Reviews

What's new in chitinase research?

J. Flach[a], P.-E. Pilet[a] and P. Jollès[b,*]

[a] *Institut de Biologie et de Physiologie Végétale de l'Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne (Switzerland), and* [b] *Laboratoire des Protéines, Unité associée CNRS 1188, Université de Paris V, 45 rue des Saints-Pères, F-75270 Paris Cedex 06 (France)*

Abstract. This review article deals with recent developments in molecular and physiological aspects of chitinases from plants, fungi, bacteria, insects and fishes.
Key words. Chitinase; plant chitinases; fungal chitinases; bacterial chitinases.

Introduction

Chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosamine (GlcNAc), is one of the most abundant polysaccharides in nature. It is a common constituent of insect exoskeletons, shells of crustaceans and fungal cell walls. These chitin-containing organisms produce chitinases (EC 3.2.1.14). Some other organisms which do not contain chitin also produce chitinases: for example, a wide variety of bacteria and higher plants. The latter develop several biochemical defence mechanisms in response to pathogens and abiotic stresses. Following pathogen attack, plants synthesize phenylpropanoid products such as lignin, low molecular weight antimicrobial compounds known as phytoalexins, and several defence-related proteins. Among these proteins are the pathogenesis-related proteins (PR-proteins) which include some of the fungal cell wall-degrading enzymes, β-1,3-glucanase and chitinase. These observations underline the significance of chitinase in ecological interactions between organisms.

A chitinase was described for the first time in 1911 by Bernard[8] who found a thermosensitive and diffusable antifungal factor in orchid bulbs, and in 1929 by Karrer and Hofmann[60] in a snail. More recently Jeuniaux's investigations[55] led to renewed interest in chitinases.

The present review will be devoted to a survey of the main achievements in the chitinase research field during the last five years, particularly plant chitinases on which much attention has recently been focused.

Definition

Chitinases are defined as enzymes cleaving a bond between the C1 and C4 of two consecutive N-acetylglucosamines of chitin. Endochitinases, exochitinases (EC 3.2.1.14), β-N-acetylglucosaminidases and chitobiases (EC 3.2.1.30) have been characterized. Usually β-N-acetylglucosaminidase is defined as an enzyme releasing N-acetylglucosamine monomers from chitin, exochitinase as an enzyme releasing chitobiose and endochitinase as an enzyme splitting within the chitin polymer. Chitobiase hydrolyses chitobiose. Some chitinases also display a more or less pronounced lysozyme activity (EC 3.2.1.17) corresponding to the cleavage of a glycosidic bond between the C1 of N-acetylmuramic acid (MurNAc) and the C4 of N-acetylglucosamine in the bacterial peptidoglycan[56]. Transglycosidase activities associated with exochitinase activities have also been detected.

Activity determination

Substrates

Natural chitin can be found in arthropod exoskeleton and fungal walls. Various commercial chitins are available, but the particle size is generally too large to permit sensitive assays, thus colloidal chitin preparations are currently used. Dyes such as remazol brillant blue or remazol brillant violet can be linked to chitin. A radioactive substrate may also be used. Glycol chitin is used for assays which need a soluble substrate[158]. A series of synthetic substrates have also been employed, some of which will be mentioned below.

Assays

Chitinase assays are numerous. Chitinase activities are assayed by monitoring changes in the molecular size of a substrate by viscosity measurement, determination of chitooligosaccharides for endochitinases or of N-acetylglucosamine for β-N-acetylglucosaminidases. These determinations can be performed by measurements of reducing power, by the Morgan and Elson reaction using 4-dimethylaminobenzaldehyde after enzymatic conversion of the oligosaccharides into monosaccharides, or using [(^{3}H)acetyl]-chitin as substrate[158].

Chitinases which have chitobiase or β-N-acetylglucosaminidase activity can hydrolyse PNP-N,N′,N″,N‴,N⁗-pentaacetyl-β-chitopentaoside[147,148] or equivalent di-, tri-, or tetraosides[6,114]. Some endochitinases have lysozyme activity, which can easily be measured by lysis of a *Micrococcus luteus* suspension.

Activity can be detected on polyacrylamide gels[87,143] or isoelectric focusing gels[46] using glycol chitin or a fluorescent derivative as substrate.

Plant chitinases

Plant chitinases are the most widely studied enzymes of this enzyme family. They are of interest partly due to the probable absence of natural substrates in the plant itself. Chitinases are therefore considered as a plant defence against pathogens.

Induction of plant chitinases

Chitinases are present either constitutively or after induction. The induction mechanisms are not completely elucidated. Infection with pathogens, treatment with chitooligosaccharides and other fungal or bacterial extracts, physical or chemical stresses and wounding, can all influence chitinase production.

Constitutive chitinases. Chitinases are sometimes observed constitutively. In *Hevea*[90], the latex contains large amounts of chitinase. Chitinases can also be produced during specific steps of plant development (refer to section on roles of chitinases in plants).

Ethylene treatment. Chitinases can be induced by ethylene treatment[15]. Abeles et al.[1] and Boller et al.[16] showed that chitinase activity in bean seedlings increased 30-fold after exposure to exogenous ethylene. Broglie et al.[21] showed that the increase in chitinase activity was associated with changes in the level of the chitinase mRNA, and that at least two different chitinases were expressed. In potato, chitinase was 30-fold[39] or 3 to 5-fold[81] inducible by ethylene. In carrot cell cultures, four chitinases were induced by ethylene, as reported by Kurosaki et al.[77].

In melon plants, Roby et al.[120,142] demonstrated that ethylene induced chitinase activity. It could be a secondary messenger after treatment of leaves or seedlings with elicitors from a fungal pathogen. Comparisons between the time-course of the appearance of ethylene and chitinase showed (see Toppan and Roby[142] and table 1) that enhancement of ethylene preceded chitinase induction by 48 hours. In the presence of aminoethoxyvinylglycine (AVG), an inhibitor of ethylene synthesis, both elicitor-induced ethylene and elicitor-induced chitinase were reduced. The reduction of elicitor-induced chitinase was overcome by adding exogenous ethylene or 1-aminocyclopropane-1-1carboxylic acid (ACC). However, the levels of ethylene and chitinase were only partially affected in the experiment using AVG or ACC, suggesting that the effect of ethylene on chitinase might be complex and that several enzymes might account for the measured chitinase activity.

Table 1. Effect of ethylene on chitinase activity of elicitor-treated leaves, after previous inhibition by aminoethoxyvinylglycine (AVG) (from Roby et al[120], with permission). Melon leaves were incubated in test tubes for 1 h in the presence of AVG or buffer. In the assay, elicitor (200 µg glucose eq/ml) was then added to the medium. Incubation lasted for 24 h in these conditions, prior to addition of ethylene (10 µl/l) to the internal atmosphere above the plant material. After an additional 24 h, ethylene and chitinase were measured. The experiment was repeated twice: each figure represents the average of 3 assays.

Treatment	Chitinase activities		
	Ethylene	Colorimetric assay	Radiochemical assay
	nl/g leaf	U/g protein	
Control			
Buffer	8.8	18.7	60.1
Buffer + AVG	7.8	21.4	52.6
Buffer + ethylene		26.0	170.3
Buffer + AVG + ethylene		24.0	140.0
Assay			
Elicitor	13.2	38.6	314.6
Elicitor + AVG	7.9	31.8	200.1
Elicitor + ethylene		45.6	362.1
Elicitor + AVG + ethylene		39.0	316.9

Viral infection. Some chitinases can be induced by virus infection. From tobacco leaves infected with TMV (tobacco mosaic virus), Legrand et al.[83] found two additional chitinases (named pathogenesis-related proteins P and Q). Up to 13 (6 acidic and 7 basic) electrophoretic forms of chitinases could be detected in Xanthi-nc tobacco leaf tissue infected with TMV[144]. Some of them were also detected in healthy tissues. In cucumber, Metraux et al.[97] also induced a chitinase by TMV infection. In maize[99,100], brome mosaic virus induced a family of chitinases, named PRmBa2 and PRm3,4,5,7.

Infection by microorganisms, wounding and fungal elicitors. Hedrick et al.[45] showed that chitinase synthesis was stimulated in bean cell suspension cultures treated with fungal cell wall elicitors and in hypocotyls after fungal infection. Elicitors caused a very rapid activation of chitinase transcription with a 10-fold stimulation after 5 minutes. Chitinase transcripts were also greatly accumulated in wounded and infected hypocotyls. In fungally infected tomato[58], four chitinases were immunologically detected. In pea, two chitinases were purified from pods after fungal infection[91]. Other chitinases were found in carrot[78] and in potato[67] after induction by fungal walls or elicitors. In *Rubus* calli[9], chitinase was induced by chitosan, chitin, peptidoglycan or infection with microorganisms. Two chitinases were described. Similar enzymes were found by Bernasconi et al. in *Parthenocissus*[12].

Roby et al.[118,119] also studied the relationships between oligosaccharide size and elicitor efficacity: for the colorimetric assay, the hexamer of chitin was the most efficient elicitor, whereas the heptamer was the most convenient one for the radiochemical assay (table 2).

Chemical induction. Chitinase activity can be induced by treatments with salicylate or mercuric chloride[100,104] but, as for most other induction means, the effects of chemical treatments are not specific; other defence mechanisms are simultaneously induced.

Cellular localization

Chitinases can be found in the vacuole as demonstrated by Boller and Vögeli[18] and by Mauch and Staehelin[93] in

Table 2. Local and systemic induction of chitinase activity in melon seedlings treated with chitin oligosaccharides (from Roby et al.[119], with permission)

Treatment	Chitinase activity (U/g protein)					
	Cotyledons		Hypocotyls		Leaves	
	Colorimetric assay	Radiochemical assay	Colorimetric assay	Radiochemical assay	Colorimetric assay	Radiochemical assay
Control	21.5	197	22.3	687	8.2	169
Oligomer 5	56.2	279	24.7	602	10.2	160
Oligomer 6	74.9	928	31.4	555	11.8	260
Oligomer 7	55.0	2622	32.7	1610	11.2	259
Oligomer 8	57.5	2201	30.8	283	11.7	346

Each value corresponds to the mean of 4 replicates. For all points, the maximal SD was 10.2%.

ethylene-treated bean leaves. Antibodies against chitinase labeled mainly vacuoles but also Golgi cisternae, suggesting that newly synthesized chitinase is processed in the Golgi apparatus. Chitinase was not present in the intercellular washing fluid collected from the same material. In maize, Nasser et al.[100] also found that PRmBa2 chitinase was not detected in the intercellular fluid.

Chitinases have been found in the extracellular compartment. Bernasconi et al.[10] showed that chitinase accumulated mainly in the medium of *Rubus* cell cultures. Cucumber chitinase was described by Boller and Métraux[17,97] as an extracellular enzyme; the specific activity of chitinase was higher in extracts of the intercellular fluid than in leaf homogenates and the specific activity of chitinase in leaf protoplasts was only 5% of that in tissue homogenates. In tobacco[105], oat leaves[32] and wheat germ[3], some chitinases were located in apoplastic compartments. In tomato roots[7], the enzymes detected with an antiserum were found to accumulate in areas where host walls were in close contact with fungal cells. In contrast, the enzyme could not be detected in vacuoles and intracellular spaces. In maize[100], PRm3,4,5,7 are extracellular enzymes.

Latex contains chitinases as described in *Hevea*[122] and *Asclepias*[85].

Chitinase purification

Chitinases can be purified from a total homogenate, from the intercellular fluid or from latex. Affinity chromatography, using chitin or regenerated colloidal chitin, is the most specific method. It was used successfully for the purification of the chitinases from barley leaf intercellular fluid[73], soybean[155], bean[16], tobacco[133] and wheat[112].

This method cannot always be used, as problems in the binding or in the release of chitinases may occur. Most chitinases have a very high or verly low isoelectric point. This characteristic has often been used to purify chitinase. Bernasconi et al.[11] described a one-step purification of *Rubus* chitinase from culture medium, using ion exchange chromatography. However, this step is generally inadequate for obtaining a pure protein. Additional steps such as hydrophobic interaction chromatography[83], gel filtration on P-100[12] or Sephacryl S-200[117] gels, or chromatofocusing[49] can be used. Alternatively, all steps can be performed by HPLC or FPLC[81,94]. Figure 1 and table 3[91], show an example of the purification of pathogenesis-related proteins.

Other examples of chitinase purifications are reported in *Methods in Enzymology*[158].

Isoelectric point

Chitinases generally have very basic or very acid isoelectric points. Some chitinases were described as basic proteins[16,83,84,100]. *Rubus* and *Parthenocissus* chitinases[9] have measured isoelectric points of 9.0 and 9.9 respectively. An *Arabidopsis* chitinase has a basic pI[129]; however, the closely related potato chitinase has a calculated pI of 7.0[81]; the signal peptide is hydrophobic and has no influence on the global charge.

Other chitinases were described as acidic proteins[97,129]. PR-P and PR-Q from tobacco have a calculated charge

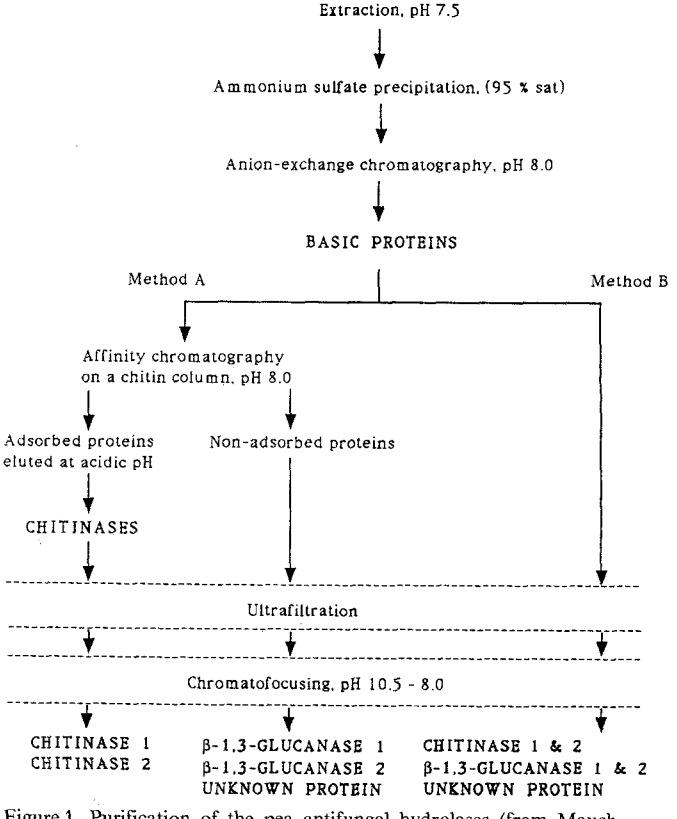

Figure 1. Purification of the pea antifungal hydrolases (from Mauch et al.[91], with permission).

Table 3. Purification of chitinases from mature and immature pea pods. The starting material consisted of 520 g of healthy, mature, and 50 g of immature pea pods which were incubated with *F. solani phaseoli* for 48 h. Purification procedure according to method A described in figure 1 (from Mauch et al.[91], with permission)

Step	Mature pods				Infected immature pods			
	Total protein mg	Activity units	Specific activity units/mg	Recovery %	Total protein mg	Activity units	Specific activity units/mg	Recovery %
Ammonium sulfate	608.0	150	0.3	100	310	132	0.4	100
Trisacryl-DEAE	22.1	117	5.3	78	ND	116		88
Chitin column pH 3.2	4.5	58	12.8	38	1.9	32	16.8	24
Chromatofocusing:								
Ch1	0.7	12	17.7	8	1.1	22	19.0	16
Ch2	2.7	34	12.7	23	0.1	1	9.5	1

ND = not detected

at pH 7.0 of −4.9 and −3.8 respectively[105] and tomato 26 kDa chitinase has a measured pI of 6.1[58].

Molecular weight

All plant chitinases are small proteins. M_r vary between 25,000 and 40,000. Measured and calculated molecular weights are situated in the same range (see section on 'Chitinase genes'). It may be noted that smaller molecular weights have been published, but they were determined by elution volume measurements on gel filtration columns. This method often gives inaccurate information, since chitinases can interact with the gel matrix. It may be that chitinases have very small molecular weights, because hen egg white lysozyme, which possesses chitinase activity, has a M_r of 14,400. However, plant chitinases have never been clearly demonstrated to be so small.

Amino acid sequences and chitinase classes

Three classes of chitinases have so far been characterized. It appears that all three can be present in the same plant and their occurrence is not correlated with phylogenetic classification, but the major chitinase produced in different materials does not always belong to the same class. As proposed by Payne et al., the definition of chitinase classes is based on their primary structure[105, 134].

Class I chitinases. Class I chitinases (fig. 2) contain an amino terminal cysteine-rich domain of about 40 amino acids, which has considerable structural homology with wheat germ agglutinin and hevein. This domain was described by Lucas et al. in bean[84] and was also found in the basic chitinases of tobacco[133], potato[39, 81], *Arabidopsis*[129] and *Populus (chiX)*[28]. It could be involved in chitin binding. A glycine- and proline-rich region (glycine- and arginine-rich in rice[164], missing in *win8* from *Populus*) separates the cysteine-rich domain from the catalytic domain. Class I chitinases possess a leucine-rich or valine-rich signal peptide. They usually have a basic isoelectric point and are located in the vacuole. For a class I chitinase from tobacco, the seven C-terminal amino acids (GLLVDTM) were shown to be necessary and sufficient for targeting to the vacuole[102b]. These amino acids were lacking at the C-terminus of the mature chitinase.

However, *win6* and *win8* from poplar[28] and PR4 from bean (see ref.[89] and fig. 3) are acidic. PR4 is extracellular. It is not serologically related to the basic protein, but has sequence homologies with the latter[4]. All these proteins possess the cysteine-rich domain. We propose

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                                                                      0
Arabidopsis                          MPPQKENHRTLNKMKTNLFLFLIFSLLLSLSSA
Nicotiana                                                    ....LSA..
Solanum                                    M.RHKEVNFVAY.LFSLLV.V.AAL.
Phaseolus                                  MKKNRMM.MIWSVGVVWML..VGG.YG
Oriza                                        MRALAVVAMVARPFLAAAVH

                                                                     40
Arabidopsis                  EQCGRQAGGALCPNGLCCSEFGWCGNTEPYCKQPGCQSQC
Nicotiana                    ....S.....R.AS.....K.......ND..GPGN.....
Solanum                      QN..S.G..KA.AS.Q...K.......ND..GSGN.....
Phaseolus                    .............G.N...Q.....S.TD..GPG---C.S
Oriza                        ....S.....V...C....Q.....S.SD..GAG-.....

                                                                     80
Arabidopsis                  -----TPGGTPPGPTGDLSGIISSSQFDDMLKHRNDAACP
Nicotiana                    -----P-..PT.PGG...GS.....M..Q.......N..Q
Solanum                      -----PG..PG...G...GSA..NSM..Q......ENS.Q
Phaseolus                    -----QC..PS.A..-...AL..R.T..Q.......G...
Oriza                        SRLRRRRPDASG.GGSGVAS.V.R.L..L..L........

                                                                    120
Arabidopsis                  ARGFYTYNAFITAAKSFPGFGTTGDTATRKKEVAAFFGQT
Nicotiana                    GK...S.....N..R.......S...TA..R.I....A..
Solanum                      GKN..S.....N..R.......S..INA..R.I....A..
Phaseolus                    AK.....D...A...AY.S..N........R.I...L...
Oriza                        .SN....D..VA..SA....AAA..AD.N.R.....LA..

                                                                    160
Arabidopsis                  SHETTGGWATAPDGPYSWGYCFKQEQNPAS-DYCEPSATW
Nicotiana                    ................A....WLR..GSPG-...T..GQ.
Solanum                      .........S......A.....LR.RGNPG-...P..SQ.
Phaseolus                    ................A.....VR.R....-T..SATPQF
Oriza                        ................T......E.NGG.GP...QQ..Q.

                                                                    200
Arabidopsis                  PCASGKRYYGRGPMQLSWNYNYGLCGRAIGVDLLNNPDLV
Nicotiana                    ...P.RK.F....I.I.H.....P.................
Solanum                      ...P.RK.F....I.I.H.....P.................
Phaseolus                    ...P.QQ.F....I.I.......Q..........K.....
Oriza                        ...A..K......I...Y.F...PA.Q...A...GD.....

                                                                    240
Arabidopsis                  ANDAVIAFKAAIWFWMTAQPPKPSCHAVIAGQWQPSDADR
Nicotiana                    .T.P..S..S.L.....P.S......D..I.R....S...
Solanum                      .T.P..S..T.L.....P.S......D..I.R.N..S...
Phaseolus                    .T.S..S..S.L.............S.D..TSR.T..S..V
Oriza                        .S..TVS.DF.F.....P.S.....N..AT...T..AD.Q

                                                                    280
Arabidopsis                  AAGRLPGYGVITNIINGGLECGRGQDGRVADRIGFYQRYC
Nicotiana                    ..N....F.................T.S..Q......R...
Solanum                      ..N....F.................T.N..Q......R...
Phaseolus                    ..R......TV..............S.........FK...
Oriza                        R...V.................H.E.D.I.......K...

                                                                    308
Arabidopsis                  NIFGVNPGGNLDCYNQRSFVNGLLEAAI
Nicotiana                    S.L..S..D....G.....G....VDTM
Solanum                      S.L..T..D....V...W.G....VDTL
Phaseolus                    DLL..GY.N.......TP.G....LSDLVTSQ
Oriza                        D.L..SY.A.....S..PSAPPK.RLPSFHTVINNH
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Figure 2. Amino acid sequences of plant class Ia chitinases (deduced from nucleotide sequences) from *Arabidopsis*[129], *Nicotiana*[133], *Solanum*[39], *Phaseolus*[21], and *Oriza*[164].

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PR4     1   MRFWALTVLSLLLSLLLGVSSDTAQCGSQAGNATCPNDLCCSSGGYCGLT   50
win8    1          MGNK.VLV.VA.ALVMGPKNVS.Q.CG.AEG....QY....TG   43

PR4    51   VAYCCAGC-----------VSQCRNCFFTESMFEQMLPNRNNDSCPGKGF   89
win8   44   ED..GT..QQGPCTTASPPP.NNV.ADILTAD.LNGIIDQADSG.A..N.   93

PR4    90   YTYDAYFVATEFYPGFGMTGDDDTRKRELAAFFAQTSQETSGRSIIGEDA  139
win8   94   ..R..FLS.LNS.TD..RV.SE.DS...I..A..HFTH..GHFCY.E.ID  143

PR4   140   PFTWGYCLVNELNPNSDYCDPKTKSSYPCVADYYGRGPLQLRWNYNYGEC  189
win8  144   GASKD..DEESI----------AQYPCSSSKG.H....I..S..F...PA  183

PR4   190   GNYLGQNLLDEPEKVATDPVLSFEAALWFWMNPHSTGAPSCHEVITGEWS  239
win8  184   .SANNFDG.GA..T.SN.V.V..KT...YW.Q------------------  215

PR4   240   PSEADIEAGRKPGFGMLTNIITNGGECTKDGKTRQQNRIDYYLRYCDMLQ  289
win8  216   ----HVRPVINQ...ATIRA.NGAL..DGANP.TV.A.VN..TE..RQ.G  261

PR4   290   VDPGDNLYCDNQETFEDNGLLKMVGTM*                        317
win8  262   .AT....T.                                           270
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Figure 3. Amino acid sequences of plant class Ib chitinases (deduced from nucleotide sequences) from *Nicotiana* (PR4)[89] and from *Populus* (*win8*)[28].

that two subclasses should be defined: class Ia for the basic chitinases and class Ib for the acidic ones.

Class II chitinases. Class II chitinases are similar to class I enzymes, but the cysteine-rich domain and the proline-rich small region that follows are missing (fig. 4)[49,82]. A dot matrix comparison of the DNA sequences for the tobacco class I and class II chitinases was published by Payne et al.[105]. The signal peptide is also different. Class II chitinases seem to be acidic proteins, as are PR-P and PR-Q proteins from tobacco[49].

Class III chitinases. Class III chitinases (fig. 5) have no sequence similarities with class I and class II chitinases. Class III chitinases were described in *Rubus*[9], *Parthenocissus*[12], *Vigna*[53] and completely sequenced in *Cucumis*[96] and *Hevea*[54]. They can be acidic (*Cucumis*) or basic (*Rubus*) proteins.

Chitinase class determination. In many studies the induction of chitinases by different means was reported, but the class was not determined. However, it could be important to know systematically to which class all described chitinases belong, in relation to localisation, induction mechanisms and possible roles.

The best method for determining to which class a chitinase belongs is the N-terminal sequence determination[31,73]. However, some information can be obtained by the compartmentation of the enzyme and its pI value. Class I chitinases can be induced by ethylene treatment as shown in bean[1,84], in tobacco[61], potato[39], and *Arabidopsis*[150]. These chitinases seem to be exclusively located in the intracellular volume and accumulated in the vacuole[18,93]. Mauch and Staehelin[93] showed that in ethylene-treated bean leaves, antibodies against a class I

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                                                       Mature prot.
                                                         0        10
Arabidopsis      MTNMTLRKHVIYFLFFISCSLSKPSDASRG-GIAIYWGQN
Cucumis                MAAHKITTT.SIFFLLS.IFRSSDAA.........
Hevea                                          G.........
Pathenocissus                                  G.........
Rubus                                          G.........
Vigna                                          G..SV.....

                                                          50
Arabidopsis      GNEGNLSATCATGRYAYVNVAFLVKFGNGQTPELNLAGHC
Cucumis          ....S.AS.....N.EF..I...SS..S..A.V.......
Hevea            ....T.TQ..S.RK.S...I...N........QI......
Pathenocissus    ....T.TQ..N..K.S...I...N.........I...
Rubus            ....T.TQ
Vigna            ....S.ADAXN..N.K...I...FT..G..

                                                          90
Arabidopsis      NPAANTCTHFGSQVKDCQSPGIKVMLSLGGGIGNYSIGSR
Cucumis          ..DN.G.AFLSDEINS.K.QNV..L..I...A.S..LS.A
Hevea            ....GG..IVSNGIRS..IQ.............S.TLA.Q
Rubus

                                                         130
Arabidopsis      EDAKVIADYLWNNFLGGKSSSRPLGDAVLDGIDFNIELGS
Cucumis          D...QV..FI..SY...Q.D.....A.....V..D..S..
Hevea            A...NV............................D..H..
Rubus                                                 ...T

                                                         170
Arabidopsis      PQHWDDLARTLSKFSHRGRKIYLTGAPQCPFPDRLMGSAL
Cucumis          G.F..V..QE.KN.GQ----VI.SA.....I..AHLDA.I
Hevea            TLY......Y..AY.KQ.K.V...A.........YL.T..
Rubus            TGY..E..GY.KE          ES H..

                                                         210
Arabidopsis      NTKRFDYVWIQFYNNPPCSYSSGNTQNLFDSWNKWTTSIA
Cucumis          K.GL..S..V........MFAD-.AD..LS...Q..A-FP
Hevea            ..GL.....VQ.......QY....IN.IIN...R.....N

                                                         250
Arabidopsis      AQKFFLGLPAAPEAADS-GYIPPDVLTSQILPTLKKSRKY
Cucumis          TS.LYM.....R...P.G.F..A...I..V...I.A.SN.
Hevea            .G.IFL.........-GS..V.....I.R...EI...P..

                                     270
Arabidopsis      GGVMLWSKFWDDKNGYSSSILASV
Cucumis          ........AF.--....DS.KG.IG
Hevea            .........Y..........D..
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Figure 5. Amino acid sequences of plant class III chitinases. Complete deduced sequences from *Arabidopsis*[129] and *Cucumis*[96], complete or partial sequences determined by protein chemical techniques from *Hevea*[54], *Parthenocissus*[12], *Rubus*[9] and *Vigna*[53].

chitinase labelled vacuoles and the Golgi apparatus. But chitinase was not present in the intercellular fluid collected from the same material. Thus, intracellular basic chitinases such as PRmBa2 protein from maize[100] could be class I chitinases.

Class II chitinases seem to be located in the extracellular volume. PR-P and PR-Q chitinases from tobacco are located in the apoplastic compartment[83]. In tomato leaves[58], enzymes detected with antiserum and accumulated near the wall could belong to class II chitinases, as does the 26-kDa protein which interacts with antibodies against PR-P from tobacco. The pathogenesis-related proteins PR-5 and PRm7 from maize could also be class II chitinases, because they are extracellular enzymes and

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Protein                                              hevein domain                            spacer                      chitinase domain

WGA                                   K....SG.K L..NN....Q W.S..LGSEF .--.GG...G ACS
hevein                                ....R....K L..NN....Q W....S.DE. .S.DH....N .KD
tobacco class I chitinase             EQCGSQAGGA RCPSGLCCSK FGWCGNTNDY CGPG-NCQSQ CPGGPTPTPP TPPGGGDLGS IISSSQFDQM LKHRNDNACQ GKGFY
tobacco class II chitinase (PR-P)                                                                          QGI.. .VT.DL.NE. ..N...GR.P AN...
tobacco class II chitinase (PR-Q)                                                                          QGI.. .VTNDL.NE. ..N...GR.P AN...
barley class II chitinase (CHI26)                                                                           ..S. .V.RA...R. .L....G..P A....
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Figure 4. Partial amino acid sequences of two lectins (wheat germ agglutinin[134b] and hevein[18b]), of a class I chitinase from tobacco[133] and of class II chitinases from tobacco[50] and barley[82].

Table 4. Amino acid composition (residues/mole) of plant chitinases; two examples from each of the 3 known classes[54, 96, 105, 129, 133]

Amino acid	Class I Tobacco[133]	Arabidopsis[129]	Class II Tobacco[105] (PR-P)	Tobacco[105] (PR-Q)	Class III Hevea[54]	Cucumis[96]
Ala	21	31	22	20	20	29
Arg	17	13	13	15	7	2
Asn	17	17	22	20	22	20
Asp	17	15	16	15	14	17
Cys	17	17	6	5	6	6
Gln	14	16	11	10	11	12
Glu	5	9	7	9	4	6
Gly	45	41	26	28	32	29
His	4	3	2	2	2	2
Ile	15	13	15	16	20	15
Leu	14	14	8	8	23	23
Lys	7	9	5	5	14	9
Met	4	3	3	3	2	3
Phe	14	14	12	12	9	14
Pro	25	23	11	10	14	13
Ser	25	16	13	11	25	31
Thr	15	17	15	17	13	6
Trp	8	8	3	3	7	7
Tyr	13	14	10	11	17	8
Val	8	9	9	9	12	14

Table 5. Some characteristics of plant chitinases

	Class I	Class II	Class III
Induction by ethylene	+	−	−
Induction by elicitors or infection	+	+	+
Induction by salicylate	+	?	+/−
Localisation	Intracellular (Ia) Extracellular (Ib)	Extracellular	Extracellular
Molecular weight (mean value)	33,000	27,000	31,000
pI	Basic (Ia) Acidic (Ib)	Acidic	Basic or acidic
Cysteine-rich domain and proline-rich spacer	+	−	−

antibodies against PRm7 reacted against PRm5, PRm7 and PRmBa2[100].

Class III chitinases seem to be compartmentalized in the extracellular volume. In cucumber leaves, class III chitinase was demonstrated to be an extracellular protein. In maize, PRm3 and PRm4 could belong to class III chitinases, as could the chitinases from *Rubus* or *Parthenocissus* cell suspension.

Although all types of chitinases can be induced by infection or by elicitor treatment, ethylene treatment seems to induce class I chitinases only. Some chitinases seem to have too high a molecular weight to belong to a known class. It is possible that they may be assigned to a fourth class. Table 4 shows the amino acid composition of several chitinases and table 5 some of their characteristics.

Chitinase genes

Class I and II chitinase genes. Broglie et al.[21] performed Southern blot analysis of bean genomic DNA using a cDNA clone encoding for a class I chitinase: chitinase is encoded by a small multigene family consisting of about four members. At least two ethylene-regulated genes are expressed, as shown by sequence analysis of additional c-DNA clones. Similar observations were made using cDNA clones from infected bean[45] and rice[103]. In barley, endochitinase genes were present as multiple copies on chromosome 1[140]. In tobacco[49], hybridization of cDNA probes to genomic blots indicated that the acidic and basic chitinases are each encoded by two to four genes in the amphidiploid genome of Samsun NN tobacco. A similar complexity was found for the genes encoding other pathogenesis-related proteins of bean[123]. In peanut[47], Herget et al. showed an elicitor-specific induction of chitinase genes (chit 1–4); chit 1 gene was activated by the yeast extract, chit 2 gene by fungal wall components; chit 3 gene was constitutively expressed and chit 4 gene induced by each tested stimulus. The structure of a tobacco class I chitinase gene was described by Shinshi et al.[134] (see also fig. 6). The gene contained two introns. The authors suggested that the sequences encoding the cysteine-rich domain were introduced into genes for class I enzymes by transposition events. In class I chitinase gene from *Arabidopsis*, the second intron is missing[129]

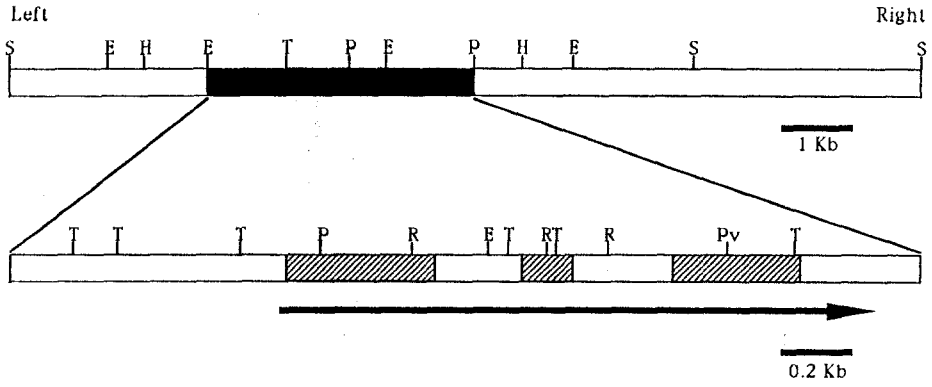

Figure 6. Partial restriction map of a tobacco chitinase genomic clone. The arrow shows the direction of the transcription and the length of the transcribed region. Shaded regions, exons. Solid bar, region which was sequenced. Restriction endonuclease sites: E, *Eco* RI; H, *Hind* III; P, *Pst* I; Pv, *Pvu* II; R, *Rsa* I; T, *Taq* I (from Shinshi et al.[134], with permission).

and the chitinase is encoded by a single copy gene. There is no intron in rice[164] and potato[40] chitinase genes. Bean class I chitinase gene was introduced into tobacco plants using Ti-plasmid[20]. Exposure to ethylene resulted in induction of bean chitinase. The promotor region was analysed. This region is characterized by two short DNA sequences that are exactly conserved in a second ethylene-regulated bean chitinase gene (fig. 7). Analyses of 5'deletion mutants indicated important DNA sequences for induction[116]. Similar experiments were performed with infection-activated promoter[115].

Class III chitinase genes. In *Arabidopsis*, class III chitinase is encoded by a single copy gene[29]. It possesses two introns. The difference in the size of restriction fragments hybridizing to both *Arabidopsis* chitinase probes suggests that the two genes (classes I and III) are not linked.

Roles of chitinases in plants

Effect against fungi and insects. Many plant chitinases are considered pathogenesis-related (PR) proteins. They are induced in the presence of pathogens, or pathogen extracts, and also after a stress. Plant chitinases are potent inhibitors of fungal growth, but other enzymes are induced simultaneously. Schlumbaum et al.[131] tested the effect of chitinase on a non-pathogenic fungus. They showed that chitinase can inhibit in vitro the growth of the fungus; commercial lectin preparations, which were previously considered as inhibitors, were shown to have an effect only when contaminated with chitinase. However, a chitin-binding lectin from stinging nettle rhizomes has antifungal properties[19]. Roberts and Selitrennikoff[114] compared the efficiency of plant and bacterial chitinases on fungal growth inhibition. In vitro antifungal activity was observed only for plant chitinases. This difference in antifungal activity was related to the different mechanisms of action of the two types of enzymes; plant chitinases are generally endochitinases and bacterial chitinases, exochitinases. However, Shapira et al.[132] showed that a cloned bacterial chitinase from *Serratia marcescens* expressed in *Escherichia coli* could protect plants against fungi when added in tap water. In vivo, bean chitinase activity increased more rapidly in response to avirulent than to virulent cells of *Pseudomonas siringae*[153], and also in incompatible rather than compatible interactions with *Colletrichum lindemuthianum*[27]. However, experiments using transgenic tobacco plants that make high levels of a class I chitinase, showed that this enzyme did not increase resistance against *Cercospora nicotiana*[102].

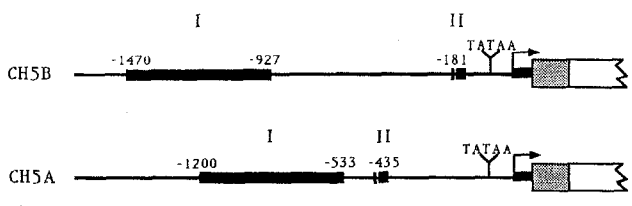

Figure 7. Schematic diagram of 5' upstream regions of two chitinase genes CH5B and CH5A. Bold lines indicate regions of conserved sequence homology (from Broglie et al.[20] with permission).

An endochitinase from seeds of Job's tears[2] inhibited an insect α-amylase. This enzyme was closely related to class I chitinases but was a dimer. This combination of functions may be relevant to protection of the grain from both insect feeding and fungal infection.

Chitinases and β-1,3-glucanases. Inhibition of most of the fungi required the presence of a combination of chitinase and β-1,3-glucanase (EC 3.2.1.6). Co-induction of these enzymes was described in potato[67], tobacco[94,152], bean[151], pea[91,92], tomato[58], oat[32], and maize[100]. In tobacco[152], the content of mRNA in TMV-infected leaves was measured by Northern blot analysis using cDNA clones of tobacco chitinase and β-1,3-glucanase as probes. There was a parallel increase in the two mRNAs following TMV infection indicating that chitinase and β-1,3-glucanase are coordinately induced at the mRNA level. The same study was carried out with bean leaves using in vitro translation and antiserum precipitation[151]. In vitro efficiency of purified chitinase and β-1,3-glucanase combinations was demonstrated for pea enzymes

by Mauch et al.[92]. Inhibition of fungal growth was caused by the lysis of the hyphal tips. Plant PR-hydrolases may be as important as phytoalexins in the defence of plants against pathogens.

Chitinases and plant hypersensitivity. Chitinase induction can be local or systemic. The local induction seems to be correlated with a hypersensitive reaction in the plant. Vögeli-Lange et al.[152] measured the increase of chitinase and β-1,3-glucanase content in TMV-infected leaves of tobacco. This induction was not observed in TMV-infected leaves of tobacco plants which do not exhibit a hypersensitive local lesion reponse, suggesting that β-1,3-glucanase and chitinase induction is part of the hypersensitive reaction. In tomato leaves inoculated with fungus, chitinase and β-1,3-glucanase activity in the apoplastic fluid increased more rapidly in incompatible interactions than in compatible ones[58]. In tobacco infected with a bacterial pathogen[94], increases in the content of both enzymes were confined to infected parts of the plant indicating that this reponse is local rather than systemic. In tomato[7], a time-course experiment revealed that chitinase accumulated earlier in the incompatible interaction than in the compatible one.

Chitinases and systemic resistance. Chitinase activity has also been observed in the systemic resistance induced by localised induction in cucumber leaves[95,130] and tobacco[145]. Roby et al.[12] showed in melon that fungal elicitors induce a systemic induction more rapidly than does infection by the pathogen. They proposed that fungal elicitors in induced systemic resistance were released from the pathogen by degrading enzymes of the host cells and that these elicitors in turn trigger the release of an endogenous elicitor.

Induction of other plant defence mechanisms. The effect of plant chitinases on pathogens could also be indirect. Fungal glycans released by the enzyme could induce chitinase itself and β-1,3-glucanase, but also phenylalanine ammonia-lyase. The role of chitinase and chitin oligosaccharides in the lignification response of cultured carrot cells treated with mycelial walls was studied by Kurosaki et al.[78,79]. After gel filtration of a chitinase digest, elicitors were distributed in many fractions; however, potent activity for inducing phenolic acid synthesis was observed in the high molecular weight fractions.

Chitinases during plant development. Chitinase production was not only observed after infection or stress. Constitutive or development-dependent presence was reported by some authors[87]. In tobacco tissues cultured in vitro[133], chitinase was regulated by the addition of combinations of the plant hormones auxin and cytokinin to the culture medium; chitinase was also regulated during development in the intact plant. Not detectable in leaves near the top of the plant, it constituted 1–4% of the soluble proteins in roots and lower leaves. As already discussed, ethylene can induce chitinase and could be involved in response to an attack by pathogens. But ethylene is also involved in the abscission process. Gomez et al.[41] identified chitinase mRNA in abscission zones from bean during ethylene-induced abscission. Chitinase was expressed in tobacco explants during flower formation[101] and in apical leaves[144]. It was also found in seeds[155]. In conclusion, chitinase could be present and hormonally regulated as a protection measure in organs and tissues particularly exposed to infection.

Chitinases and mycorrhizal interactions. Spanu et al.[136] measured chitinase activity in mycorrhizal and non-mycorrhizal roots of *Allium*. Between 10 and 20 days after inoculation, specific activity was higher in mycorrhizal roots than in control ones. However, 60–90 days after inoculation, when the symbiosis was fully established, the mycorrhizal roots contained less chitinase than control roots. An antiserum against bean leaf chitinase was used for the immunocytochemical localization of the enzyme. Chitinase was localized in the vacuoles and in the extracellular spaces of non-mycorrhizal and mycorrhizal roots. There was no labelling of the fungal cell walls. It was concluded that chitin in the fungal walls was inaccessible to plant chitinase. Thus, if fungal penetration appeared to cause a typical defence response in the first stages, this effect was later suppressed.

Potential substrates in plants. Potential substrates in the plant itself were also investigated. Chamberland et al.[24] used a microbial chitinase-gold complex to localize chitin ultrastructurally in infected tomato root cells. They found labelling over the fungal wall, but also in secondary walls of vessels and occasionally of adjoining parenchyma cells. Using the same technique, Benhamou and Asselin[6] showed that abundant N-acetylglucosamine residues were present in secondary walls of plant cells. Enzymatic digestion of plant tissues with chitinase from *Streptomyces griseus* abolished the labelling of the fungal cell wall but did not interfere with that of secondary cell walls, suggesting that polymers analogous to fungal chitin were absent in the plant cell walls. On the contrary, lipase digestion abolished the plant cell wall labelling, suggesting that N-acetylglucosamine residues may be linked to lipids. If plant chitinases cannot hydrolyse such substrates, they could perhaps act as lectins[6].

Fungal chitinases

Fungal wall-degrading enzymes could be involved in the growth of the fungus itself. All types of chitin-degrading activities were found in fungi. For example, an endochitinase and a β-N-acetylglucosaminidase in *Aspergillus nidulans*[11], and an exochitinase in *Mucor rouxii*[106] have been described. Overproducing mutants can be selected[149].

Cellular localization

Chitinases can be soluble, probably sequestered in lysosomal vacuoles, membrane-bound or wall-bound. In

Mucor rouxii[23], chitinase was demonstrated in the three compartments. Nine chitinase species were detected in germinating cells[107]. In *Mucor mucedo*[51,52] and *Candida albicans*[29,30], microsomal and supernatant chitinases were described.

Purification

The purification of supernatant chitinase can be performed by classic techniques, essentially by the same techniques as used for plant chitinases; for example, the purification of the chitinases from *Mucor rouxii* required a series of steps as shown by Pedraza-Reyes et al.[106]. Examples have also been described in *Methods in Enzymology*[158].

Some characteristics

Myrothecium verrucaria chitinase[154] showed broad temperature (25–55 °C) and pH (4.0–6.5) activity profiles. *Candida albicans* supernatant and microsomal chitinases had apparent temperature optima at 45 °C, pH optima at 6.5, and Km = 2.9 and 2.1 mg chitin/ml, respectively[29,30].

Microsomal chitinase activity was shown to be dependent on the phospholipid environment[52] and can be stimulated by dimyristoyl phosphatidylcholine. In contrast to wall-bound chitinase, membrane-bound chitinase could not be extracted by digitonin. Humphrey and Gooday[51] showed that chitinase activity could be enhanced by treatment with commercial proteases such as trypsin. This effect was due to an activation of microsomal chitinase. Longer enzyme treatments inhibited microsomal chitinase, while supernatant chitinase was always inhibited. Manocha and Balasubramanian[88] observed the same induction using partially purified proteases from the fungus itself. Both chitinase and chitin synthase were membrane-bound, zymogenic and activated by partial proteolysis. This suggests that microsomal chitinase was co-regulated with chitin synthase and implicated in cell growth regulation. However, Humphrey and Gooday[51] showed that chitinase was not zymogenic when solubilised with low Triton X-100 concentrations, while non-solubilised chitinase was zymogenic. They suggest that the membrane-bound proteolytic activities responsible for chitinase activation may activate solubilised chitinase only. At high Triton X-100 concentration the proteolytic activity may be solubilised and would activate both the soluble and insoluble forms of chitinases.

A yeast chitinase from *Saccharomyces cerevisiae* was cloned and sequenced[76]. Analysis of the derived amino acid sequence suggests that the protein contains four domains: a signal sequence, a catalytic domain, a serine/threonine-rich region, and a chitin-binding domain (fig. 8). Two short sequences of the catalytic domain have homologies with other chitinases and glycosidases (fig. 9).

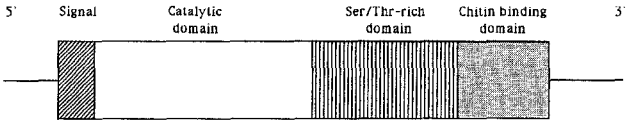

Figure 8. Schematic representation of a yeast chitinase from *Saccharomyces cerevisiae*[76].

		Region 1		Region 2
Saccharomyces cervisiae	102	KVLLSLGGASGSYLF	150	DGFDFDIENNNEVG
Cucumis sativus	98	.....I..GA...SL	145	..V.....SGSGQF
Serratia marcescens	267	.I.P.I..WTL.DP.	300	..V.I.W.FPGGK.
Streptomyces Endo-H	128	.....VL.NHQGAG.	160	..V...D.YAEYGN
Kluyveromyces lactis	441	.KIF.F..WDF.TSP	488	..I.L.W.YPGAPD
rat/human chitobiase			121	..INI...QEVDCS

Figure 9. Sequence comparisons of *Saccharomyces* chitinase[76] with chitinases from *Cucumis*[96] and *Serratia*[57], with a toxin from *Kluyveromyces*[139b], with an endo-H from *Streptomyces*[139b] and with a chitobiase from rat (unpublished data in Kuranda and Robbins[76]).

Roles of fungal chitinases

Fungal chitinases could be involved in growth regulation as already discussed. The chitinase from *Saccharomyces cerevisiae* seems required for cell separation[76]. Saprophyte chitinases are anticipated to have tropic functions. In vitro, *Myrothecium verrucaria*, a very common fungus from soil, can be cultivated in a medium containing chitin as sole carbon source[154]. Chitinases could also be involved in the penetration of a host by mycoparasites[5,139] or by entomopathogenic fungi[30b]. In *Nomuraea rileyi*[30b], high levels of both endo- and exochitinase activities were detected in virulent isolates but not in an avirulent one. The greatest difference in chitinase activity occurred at germination.

A chitinase activity was found associated with the biological activity of the *Kluyveromyces lactis* toxin[22]. This protein contains three subunits (α, β, γ). The γ subunit appeared to be the only component required to arrest proliferation of sensitive yeast cells. The α subunit was an endochitinase: it was absolutely required for the initial interaction of the toxin with sensitive cells.

Streptomyces chitinases

Exo- and endoactivities were described in a *Streptomyces* sp. and *Streptomyces plicatus*[13,113]; *Streptomyces erythraeus* chitinase had a M_r of 30,000, a pI of 3.7 and showed optimal activity at pH 5.0 in the presence of a $\leq$ 0.2 M buffer[43]. Using chitooligosaccharides and their derivatives, the binding mode of the *Streptomyces erythraeus* chitinase to the substrate seems similar to that of hen egg white or *Streptomyces erythraeus* lysozymes. The *Streptomyces erythraeus* chitinase was completely sequenced by the conventional method[59]. It consists of 290 amino acid residues (M_r = 30,000) and has two disulfide bridges at Cys 45-Cys 49 and Cys 265-Cys 272. Chitinase 63 from *Streptomyces plicatus*[113] was cloned (fig. 10). The partial DNA sequence showed that the protein possesses a signal sequence of 30 amino acids. This chitinase exhibits no sequence homology with the *Streptomyces erythraeus* chitinase.

```
                                                                50
EAAGVVSASP YLYNGWGNPP SPTEVMNASG IKNFTLAFIL ADGTCNPAWD

                                                               100
GNRPLDGQDK ATIDAIRGAG GDVIPSIGGY SGSKLGEVCQ DSQSLAGAYQ

                                                               150
KVIDAYGLKA IDVDIEATEF ENDASETRVL EALKIVKEAN PGLRTVVTFP

                                                               200
TLVNGPNDVG KRMIDAKAAR IGSDVDVWTQ MPFNFGGGDM AATITSTEGL

                                                               250
VAHLKSAFGY DDATAYAHAG ISSMNGKSDT GETVDQAAFQ KMADYAGEKG

                                                       290
LGRLSFWSVN RDRPCDGAPD ACGGIDQQWD FTKIVAGLQS
```

Figure 10. Amino acid sequence of a chitinase from *Streptomyces erythraeus*[59].

Bacterial chitinases

Chitinase production

Bacteria play a large role in chitin mineralisation, for example in marine waters and sediments[48,108], but not all species are able to hydrolyse chitin. Cody et al. studied the distribution of chitinase and chitobiase in strains of *Bacillus*[26]. Chitinase activities were found in 10 out of 29 species tested and chitobiase activities in 15 species. Thermostable chitinases are produced by *Bacillus licheniformis*[141].

In bacteria, chitinases were shown to be extracellular enzymes. In *Serratia marcescens*[66] cultured in liquid medium with chitin as the sole carbon source, chitinase was secreted into the medium. Washed, sonicated cells showed no detectable chitinase activity. A chitobiase of *Serratia marcescens* cloned in *E. coli* was also secreted into the periplasm[62].

The time-course of chitinase production during growth of *Serratia marcescens* on chitin was studied by Young et al.[163], who proposed a mathematical model. Purification can be performed with methods used for chitinase purifications from other organsims. Examples have been described in *Methods in Enzymology*[158].

Characteristics

In *Aeromonas hydrophila*[162], sulfhydryl groups appeared to be involved in the expression of the activities. The Km value of the chitinase was 2.8 mg chitin/ml and of the chitobiase 1 mM of PNP-β-N-acetylglucosamine. The optimum pH and temperature were 7.0 and 45 °C for chitinase, and 7.0 and 50 °C for chitobiase, respectively. The relation between the decrease in the absorbance of the chitin suspension and the increase in the amount of liberated N-acetylglucosamine during hydrolysis by purified chitinase suggested that chitinase acted in an endo-splitting manner.

Nanjo et al.[98] examined the hydrolytic products resulting from the action of a chitinase purified from *Nocardia orientalis* on reduced chitooligomers (GlcNAc)n, n = 2–6. The rate of hydrolysis on reduced (GlcNAc)4–6 increased with increasing chain length of N-acetylglucosamine residues, but the enzyme did not act on reduced (GlcNAc)2–3. It was shown to release predominantly (GlcNAc)2 from the nonreducing end of each substrate (exochitinase), such as a chitinase from *Bacillus circulans*[157].

Transglycosylation reactions

The *Nocardia orientalis* chitinase[98,146], which was essentially a hydrolase, also catalysed a transglycosylation reaction on (GlcNAc)4 and (GlcNAc)5. In the presence of ammonium sulfate, the enzyme converted the tetrasaccharide into (GlcNAc)6 and (GlcNAc)2 as the major products. It converted the pentaoligosaccharide into (GlcNAc)7 and (GlcNAc)3. The rate of the transglycosylation depended on the temperature, the concentration of substrate and the pH. This method can be used for preparative-scale synthesis of (GlcNAc)6–7, which are biologically active oligosaccharides of use in medicine and biology.

Bacterial chitinase genes

Serratia marcescens was shown to produce five chitinolytic proteins with subunit molecular weights of 21,000, 36,000, 48,000, 52,000 and 57,000. Fuchs et al.[33] cloned the 57,000 chitinase, which was the most abundant one. Jones et al.[57] isolated and characterized genes encoding two chitinases from the same microorganism. These genes, named ChiA and ChiB, showed no detectable homology to each other. DNA sequence analysis of ChiA predicted a N-terminal signal peptide typical of genes encoding secreted bacterial proteins. The ChiA gene coded for a protein with a molecular weight of 58,000 (fig. 11). DNA sequence analysis of ChiB[44] predicted a

```
                                                                50
MRKFNKPLLA LLIGSTLCSA AGAAAPGKPT IAWGNTKFAI VEVDQAATAY

                                                               100
NNLVKVKNAA DVSVSWNLWN GDAGYGPKIL LNGKEAWSGP STGSSGTANF

                                                               150
LVNKGGRYQM QVALCNADGC TASDATEIVV ADTDGSHLPP LKEPLLEKNK

                                                               200
PYKQNSGKVV GSYFVEWGVY GRNFTVDKIP AQNLTHLLYG FIPICGGNGI

                                                               250
NDSLKEIEGS FQALQRSCQG REDFKISIHD PFAALQKAQK GVTAWDDPYK

                                                               300
GNFGNLMALK QAHPDLKILP SIGGWTLSDP FFFMGDKVKR DRFVGSVKEF

                                                               350
LQTWKFFDGV DIDWEFPGGK GANPNLGSPQ DGETYVLLMK ELRAMLDQLS

                                                               400
AGTGRKYELT SAISAGKDKI DKVAYNVAQN SMDHIFLMSY DFYGAFDLKN

                                                               450
LGHQTALQAR PGSRHRLHHG ERRECAAGQG VKPGKIVVGT AMYGRGWTGV

                                                               500
NGYQNNIPFT GTHRAVKGTW ENGIVDYRQI ASQFMSGEWQ YTYDATAEAP

                                                               550
YVFKPSTGDL ITFDDARSVQ AKGKYVLDKQ LGGLFSWEID ADNGDILNSM

NASLGNSAGV Q
```

Figure 11. Predicted amino acid sequence deduced from the bacterial chitinase gene chiA from *Serratia marcescens*[57].

52,000 mature protein. A chitobiase was also cloned[62]. The molecular weight of this periplasmic protein was about 95,000.

A *Vibrio vulnicius* chitinase was cloned[159]. In *Cellvibrio mixtus*, Wynne et al.[161] cloned a gene cluster coding for cellulase, chitinase, amylase and pectinase. In *Bacillus circulans*[156], a chitinase gene was cloned which showed an N-terminal homology with the ChiA gene of *Serratia marcescens*. This region was immediately followed by tandemly repeated 95-amino acid segments which were 70% homologous to each other. These repeated segments were homologous to the type III homology units of fibronectin, a multifunctional extracellular matrix and plasma protein of higher eukaryotes[156].

Insect chitinases

Chitinase distribution and characteristics

In insects, chitinolytic activities were found principally in the integument, moulting fluid, haemolymph and alimentary canal. The molecular weights of chitinases and β-N-acetylglucosaminidases from invertebrates usually range between 40,000 and 75,000 and between 60,000 and 150,000 respectively. Several insect chitinases are glycoproteins[38, 75]. In *Bombyx mori*, chitinase is synthesized as an inactive precursor which is activated by limited proteolysis[63]. β-N-acetylglucosaminidases can be dimeric. The isoelectric points of chitinases range between 4 and 5 (between 4.8 and 5.9 for β-N-acetylglucosaminidases), the optimum pH between 4.5 and 6.0 and Km values with chitin between 0.2 mg/ml and 5 mg/ml. The characteristics of the main insect chitinases and β-N-acetylglucosaminidases were listed in a review by Kramer and Koga concerning insect chitin[74].

Roles of insect chitinases

Chitinases and β-N-acetylglucosaminidases which are localized in moulting fluid and integument may participate directly in cuticular chitin degradation. The GlcNAc supplied by chitonolysis of the old cuticle is reabsorbed with the moulting fluid and recycled for synthesis of the new cuticle. In *Manduca sexta*[34, 35], chitin degradation was carried out by a binary mixture of chitinase and β-N-acetylglucosaminidase. The enzymes showed a synergistic effect that was as much as six times faster than the sum of the individual enzyme rates. The greatest synergism occurred at a ratio of enzymes (6:1) typically found in moulting fluid. The catalysis was dominated by the endo-splitting chitinase which initiated hydrolysis. Intermediate oligosaccharides were converted to GlcNAc by the exo-splitting β-N-acetylglucosaminidase. However, exo-chitinases were also described in insects[14] and could play a role in cuticle degradation. Chitinase activities in insect moulting fluid seem to be regulated by the moulting ecdysteroid hormones[36, 138], particularly during larval-pupal transformation[63, 125].

Chitinases could also play a trophic role in mycophagous insects[37]. Koga et al.[65] compared β-N-acetylglucosaminidases from integument of *Bombyx mori* with the same enzyme from pupal alimentary canal. The physiological role of the β-N-acetylglucosaminidases which are present in haemolymph is probably related to the hydrolysis of GlcNAc of glycoproteins or glycolipids.

Allosamidin and insect chitinases

Allosamidin production and structure. Allosamidin was purified from the mycelial extract of *Streptomyces* sp 1713[125, 126]. Methylallosamidin was purified from the mycelium of an unidentified actinomycete. The structure of these molecules was determined by nuclear magnetic resonance[124]. Allosamidins consist of two D-allosamin and one aminocyclitol derivative named allosamizoline. Absolute configuration of allosamizoline was determined by circular dichroism (fig. 12 and Sakuda et al.[127]).

Allosamidin effects on chitinase. Allosamidin strongly inhibits insect chitinases in vitro[42, 125, 135]. In vivo, allosamidin had insecticidal action due to its ecdysis-preventing activity[125]. Methylallosamidin showed slightly less insecticidal activity[124]. The inhibition was competitive and Ki values were 0.07 mM and 0.11 mM for chitinases from the alimentary canal and larval integument of *Bombyx mori*, respectively. Allosamidin did not inhibit yam chitinase, lysozymes of hen egg white or human urine or *Bombyx mori* β-N-acetylglucosaminidase[64].

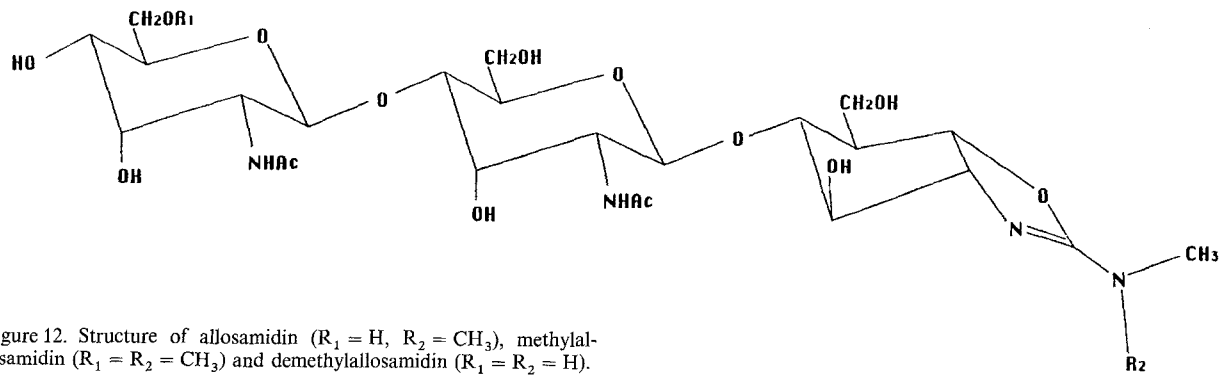

Figure 12. Structure of allosamidin ($R_1 = H$, $R_2 = CH_3$), methylallosamidin ($R_1 = R_2 = CH_3$) and demethylallosamidin ($R_1 = R_2 = H$).

However, it inhibited *Candida albicans* chitinase (yeast) competitively (Ki = 0.23 mM and 0.29 mM for supernatant and microsomal chitinase, respectively)[29,30], and demethylallosamidin (after demethylation of the allosamidin aminocyclitol) inhibited *Saccharomyces cerevisiae* chitinase[128]. Allosamidin could also enhance chitin biosynthesis[109].

Marine invertebrate chitinases

Chitinases have been characterized in marine invertebrates, molluscs and crustaceans, such as oysters[160], prawns[72], lobsters[86], and krills[137]. In *Euphasia superba* and *Meganyctiphanes norvegica*[137], a chitinase and a β-N-acetylglucosaminidase have been demonstrated. Both enzymes of both species had broad pH optima around 5.0 and temperature optima between 40 and 50 °C; enzyme activities in the lower temperature range were still high, suggesting a functional adaptation to low temperature in seawater.

Fish chitinases

Chitinases have also been described in the digestive tract of some fishes feeding on invertebrates, such as antarctic fishes feeding on krill[110] or in Dover sole[25]. A chitinase was purified from the stomach of red sea bream[69]. Its molecular weight was about 46,000, its pI 8.3, its optimal temperature and pH were 50 °C and 5.5, respectively. The activity was strongly inhibited by Hg^{2+}, Fe^{2+} and Sn^{2+}. The final hydrolysis products of chitin were N-acetylglucosamine and chitobiose. The chitinase was synthesized within the egg[68] and could be induced during the larval period by the consumption of exogenous foods. In Japanese eel[71], chitinase was found in the stomach. However, the digestive tract of eel contained also chitin-decomposing bacteria[70].

A protozoan chitinase

Malaria parasites (ookinetes) appeared to digest the peritrophic membrane in the mosquito midgut during penetration[50]. A chitinase from the parasite was detected, which might be involved in the digestion of the peritrophic membrane.

Conclusion

In addition to the interesting properties of chitinases themselves, we should also point out the importance of their substrates: chitin and chitosan. The uses of these abundant, renewable and polyvalent substances are numerous.

Chitosan occurs in the composition of threads, fibers, films and gels. In the agricultural industry, seeds can be protected from fungi using a capsule containing chitosan derivatives. In the food industry, chitosan is used in the preparation of fruit juices or soluble coffee. The cosmetic industry makes shampoos, gels, creams and even sponges with chitosan. In the pharmaceutical industry and in medicine, chitosan occurs in the making of contact lenses, of drug excipient and of dressings for burns. In addition, some shorter chitin oligosaccharides could have an inhibitory effect on the development of cancerous tumors.

* Corresponding author.

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Research Articles

Effects of vitamin B12 on plasma melatonin rhythm in humans: increased light sensitivity phase-advances the circadian clock?

K. Honma, M. Kohsaka[a], N. Fukuda[b], N. Morita[a] and S. Honma

Department of Physiology , and [a]Department of Psychiatry, Hokkaido University School of Medicine, and [b]College of Medical Technology, Hokkaido University, Sapporo 060 (Japan)
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Abstract. Vitamin B12 (methylcobalamine) was administered orally (3 mg/day) to 9 healthy subjects for 4 weeks. Nocturnal melatonin levels after exposure to bright light (ca. 2500 lx) were determined, as well as the levels of plasma melatonin over 24 h. The timing of sleep was also recorded. Vitamin B12 was given blind to the subjects and crossed over with placebo. We found that the 24-h melatonin rhythm was significantly phase-advanced (1.1 h) in the vitamin B12 trial as compared with that in the placebo trial. In addition, the 24-h mean of plasma melatonin level was much lower in the vitamin B12 trial than with the placebo. Furthermore, the nocturnal melatonin levels during bright light exposure were significantly lower in the vitamin B12 trial than with the placebo. On the other hand, vitamin B12 did not affect the timing of sleep. These findings raise the possibility that vitamin B12 phase-advances the human circadian rhythm by increasing the light sensitivity of the circadian clock.
Key words. Circadian rhythm; melatonin; bright light; vitamin B12; entrainment.

Vitamin B12 has been reported to normalize the entrainment of circadian rhythms in delayed sleep phase insomnia (DSPI) and in non-24-h sleep-wake cycle [1–3], where disturbance of the entrainment of the circadian clock is assumed to be involved. DSPI is a sleep disorder in which sleep occurs regularly but is extremely delayed, and is thought to be a state where the circadian clock entrains at the border of the entrainment range [4]. Non-24-h sleep-wake cycle is a sleep disorder in which the timing of sleep is increasingly delayed, and is thought to be a state where the circadian clock is free-running in the presence of zeitgebers [5].

The effect of vitamin B12 can be explained theoretically by three mechanisms. First, vitamin B12 changes the free-running period of the circadian clock and thereby facilitates the entrainment to zeitgebers. Second, vitamin B12 increases the sensitivity of the circadian clock to photic or social zeitgebers. Third, vitamin B12 changes the quality of sleep or wakefulness, improving the internal organization of the circadian system. In the present study, we wanted to know whether vitamin B12 affects human circadian rhythms in normal subjects, and whether vitamin B12 increases the light sensitivity of plasma melatonin, in order to gain an insight into the mechanism of vitamin B12 action.

Materials and methods

Subjects were 10 male students (20–28 years old) who had been living in Sapporo City for at least 3 years. Before the start of the experiment, medical examinations