World Journal of Microbiology and Biotechnology, 9, 468–475

Review

Chitinolytic enzymes: their contribution to basic and applied research

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After cellulose, chitin is the second most abundant renewable resource available in nature. Marine invertebrates and fungal biomass are the two main sources of chitinous waste, which is commercially exploited. The enzymes involved in chitin degradation have been particularly well studied. Such enzymes have applications in ultrastructural studies, in the preparation of chitooligosaccharides which show anti-tumour activity, as biocontrol agents and in single-cell protein production. Here, the contribution chitin enzymology can make to basic and applied research is discussed.

Key words: Biocontrol agents, chitinase/chitosanase, chitinous waste, single-cell protein, ultrastructural studies.

Chitin is the most abundant renewable natural resource after cellulose (Deshpande 1986; Gooday 1991; Nicol 1991). It is widely distributed in nature, particularly in marine invertebrates, insects, fungi and algae (Muzzarelli 1977). It is estimated that the worldwide annual recovery of chitin from the processing of marine invertebrates, for example, is 37,300 tonnes (Nicol 1991). Due to chitin's important biological role as a structural component, its synthesis and degradation have been the subject of extensive research. Most of the studies have focussed on fungal morphogenesis, bio-insecticides and bio-fungicides, and the commercial utilization of chitinous wastes for single-cell protein (SCP) production (Zikakis 1989).

Chitin is a polymer of unbranched chains of β-1,4-linked 2-acetamido-2-deoxy-D-glucose (GlcNAc; *N*-acetylglucosamine) and is related to cellulose, with the C-2 hydroxyl groups replaced by acetamido residues. The partially deacetylated derivative of chitin is chitosan (the corresponding glucosamine polymer). Chitin synthesis is known to occur in many fungi, of various taxonomic groups (Cabib 1987). Chitin synthase (CS; EC 2.4.1.16) is a key enzyme, catalysing the final step in which the GlcNAc from UDP-GlcNAc is transferred to a growing chain. The biochemical properties of this enzyme appear to be rather similar whatever its fungal source. CS activity can be measured by a radiometric assay in which the incorporation of labelled GlcNAc from a reaction mixture (containing UDP-^{14}C-GlcNAc, Mg^{2+}/Co^{2+} and unlabelled GlcNAc) into chitin is monitored (Kang *et al.* 1984).

The enzymatic degradation of chitin to free GlcNAc is performed by a chitinolytic system, which has been found in microorganisms, plants and animals (Flach *et al.* 1992). Some of the potent microbial chitinase producers are *Serratia marcescens* (Horwitz *et al.* 1984), *Serratia liquefaciens* (Joshi *et al.* 1988), *Vibrio vulnificus* (Wortman *et al.* 1986), *Streptomyces* spp. (Ueno *et al.* 1990), *Trichoderma harzianum* (Ridout *et al.* 1988) and *Myrothecium verrucaria* (Vyas & Deshpande 1989). The chitinolytic enzymes are generally induced as a multi-enzyme complex and are traditionally divided into two main classes: (1) endo-chitinases (EC 3.2.1.14) and (2) *N*-acetyl glucosaminidases (sometimes termed 'chitobiase'; EC 3.2.1.30) or *N*-acetyl hexosaminidases (EC 3.2.1.52). The existence of a third class of enzyme, exo-chitinases, has also been suggested (Robbins *et al.* 1988). Endo-chitinases randomly hydrolyse GlcNAc polymers, eventually giving diacetylchitobiose as the major product together with some triacetylchitotriose. Though the *N*-acetyl glucosaminidases act preferentially on a dimer, diacetylchitobiose, some enzymes cleave GlcNAc units from the non-reducing ends of chitin chains (Gooday 1990). The exo-chitinases also catalyse processive release of diacetylchitobiose (or GlcNAc?) units from the non-reducing ends of chitin chains. The classification of exo- and endo-enzyme depends mainly on the nature of the substrate. For example, the *Streptomyces* chitinase complex degrades pure crystalline β-chitin of

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NCL Communication No. 5691.

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diatom spines only from the non-reducing ends to yield diacetylchitobiose, whereas colloidal chitin is degraded to a mixture of oligomers and diacetylchitobiose (Lindsay & Gooday 1985).

Like cellulose, chitin possesses considerable crystalline structure and usually forms part of a very complex system. Insect exoskeletons, for instance, are largely composed of chitin-protein complexes, and the chitin of fungal cell walls is closely associated with proteins and glucans. Therefore, although all chitinases act on β-1,4- glycosidic bonds, the variation in the micro-environment of these bonds in natural substrates means that most chitinolytic organisms produce a family of different chitinases, each with different substrate and product specificities.

Several microorganisms produce enzymes which degrade chitosan, a major cell wall constituent of Zygomycetes (Alfonso *et al.* 1991; Ohtakara 1988b; Uchida & Ohtakara 1988; Pelletier & Sygusch 1990). Some plants also produce extracellular chitosanases when stressed (Grenier *et al.* 1991; El Ouakfaoui & Asselin 1992). Chitosanases (EC 3.2.1.99) are a class of endo-acting enzymes which hydrolyse chitosan to a mixture of glucosamine oligomers in which the degree of polymerization (DP) is between two and eight (Ohtakara 1988b; Uchida & Ohtakara 1988). Chitosanases are distinguished from chitinases by their substrate specificity. Most of the plant chitosanases have molecular weights in the range 10,000 to 23,000 (El Ouakfaoui & Asselin 1992), whereas microbial chitosanases have molecular weights from 20,000 to 43,000 (Ohtakara 1988b; Uchida & Ohtakara 1988; Pelletier & Sygusch 1990).

The hydrolysis of chitin, the natural substrate of chitinases, is inconvenient to monitor because of its insolubility and the variable degree of acetylation retained during its isolation. Moreover, this insoluble substrate probably changes, during enzymatic hydrolysis, with respect to its deacetylation and the number of susceptible regions for the enzymes to act. For this reason, a wide variety of substrates, ranging from locust cuticle to chemically synthesized *p*-nitrophenyl derivatives, has been used for the measurement of endochitinase and *N*-acetyl glucosaminidase activity (Aribisala & Gooday 1978; Boller & Mauch 1988; Cabib 1988; Ohtakara 1988a; Hood 1991). Deshpande *et al.* (1984) used *p*-nitrophenyl-β-D-cellobioside as a selective substrate for the estimation of cellobiohydrolase (an enzyme which preferentially removes cellobiose from the non-reducing end of cellulose chains) activity. As chitin is regarded as a modified cellulose, a similar type of substrate, i.e. *p*-nitrophenyl-β-D-chitobioside, can be used for the specific determination of exochitinases.

Binding Mode of Chitinases

The chito-oligosaccharides and their reduced or methylated derivatives have been used to elucidate the mode of binding of chitinases and their action pattern. For instance, from the analysis of the hydrolysis products of the oligosaccharides, it has been suggested that the chitinase from *Pycnoporus cinnabarinus* had an exo-type action, predominantly hydrolysing the second β-*N*-acetylglucosaminide linkage from the non-reducing end (Ohtakara & Mitsutomi 1988).

Most of the carbohydrases have similar protein-saccharide interactions although they show widely diverse three-dimensional structures and binding site topologies. The structure and functioning of lysozyme's active site are well known. According to Hara *et al.* (1989) a chitinase from *Streptomyces erythraeus* and hen egg-white lysozyme have a similar binding mode to *N,N'*-diacetylchitobiose. Using various partially O-methylated disaccharides to elucidate the substrate-binding mode of the chitinase, they found that the hydroxyl groups in the C6 position of the reducing-end sugar and in the C3 and C4 positions of the non-reducing-end sugar are not important for the enzymatic action, whereas the acetamide group in the C2 position and the hydroxyl groups in the C3 position of the reducing-end sugar and in the C6 position of the non-reducing-end sugar are oriented towards the enzyme molecule.

Genetic Improvement in Chitinase Production

In recent years, renewed interest in the degradation of chitin to exploit its commercial potential and research on the involvement of chitinases in the chemical defence of plants has led to genetic improvement programmes for chitinase production. *S. marcescens* is one of the most extensively studied organisms for genetic improvement. This species produces high levels of chitinases which can act on milled and colloidal chitin and affinity purification procedures for these chitinases are known. Genes from this species which encode chitinases and their associated regulatory signals can be expressed in *Escherichia coli* and many molecular cloning techniques possible with *E. coli* could be adapted for use with *S. marcescens*. (Horwitz *et al.* 1984; Fuchs *et al.* 1986; Jones *et al.* 1986; Sundheim *et al.* 1988; Kless *et al.* 1989; Shapira *et al.* 1989).

Initially, genetic improvement of chitinase production was attempted by mutagenesis of *S. marcescens* QMB 1466 (Reid & Ogrydziak 1981). A mutant, IMR-1E1, was isolated after UV and subsequently EMS treatment and produced two to three times higher levels of all chitinolytic activities than the parental strain. The proportional increase in all activities suggested the possibility of coordinate control of unlinked genes, with IMR-1E1 being a regulatory mutation. However, the high rate of reversion of IMR-1E1 to forms with decreased chitinase activities indicated tandem gene duplication of the chitinase genes.

Fungal protoplast fusion has now been established as a means for genetic manipulation and strain improvement

(Pe'er & Chet 1990). Protoplast fusion has been employed to obtain improved biocontrol strains, including *Trichoderma* sp. (Pe'er & Chet 1990).

Molecular cloning to achieve overproduction, change in the induction pattern or change in the localization (periplasmic/extracellular) of chitinases has been reported in many systems (Table 1). Fink *et al.* (1991) reported cloning a chitosanase gene. Most chitinase genes cloned so far have

Table 1. Summary description of some chitinase genes.

Source	Host	Genes cloned	Remarks	Reference
Serratia marcescens	*Escherichia coli*	*N*-acetylglucosaminidase	Constitutive and periplasmic expression of the gene	Kless *et al.* 1989
	Pseudomonas fluorescens	Two chitinases	Fungicidal activity against *Fusarium oxysporum* has been tested	Sundheim *et al.* 1988
	E. coli & *P. fluorescens*	Four chitinases	High level of expression in *E. coli* compared with *P. fluorescens*	Jones *et al.* 1986
Serratia liquefaciens	*E. coli*	Two chitinases (*Chi* A and *Chi* B), *N*-Acetylglucosaminidase (*Chi* C)	Repressor gene *Chi* D for chitinase has been identified	Joshi *et al.* 1988
Vibrio harveyi	*E. coli*	Chitinase, *N*-Acetylglucosaminidase	Chitinase and *N*-acetylglucosaminidase are not under coordinate control. The *N*-acetylglucosaminidase gene is expressed constitutively and in outer membrane of *E. coli.* Lipoprotein nature of pre-*N*-acetylglucosaminidase has been suggested	Jannatipour *et al.* 1987
Vibrio vulnificus	*E. coli*	Chitinase, *N*-Acetylglucosaminidase	Contains natural promotor signal. Location in *E. coli* is changed from extracellular to a cell bound nature	Wortman *et al.* 1986
Streptomyces lividans	*E. coli* *S. lividans* *S. coelicolor*	Four chitinases	Cloned genes are induced in the presence of chitin and repressed by glucose. Contains natural promotor signal	Miyashita *et al.* 1991
Streptomyces plicatus	*E. coli* *S. lividans*	Two chitinases, *N*-Acetylglucosaminidase	Cloned genes are induced in the presence of chitin and repressed by glucose. Gene duplication possibility is suggested	Robbins *et al.* 1988
Kitasatosporia	*S. lividans*	Chitosanase	High extracellular chitosanase activity and no detectable chitinase activity compared with the source is expressed	Fink *et al.* 1991
Saccharomyces cerevisiae		Chitinase	Amino acid analysis suggests four domains–signal sequence, catalytic domain, serine/threonine rich region and carboxyterminal with high binding affinity for chitin	Kuranda & Robbins 1991
Barley		Three chitinases	Chitinases are encoded by a small family of three to eight genes	Leah *et al.* 1991
Rice		Chitinase	Class I basic chitinase, shares a high sequence homology with barley chitinase	Anuratha *et al.* 1992
Maize		Chitinase (*Chit* A & *Chit* B)	Class I basic chitinases play a significant role in protecting seeds during germination against phytopathogen attack	Huynh *et al.* 1992
Bean	*E. coli*	Chitinase	Chitinase production at the level of gene transcription is induced by ethylene and elicitors. Differential regulation is suggested	Broglie *et al.* 1986
Tobacco		Two chitinases	97% homology was observed	Fukuda *et al.* 1991
Potato		Chitinase	Gene is homologous with other Solanaceae and Leguminosae plant chitinase genes	Gaynor 1988
Populus		Two chitinases (*win* 6 and 8, *chi*X)	Class I basic chitinase; clustering of *chi*X and *win* 6 genes is suggested; *win* 6 has four domains including hinge region, while *win* 8 lacks hinge region	Davis *et al.* 1991

been isolated by expression in *E. coli*, *Pseudomonas* sp. or their natural hosts.

The cloning of the set of genes (*chi*A, *chi*B and *chi*C) that determine the expression of the chitinase complex and the regulatory genes (*chi*D and *chi*E) of *S. liquefaciens* has been reported (Joshi *et al.* 1988). The chitinase (*Cht63*)-encoding gene from *Streptomyces plicatus* has been sequenced and compared with the sequences of *S. marcescens ChtA* and *Bacillus circulans ChtA1* genes reported earlier (Robbins *et al.* 1992); the three genes were strongly homologous. In both the *Streptomyces* and *Bacillus* enzymes the putative binding and catalytic domains were adjacent to each other; Kuranda & Robbins (1991) observed a glycosylated 'hinge' between these two regions of the chitinase from *Saccharomyces cerevisiae*.

Several chitinase genes have been cloned from many plants (Broglie *et al.* 1986; Shinshi *et al.* 1990; Flach *et al.* 1992; Huynh *et al.* 1992) and expressed in transgenic plants (Neuhaus *et al.* 1991). Based on the structural analysis of the plant chitinase genes, three classes of the chitinases are suggested (Shinshi *et al.* 1990). Class I enzymes are basic proteins with an amino terminal comprising of a cysteine-rich domain and a highly conserved catalytic domain, located primarily in the vacuole. Class II enzymes are acidic proteins devoid of a cysteine-rich domain, but having a catalytic domain homologous to that of class I chitinases. It has also been reported that most of the basic chitinases have tyrosine as an essential residue at or near the catalytic site (Verburg *et al.* 1992). Class III chitinases are acidic extracellular proteins with no homology to either class I or II. Huynh *et al.* (1992) reported two chitinases from maize seeds (*chit*A and *chit*B) belonging to class I; these play a role in protecting the seeds against fungal attack during germination.

Applications of Chitinolytic Enzymes

Cytochemical Localization of Chitin/chitosan using Chitinase-Chitosanase-gold Complexes

In most fungi, polysaccharides represent >80% of the dry matter of cell walls and contribute to morphology, mediation of growth, cell attachment and host wall penetration (Benhamou 1988, 1989). Chitin and chitosan are the most ubiquitous polymers of fungal cell walls and although biochemical analyses can provide precise information about their structure, cytochemical localization studies can reveal the functional specializations of these polymers.

The use of lectins, with specific binding affinities for monosaccharides, in conjunction with colloidal gold has permitted the localization of sugar residues in thin sections of plants and fungi (Benhamou 1988). Wheat germ agglutinin-gold complex and chitinase-gold complex have been used as probes for the detection of GlcNAc residues in the secondary cell walls of plants and in pathogenic fungi (Benhamou 1988, 1989; Benhamou & Asselin 1989). Grenier *et al.* (1991) report the tagging of a barley chitosanase with colloidal gold particles for the localization of chitosan in spore and hyphal cell walls of fungi. Using this technique, these authors demonstrated the presence of chitosan in the cell walls of *Ophiostoma ulmi* and *Aspergillus niger*.

Fungal Protoplast Technology

Isolation of fungal protoplasts has gained considerable importance over the past decade, in view of its various biotechnological applications. Protoplasts are used for studying cell wall synthesis, enzyme secretion, steroid transformation and mutagenesis (Kelkar *et al.* 1990). Since chitin is the major structural component in the cell walls of most fungi, chitinolytic enzymes play a significant role in protoplast isolation (Peberdy 1985; Koga *et al.* 1988; Vyas & Deshpande 1989). Hamlyn *et al.* (1981) evaluated various commercial mycolytic preparations for protoplast isolation and found that high chitinase levels permit effective mycelia degradation. Yanagi & Takebe (1984), who used various enzyme preparations, singly or in combination, to isolate protoplasts from *Coprinus macrorhizus* and other basidiomycetes, reported the notable contribution of chitinase activity to efficient protoplast release. Protoplast isolation procedures are often slow and extensive mycelial digestion can lead to a marked heterogeneity of the protoplast preparation. Use of mycolytic enzyme mixtures with high chitinase activity, which cause rapid release of large numbers of genetically homogenous protoplasts, is one of the main requirements of today's protoplast technology (Kawasumi *et al.* 1987).

Preparation of Chito-oligosaccharides

There is a growing appreciation of the potential of biologically active oligosaccharides. For example, chitohexaose and chitoheptaose show anti-tumour activity and are efficient elicitors of chitinase activity in melon plants. Due to hydrolytic *N*-deacetylation, conventional partial acid hydrolysis of chitin gives only low yields of the desired pentamers, hexamers and heptamers. *Bacillus* sp. chitosanase has been used for chitosan oligosaccharide production and gave high yields (>60%) of oligomers (dimers to pentamers) (Izume & Ohtakara 1987). Murao *et al.* (1992) reported a novel chitinase from *Vibrio alginolyticus* which yielded chitotriose and chitopentaose from colloidal chitin.

Alternatively, an efficient enzymic synthesis of oligosaccharides has been reported in the literature. Many of the carbohydrases exhibit a transglycosylation reaction, with the formation of new glycosidic linkages. Nanjo *et al.* (1989) described the transglycosylation reaction of *Nocardia orientalis* chitinases. When the tetramer or pentamer were incubated with the enzyme, accumulation of the hexamer or

heptamer, respectively, was observed. However, no chain elongation was obtained with the hexamer as the initial substrate, which may be due to its inability to function as an acceptor. A chitinase from *Trichoderma reesei* also showed efficient transglycosylation reaction with the tetramer, giving the hexamer and dimer as the major products (Usui *et al.* 1990). Usui *et al.* (1990) also observed chain elongation from dimer to hexamer and heptamer, using lysozyme catalysis in the presence of 30% ammonium sulphate in a buffer medium.

The *N*-acetylglucosaminidase purified from *N. orientalis* also showed transglycosylation activity (Nanjo *et al.* 1990); the β-1,6-linked disaccharide of GlcNAc and tri-*N*-acetylchitotriose were synthesized during the hydrolysis of di-*N*-acetyl chitobiose.

Chitinases as Biocontrol Agents

Chitin is an essential structural component of the fungal and insect pathogens of vascular plants. As it is absent from the vascular plants themselves, chitin could be used as a target molecule for fungicidal and insecticidal agents. In this regard, the role of chitinases has been studied extensively (Tanaka *et al.* 1970; Barrows-Broaddus & Kerr, 1981; Shapiro *et al.* 1989).

The chitinases occur in different organisms for many reasons: in fungi and insects, for either cell wall morphogenesis or exoskeleton development; and in bacteria, for nutrition (Roberts & Selitrennikoff 1988). In plants, microbial infections, other injuries or treatment with ethylene or salicylic acid, induce chitinases (Mauch & Staehelin 1989; Koga *et al.* 1992). The plant and bacterial chitinases differ markedly in anti-fungal activity; this difference may be attributed to differences in their substrate specificities and/or modes of action (Schlumbaum *et al.* 1986; Roberts & Selitrennikoff 1988). The products of plant chitinase action on fungal cell walls, such as partial mycelial hydrolysate containing chito-oligosaccharides, stimulate the biosynthesis of phenolics and lignification in plant cells (Verburg & Huynh 1991). Several microbial chitinases have been used as anti-fungal agents, e.g. *S. marcescens* chitinase, with β-glucanase, propan-2-ol and polyoxyethylene lauryl ether, has been sprayed on a rice field to control rice-blight caused by *Pyricularia oryzae* (Tanaka *et al.* 1970). It has also been reported that *S. marcescens* is an effective biocontrol agent, of *Sclerotium rolfsii* infecting beans and of *Rhizoctonia solani* infecting cotton, under greenhouse conditions (Ordentlich *et al.* 1988; Shapiro *et al.* 1989). Similarly, a chitinase-producing *Arthrobacter* sp. inhibited the growth of *Fusarium moniliforme* var. *subglutinans*, the causal agent of pine pitch canker (Barrows-Broaddus & Kerr 1981).

Trichoderma harzianum is a potential biocontrol agent against a wide variety of plant pathogens encountered in commercial agriculture. The mycoparasitism of pathogenic fungi involves volatile/non-volatile antibiotics and hydrolytic enzymes such as glucanases, proteinases and chitinases (Ridout *et al.* 1988). Protoplast fusion has been reported to produce improved *T. harzianum* strains for direct mycoparasitism against *R. solani*, *S. rolfsii* and *Phythium ultimum* (Pe'er & Chet 1990).

Entomopathogenic fungi (e.g. *Beauveria bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii*) are parasites of various pests, including potato beetle, sugarcane frog hopper and aphids. Entry of the parasite through the insect cuticle is a combination of mechanical pressure and enzymatic digestion. Chitinases and proteases play a role in the cuticle degradation (St. Leger *et al.* 1986). Fungal formulations containing mycelial fragments and conidia are perceived as safe alternatives to chemical pesticides (Thakur *et al.* 1991).

Smirnoff (1975) reported the contribution of chitinase in the control of spruce budworm infestation of balsam fir trees; the toxin from *Bacillus thuringiensis* arrested the feeding of larvae more rapidly and with higher mortality rate and lesser defoliation when mixed with chitinase than when used alone.

Chitinases in Single-Cell Protein Production

The enzymatic conversion of waste chitin, produced by the shellfish processing industry, to yeast single-cell protein (SCP), has been investigated (Revah-Moiseev & Carroad 1981; Deshpande 1986; Zikakis 1989; Vyas & Deshpande 1991). The SCP can be used as an animal and aquaculture feed supplement. Revah-Moiseev & Carroad (1981) used the *S. marcescens* chitinase system to hydrolyse the chitin and *Pichia kudriavezevii* to yield the SCP (with 45% protein and 8 to 11% nucleic acids). Vyas & Deshpande (1991) showed that the *M. verrucaria* chitinase complex and *S. cerevisiae* can also be used for SCP production from chitinous waste. The total protein and nucleic acid contents of their SCP were 61% and 3.1%, respectively.

Future Prospects

Chitinases are widely distributed in living organisms and contribute significantly to basic and applied research. Bacterial chitinases, for instance, are involved in chitin mineralization (Herwig *et al.* 1988) and fungal chitinases play major roles in protoplast technology and as biocontrol agents (Gooday 1990). Although plant chitinases act as natural defensive agents against fungal infections, we need to know more about plant–microbe interactions, the genetics of microbial populations and the factors involved in the control of pathogens before chitinases can be used in efficient biocontrol (Gabriel & Cook 1990). Currently, genetic improvement studies are being carried out on a number of bacterial, fungal and plant chitinases. This information will

give us new insights into the kinetics of these enzymes and will help us explore a wide range of novel applications, such as the use of chitinases against clinically-important fungi (Gooday 1990). However, any immunological problems should be considered before developing chitinase as a therapeutic agent.

Chitin and chitosan and their respective monomers have significant potential applications in medicine. However, there are very few reports on the commercial production of GlcNAc and glucosamine by enzymatic hydrolysis of their respective polymers, since the progress of the reaction depends on the synergistic and consecutive action of various chitinolytic enzymes present in the complex. To make the process commercially viable, Sakai *et al.* (1991) immobilized *N. orientalis* chitinase complex on tannin-chitosan for the continuous production of high yields of GlcNAc (>90% over 130 days). The presence of dimer and trimer in the final product may be attributed to the end product inhibition of *N*-acetyl hexosaminidase activity of the bound enzyme. Use of chitinase complex with high *N*-acetyl glucosaminidase activity and improved immobilization procedures could give an economically viable process for the production of GlcNAc free of contamination.

Acknowledgements

The authors are thankful to Dr V. Shankar for helpful discussions. SS is grateful to the Director, NCL, Pune, for kind permission to work as a Guest Worker.

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(Received in revised form 28 February 1993; accepted 8 March 1993)