible for clearance of the peripheral microfilariae in the gerbils. Sequencing the cDNA of the MF1 antigen showed homologies with known chitinase genes (cf. Table 2). The microfilarial chitinase may play a role in the regulation of stretching of the chitinous sheath, or it may aid the penetration of the mosquito gut peritrophic membrane.

A further example of an insect pathogen producing chitinase is the baculovirus *Autographa californica* nuclear polyhedrosis virus (NPV). This virus is used for biological control of insect pests and by molecular biologists as a system for the expression of heterologous proteins in infected cell cultures. The insect cell cultures produce their own chitinases, at a low activity, but on infection with *A. californica* NPV, an enormous increase in chitinase activity is observed (Hawtin et al. 1993). This is encoded by the virus genome. The amino acid sequence shows very high homology to that for the chitinase A from

Serratia marcescens (Table 2). This suggests that there has been lateral gene transfer relatively recently, especially as *S. marcescens* is itself an insect gut pathogen. The more likely direction is bacterium to virus, as other baculoviruses do not have an homologous gene. A strain of *A. californica* NPV from which the chitinase gene had been deleted was less pathogenic to larvae of the cabbage looper, *Trichoplusia ni*, but the insects still died. A dramatic difference, however, was that after death the insects infected by the chitinolytic virus were totally liquefied, whereas those infected by the mutant strain were dry cadavers. Thus, as with the bacterial, protozoal and microfilarial chitinases, this baculovirus chitinase may aid penetration of the peritrophic membrane of the insect host but its major significance is in aiding release of viruses from the dead host.

Another example of a chitinase activity involved in microbial interactions is that of the yeast killer toxin produced by the yeast *Kluyveromyces lactis* (Butler et al. 1991). This is a trimeric protein, of α, β and γ subunits. Intracellular γ subunit is responsible for killing a susceptible cell of *Saccharomyces cerevisiae*, the β subunit has no known role, and the α subunit has exochitinase activity that is essential for the action of toxin. This is shown by the inhibition of toxin activity by the specific inhibitor of chitinase, allosamidin. The significance of this chitinase activity remains unclear, but may involve the binding of toxin to the susceptible yeast cell surface to facilitate the uptake of the γ subunit. The amino acid sequence of the α subunit has striking homologies to other chitinases in two regions, one corresponding to the catalytic domain of microbial and some acidic plant chitinases (Table 2) and another corresponding to the cysteine-rich chitin-binding domain of some basic plant chitinases and lectins (Butler et al. 1991).

Chitinase production by the entomopathogenic fungi is inducible by chitin oligomers, *N*-acetylglucosamine and glucosamine (Smith and Grula 1983; St Leger et al. 1986, 1991). St Leger et al. (1986) also reported that chitosanase is co-induced with chitinase in *Metarhizium anisopliae*. In insect pathogenesis, any chitinase will be in acting in synergism with proteases and Bidochka and Khachatourians (1988) have suggested that both activities are coordinately regulated. They showed that low levels of *N*-acetylglucosamine will induce a serine protease in *B. bassiana* and suggested that an initial constitutive chitinase attack on the insect cuticle would yield *N*-acetylglucosamine, leading to the coordinate induction of chitinases and proteases. St Leger et al. (1987) questioned the importance of chitinase as in their experiments *M. anisopliae* did not appear to produce chitinase during penetration of cuticle of *Manduca sexta*. Bidochka and Khachatourians (1992) have investigated the growth of *B. bassiana* on cuticular components from the migratory grasshopper. After removal of lipids and protein, the residual chitin (about 30% w/w) was a relatively poor source of nutrients for germination and fungal growth, but their electron micrographs clearly showed penetration of the chitinous material by germ tubes. More positive evidence for the importance of fungal chitinase activities in insect pathogenesis, particularly during spore germination, was

provided by El-Sayed et al. (1989) in a comparative study of exo- and endo-chitinase activities of three isolates of *Nomuraea rileyi*. The two virulent isolates had much higher chitinase activities during early growth than an avirulent isolate.

Chitin in fungi and invertebrates comprises 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 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 in the same way that the rumen symbionts are regarded with respect to cellulose degradation (Lindsay et al. 1984; Lindsay and 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 and Taga 1965; Herwig et al. 1984); Patton and Chandler (1975) described 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 and Insam (1990), working with the soil springtail, *Folsomia candida*, concluded 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 animals' faeces, suggesting that some pre-digestion of the chitin was aiding its utilization by the animal.

VII. 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 of *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 and 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 and Wolfe 1974). Mitsutomi et al. (1990) and Ohtakara et al. (1990) reported the action patterns of chitinases from *Aeromonas hydrophila* and *Streptomyces griseus*, respectively, on partially *N*-acetylated chitosan. In both cases, but especially for *S. griseus*, there was specificity for cleavage of the *N*-acetyl-β-D-glucosaminidic linkages. In contrast, a purified chitosanase from *Nocardia orientalis* attacked 33% acetylated chitosan by hydrolysing between glucosamine and either glucosamine or *N*-acetylglucosamine, but not between *N*-acetylglucosamine and glucosamine (Sakai et al. 1991a). Sakai et al. (1991a) proposed a scheme for the total hydrolysis of partially acetylated chitosans by *N. orientalis* by the cooperative action of chitosanase, β-*N*-acetylhexosaminidase, and a novel exo-β-glucosaminidase characterized by Nanjo et al. (1990). Seino et al. (1991) described the cleavage pattern of a purified *Bacillis* chitosanase on a series of glucosamine oligomers, as measured by HPLC analysis of products, and concluded that the enzyme mainly hydrolyses chitosan in a random fashion.

Pelletier and Sygusch (1990) have purified three chitosanase activities from *Bacillus megaterium* P1. The major activity, chitosanase A, had a high specificity for chitosan, with just a trace of activity against carboxymethyl-cellulose, while chitosanases B and C had much lower activity against chitosan, and also activities against chitin, carboxymethyl-cellulose and cellulase. None had lysozyme activity. These broad specificities shown by enzymes B and C are remarkable and deserve further investigation. Somashekar and Joseph (1992) described a chitosanase activity secreted constitutively into the medium by the yeast, *Rhodotorula gracilis*. This activity was measured by decrease in viscosity of a chitosan solution and yielded a detectable chitosan oligomers. In view of this, and the observation that growth of the yeast was inhibited by even low amounts of chitosan, Somashekar and Joseph (1992) suggested that this enzyme is involved in morphogenesis of the cell wall.

VIII. Biotechnology of chitinases and chitosanases

With chitin and chitosan being an enormous renewable resource, much of which from the shellfish and fungal fermentation industries currently goes to waste, and with their essential roles in fungi and invertebrates, it is not surprising that there is a great deal of current interest in these polysaccharides and in their degradative enzymes (Muzzarelli and Pariser 1978; Hirano and Tokura 1982; Zikakis 1984; Muzzarelli et al. 1986; Deshpande 1986;

Skjak-Braek et al. 1989; Roberts 1992). The use of chitinolytic microbes in the production of single cell protein or ethanol from chitinous wastes has been investigated (Tom and Carroad 1981; Vyas and Deshpande 1991; Cody et al. 1990) but much further work is required to evaluate these ideas.

A. Cloning of chitinase genes

Genes coding for various chitinases from bacteria, fungi and plants have been cloned. Of many bacterial isolates, Monreal and Reese (1969) found *Serratia marcescens* and *Serratia liquefaciens* (*Enterobacter liquefaciens*) to be the most active producers of chitinases. Roberts and Cabib (1982) describe purification of the chitinases and mutant strains with increased production of chitinase have been produced (Kole and Altosaar 1985; Reid and Ogrydziak 1981). Two chitinase 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 and Jones 1988). The rationale to this work was 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 and 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 suggested that the bacterial signal sequence is functioning in the plant cells and that the chitinase is *N*-glycosylated through the secretory pathway. Lund and Dunsmuir (1992) have investigated the relative effects of plant versus bacterial signal sequences on secretion of *S. marcescens* chitinase A by transgenic tobacco cultures. Only a fraction of the chitinase with the bacterial sequence was secreted and glycosylated, while replacement by a plant signal sequence resulted in efficient glycosylation and secretion. The glycosylation was not, however, essential for secretion as the non-glycosylated protein was also secreted. 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 of pathogens and pests by enhancing chitinase activities of phylloplane and rhizoplane bacteria. Horwitz et al. (1984) described 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 and 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 showed 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: it has 290 amino acid residues, a molecular weight of 30,400 and two disulphide bridges (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, 1992). The *Streptomyces* chitinase was secreted into the periplasmic space of *E. coli* with its signal sequence having been removed by the *E. coli* signal peptidase. A gene for chitinase from *Vibrio vulnificus* has also been cloned into *E. coli* and was expressed but the protein was not secreted into the medium (Wortman et al. 1986). Similarly, Roffey and Pemberton (1990) expressed a chitinase gene from *Aeromonas hydrophila* in *E. coli* and found the resultant enzyme to be accumulated in the periplasmic space. In contrast, Chen et al. (1991) reported the excretion of an *A. hydrophila* chitinase cloned in *E. coli*. Watanabe et al. (1990b, 1992) described the cloning of chitinase genes from *Bacillus circulans*, the properties of which are described later. A gene for chitinase from *Saccharomyces cerevisiae* has been cloned by transforming the yeast with vector plasmids containing a genomic library and then screening for over-producing transformants (Kuranda and Robbins 1988, 1991) Again this is described later. Fink et al. (1991) reported the cloning of a chitosanase-encoding gene from the actinomycete, *Kitasatosporia*, into *Streptomyces lividans*.

Expression of microbial chitinase genes is typically induced by chitin but repressed by glucose. Delic et al. (1992) described the characterization of promoters for two chitinase genes from *Streptomyces plicatus*. Each one had a pair of perfect 12 base-pair, direct repeat sequences which overlapped the putative RNA polymerase binding site. Similar promoters were also found for chitinase genes for *Streptomyces lividans* (Miyashita et al. 1991).

Plants produce chitinases as major component of their "pathogenesis-related proteins" induced following attack by potential pathogens or treatment with ethylene (Mauch and Staehelin 1989). Some of these plant chitinases have antifungal activity (Mauch et al. 1988; Broekaert et al. 1988) greater than that of some bacterial chitinases (Roberts and Selitrennikoff 1988). Leah et al. (1991) have used a microtitre-well assay to assess the antifungal activity of a purified chitinase from barley seeds. Treatment of both *Trichoderma reesei* and *Fusarium sporotrichiodes* with 375 nM protein resulted in about 50% inhibition of growth but there were strong synergistic inhibitions with either or both of a ribosome-inactivating protein and a glucanase from the barley seeds.

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 other plants (Linthorst et al. 1990) with the aim of increasing the plants' resistance to fungal pathogens.

B. 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) described 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 anti-tumour activity (Suzuki et al. 1986). The transglycosylase activity is favoured by a high substrate concentration and a lowered water activity, e.g. in increasing concentrations of ammonium sulphate. Takayanagi et al. (1991) described transglycosylase activities of thermostable chitinases produced by a thermophilic strain of *Bacillus licheniformis*. When incubated with a 5% (w/v) solution of $(GlcNAc)_4$ at 50°C, the chitinases produced yields of about 10% $(GlcNAc)_6$ after a few minutes. The production of the disaccharide, *N*,*N*′-diacetylchitobiose, from chitin was described by Takiguchi and Shimahara (1988, 1989). They isolated two bacteria, *Vibrio anguillarum* strain E-383a and *Bacillus licheniformis* strain X-Fu, whose growth in chitin-containing medium resulted in the accumulation of 40 and 46%, respectively, conversion of chitin to diacetylchitobiose.

Sakai et al. (1991b) report the use of a column reactor of immobilised chitinase and *N*-acetylhexosaminidase from *Nocardia orientalis* for the continuous production of *N*-acetylglucosamine from soluble chitin oligomers. Pelletier and Sygusch (1990) and Pelletier et al. (1990) described the characterization of chitosanases from *Bacillus megaterium* and their use in the assay of the degree of deacetylation in samples of chitosan. Nanjo et al. (1991) also described the analysis of chitosan using the chitosanase, exo-glucosaminidase and *N*-acetylhexosaminidase activities from *N. orientalis*. A direct medical use has been suggested for chitinases in the therapy of fungal diseases in potentiating the activity of antifungal drugs (Pope and Davies 1979; Orunsi and Trinci 1985). Immunological problems however, probably debar this until anti-iodiotypic antibodies for appropriate chitinases are developed.

Chitinases have extensive uses 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* species (Beyer and Diekmann 1985; Tagawa and Okazaki 1991). Chitosanases are required to

make protoplasts from species of the Mucorales (Reyes et al. 1985).

C. 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 and Chet 1989). Dackman et al. (1989) reported that chitinase activity is required for soil fungi to infect eggs of cyst nematodes. Sneh (1981) discussed the use of rhizophere chitinolytic bacteria for biological control. Inbar and Chet (1991) suggested that rhizosphere colonization by *Aeromonas caviae* gives biocontrol against soil-borne fungal pathogens by increasing the chitinolytic activity of the rhizosphere. They demonstrated chitinolysis around the roots by staining for cleaving in chitin agar with Congo red. 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).

IX. Specific inhibitors of chitinases

Allosamidin is an antibiotic 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 development 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 pseudo-trisaccharide, being a disaccharide of *N*-acetylallosamine (until now unknown in nature) linked to a novel aminocyclitol derivative, allosamizoline (Sakuda et al. 1987b; Fig. 2). 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). Allosamidin inhibits chitinases from the fungi *Candida albicans* (Dickinson et al. 1989; Milewski et al. 1992), *Neurospora crassa* (McNab and Glover 1991) and *Mucor rouxii* (Pedraza-Reyes and Lopez-Romero 1991a) and the nematode *Onchocerca*

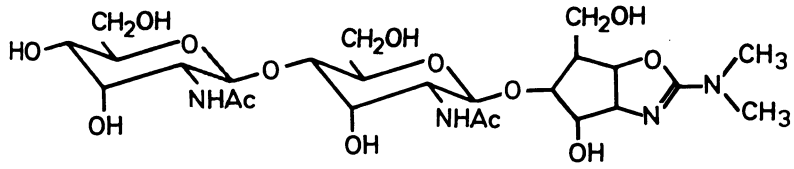

Fig. 2. Structure of allosamidin.

gibsonii (Gooday et al. 1988). *In vivo*, however, reports of its activities are very limited.

As discussed earlier, treatment with allosamidin and demethylallosamidin inhibits cell separation in budding yeasts, such as *S. cerevisiae* and *C. albicans*, and delays germination of spores of *M. rouxii*, hatching of nematode eggs and encystment of *E. invadens*. Nishimoto et al. (1991) described further minor co-metabolites of allosamidin and reported the comparative activities of six allosamidins against chitinase preparations from three fungi; *Candida albicans*, *S. cerevisiae* and *Trichoderma* sp. (Table 1). Distinctly different patterns of inhibition were apparent, with the *S. cerevisiae* activity showing a hundred-fold variation in susceptibility to the different metabolites, while the *C. albicans* and *Trichoderma* activities showed a ten-fold and a two-fold variation, respectively. Mild alkaline hydrolysis of allosamidin and glucoallosamidin A yielded pseudo-disaccharides that retained their inhibition against the *C. albicans* activity but were no longer inhibitory against activities from *S. cerevisiae* and *Trichoderma* sp. Milewski et al. (1992) presented a detailed account of the competitive inhibition of chitinase from *C. albicans* showing that it is strongly pH-dependent, with IC_{50} values of 280 nM at pH 5.0 and 21 nM at pH 7.5. At higher, micromolar concentrations allosamidin inactivates this chitinase in a time- and concentration-dependent manner. Kinetic studies of this inactivation provide evidence for the formation of a reversible complex between allosamidin and chitinase, characterized by $K_{inact} = 5\ \mu M$, followed by irreversible modification of the enzyme consistent with an active site-directed, covalent enzyme modification.

Rast et al. (1991) described the inhibition of a range of chitinase activities of *M. rouxii* by a synthetic analogue of *N,N'*-diacetylchitobiose, *N,N'*-diacetychitobiono-1,5-lactone oxime. This was a competitive inhibitor with a K_i value of around 175 μM, compared to slight inhibition by *N,N'*-diacetylchitobiose (IC_{50} value of about 20 mM).

X. Sequence homologies of chitinases

There is a growing number of amino acid sequences of chitinases. Homologies between them have been classified by Henrissat (1990, 1991, and personal

Table 1. Inhibitory activity of allosamidins and derivatives against chitinase preparations from three fungi.

	IC_{50} (μg/ml)		
	Candida albicans	*Sacharomyces cerevisiae*	*Trichoderma* sp.
Allosamidin	6.2	33.8	0.8
Demethyl allosamidin	0.7	0.3	0.8
Methylallosamidin	8.8	37.2	1.2
Methyl-*N*-demethyl allosamidin	0.6	0.4	1.3
Glucoallosamidin A	3.4	31.3	0.8
Glucoallosamidin B	0.8	0.5	1.6
Hydrolysed allosomidin	1.3	>200	>50
Hydrolysed glucoallosamidin A	5.7	>200	>50

IC_{50} is the concentration causing 50% inhibition.
Compiled from Nishimoto et al. (1991).

communication). The microbial chitinases and the plant acidic chitinases form one group (family 18 in a classification of glycosyl hydrolases) distinct from the plant basic chitinases (family 19). All glycosyl hydrolases were thought to act by an acid catalysis mechanism in which two amino acid residues participate in a displacement reaction. Henrissat's analysis identifies two invariant residues, an aspartate and a glutamate, separated by three amino acids in all chitinases of family 18 examined to date (Table 2). In agreement with this, chemical modification studies of the active centre of the chitinase from *Candida albicans* show specific inactivation by the carboxyl-specific reagent, 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide (EDC), in a single step process (Milewski et al. 1992). In contrast, Verburg et al. (1992) surprisingly reported inactivation of the basic chitinase from maize, *Zea mays*, by reaction of EDC with a tyrosine residue. This residue, however, is conserved in other basic plant chitinases. Table 2 shows homologies of just a short stretch of a range of chitinases and of endo-β-*N*-acetylglucosaminidase H from *Streptomyces plicatus* in the region most likely to contain the active site. The significance of the remarkable sequence homology between the chitinases of the virus *A. californica* NPV and *S. marcescens* has been discussed earlier, as has the chitinase activity of the α-subunit of the toxin from *Kluyveromyces lactis*. Watanabe et al. (1992), Fuhrman et al. (1992) and Kuranda and Robbins (1991) also have discussed homologies at other regions of the chitinase sequences.

Kuranda and Robbins (1991) presented a model of endochitinase encoded by *CTS1* of *S. cerevisiae*, with four functional regions:

Signal – Hydrolytic – Ser,Thr-rich – Chitin-binding

The signal sequence (amino acids 1 to 20) is recognised and cleaved by the usual secretion pathway. The hydrolytic region (amino acids 21 to 237) contains the conserved region shown in Table 2, with the invariant aspartate and glutamate residues, and another conserved region (amino acids 102 to 116). The serine-

Table 2. Alignment of the putative active site region in microbial and plant chitinases.

											*				*					
Gram positive bacteria																				
Bacillus circulans A	(190)	L	R	K	Y	N	F	D	G	V	D	L	D	W	E	Y	P	V	S	(207)
Bacillus circulans D	(290)	I	S	T	Y	G	F	N	G	L	D	I	D	L	E	G	S	S	L	(307)
Flavobacterium sp. (a)	(115)	V	S	K	Y	G	L	D	G	V	D	L	D	D	E	Y	S	D	Y	(132)
Streptomyces plicatus (a)	(161)	V	A	K	Y	G	L	D	G	V	D	F	D	D	E	Y	A	E	Y	(178)
Streptomyces plicatus	(370)	R	W	A	D	V	F	D	G	I	D	L	D	W	E	Y	P	N	A	(387)
Streptomyces erythraeus	(103)	I	D	A	Y	G	L	K	A	I	D	V	D	I	E	A	T	E	F	(120)
Gram negative bacteria																				
Serratia marcescens B	(131)	M	K	D	Y	G	F	D	G	B	D	I	D	W	E	Y	P	Q	A	(148)
Serratia marcescens A	(302)	Q	T	W	K	F	F	D	G	V	D	I	D	W	E	F	P	G	G	(319)
Viruses																				
Autographa californica NPV	(292)	Q	V	W	K	F	F	D	G	V	D	I	D	W	E	F	P	G	G	(309)
Fungi																				
Kluyveromyces lactis (b)	(482)	M	N	K	Y	N	L	D	G	I	D	L	D	W	E	Y	P	G	A	(499)
Saccharomyces cervisiae	(144)	F	D	S	A	V	V	D	G	F	D	F	D	I	E	N	N	N	E	(161)
Plants																				
Cucumis sativis	(139)	L	G	A	A	V	L	D	G	V	D	F	D	I	E	S	G	S	G	(156)
Hevea brasiliensis	(114)	L	G	D	A	V	L	D	G	I	D	F	D	I	E	H	G	S	T	(131)
Arabidopsis thaliana A	(143)	L	G	D	A	V	L	D	G	I	D	F	N	I	E	L	G	S	P	(160)
Nematode																				
Brugia malayi	(135)	L	R	K	N	N	F	D	G	F	D	L	D	W	E	Y	P	V	G	(162)

Asterisks indicate the two invariant aspartate and glutamate residues. Left hand number in parentheses represents position of amino acid from amino-terminus of protein.

(a) *N*-Acetylglucosaminidases; (b) toxin-α-chain.

From Henrissat (1990), with additions: B. Henrissat, personal communication (*B.circulans, K.lactis, H.brasiliensis, A.thaliana, Flavobacterium* sp.); *S.cerevisiae* (Karanda and Robbins, 1991); *A.californica* NPV (Hawtin et al. 1993) *B.malayi* (Fuhrman et al. 1992), *S.plicatus* (Delic et al. 1992).

One letter symbols for amino acids are: A *Ala*, B *Asx*, D *Asp*, E *Glu*, F *Phe*, G *Gly*, H *His*, I *Ile*, K *Lys*, L *Leu*, M *Met*, N *Asn*, P *Pro*, Q *Gln*, R *Arg*, S *Ser*, T *Thr*, V *Val*, W *Trp*, Y *Tyr*.

threonine-rich domain (amino acids 328 to 480) is glycosylated with sugar chains containing from 2 to 5 mannose residues. It may act as a "hinge" region between the catalytic and chitin-binding domains. The high affinity chitin-binding domain (amino acids 481 to 562) has conservation with a cellulose-binding sequence of *Trichoderma reesei* cellulase, with an exact match of a block of 7 amino acids flanked by 2 cysteines. The chitin-binding domain, however, does not display significant affinity for cellulose. Its chitin-binding properties were directly demonstrated in four ways. 1) A carboxyl-terminal deletion product of *CTS1* did not bind to chitin, but retained its catalytic properties. 2) Controlled hydrolysis of wild-type enzyme bound to chitin resulted in an undigested chitin-bound peptide with the sequence starting at amino acid 480. 3) Selective deletion of *CTS1* to remove amino acids 21 to 481 gave direct fusion of the signal sequence to the chitin-binding domain and resulted in secretion of an 18 kDa peptide with high affinity binding to chitin. 4) Expression of a fusion protein between yeast invertase and the chitin-binding domain led to secretion of an invertase that bound efficiently to chitin.

There are strong homologies in the catalytic sites of bacterial chitinases, such as those from *Streptomyces plicatus*, (Robbins et al. 1992), *Streptomyces erythrasus* (Kamei et al. 1989), *Serratia marcescens* (Jones et al. 1986) and *Bacillus circulans* (Watanabe et al. 1990a,b, 1992) (Table 2). *B. circulans* produces at least 6 distinct chitinases. Chitinases A1 has the structure: signal sequence – hydrolytic domain – chitin-binding domain – short carboxyl terminus. The hydrolytic domain, i.e. the *N*-terminal two-thirds of the molecule, has 33% amino acid match to chitinase A from *S. marcescens*. The chitin-binding domain has a tandem repeat of 95-amino acid sequences that are 70% homologous to each other but also have homology to the "type III homology units" of fibronectin, a mammalian cell adhesion molecule (Watanabe et al. 1990b). *S. plicatus* chitinase 63 has a single sequence near the C terminus which is 40% identical to the "type III homology units" of *B. circulans* chitinase A1. The *N*-terminal one-third of *B. circulans* chitinase D shows remarkable similarity to the *C*-terminal one-third of chitinase A and it is immediately upstream of the *ChiA* gene. Watanabe et al. (1992) suggested that this is a result of a complex gene duplication. Thus, the structure of chitinase D contains an *N*-terminal 47 amino acid segment with 62% amino acid match with the *C*-terminus of chitinase A1; a 95 amino acid segment with 63 and 61% matches, respectively, with the "type III homology units" of chitinase 1, and a 73 amino acid segment with the active site with considerable homology to other chitinases (cf. Table 2).

XI. 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.

Note added in proof

Further microbial chitinases and their genes that have been characterised are: from the marine bacterium *Alteromonas* sp. Strain 0–7 (Tsujibo H, Orikoshi H, Tanno H, Fujimoto K, Miyamoto K, Imada C, Okami Y and Inamori Y (1993) J. Bacteriol. 175: 176–181); from *Streptomyces lividans* (Fujii T and Miyashita K (1993) J. Gen. Microbiol. 139: 677–686); from the Zygomycete *Rhizopus oligosporus* (Yanai K, Takaya N, Kojima N, Horiuchi H, Ohta A and Takagi M (1992) J. Bacteriol. 174: 7398–7406); and from the Deuteromycete *Aphanocladium album* (Blaiseau P and Lafay J (1992) Gene 120: 243–248).

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