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Review

Cell wall-associated enzymes in fungi

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

This review compiles and discusses previous reports on the identity of wall-associated enzymes (WAEs) in fungi and addresses critically the widely different terminologies used in the literature to specify the type of bonding of WAEs to other entities of the cell wall compartment, the extracellular matrix (ECM). A facile and rapid fractionation protocol for catalytically active WAEs is presented, which uses crude cell walls as the experimental material, a variety of test enzymes (including representatives of polysaccharide synthases and hydrolases, phosphatases, γ-glutamyltransferases, pyridine-nucleotide dehydrogenases and phenol-oxidising enzymes) and a combination of simple hydrophilic and hydrophobic extractants. The protocol provides four fully operationally defined classes of WAEs, with constituent members of each class displaying the same basic type of physicochemical interaction with binding partners in situ. The routine application of the protocol to different species and cell types could yield easily accessible data useful for building-up a general objective information retrieval system of WAEs, suitable as an heuristic basis both for the unravelling of the role and for the biotechnological potentialities of WAEs. A detailed account is given of the function played in the ECM by WAEs in the metabolism of chitin (chitin synthase, chitinase and β-*N*-acetylhexosaminidase) and of phenols (tyrosinase).

Keywords: *Agaricus bisporus*; *Mucor rouxii*; Fungi; Plants; Wall; Enzymes; Digitonin; Laccase; Chitin; Tyrosinase

Contents

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1. Introduction

According to a pioneer of research on the present topic (Fleet, 1991), the cell wall is considered as that complex of macromolecules which envelopes the cell external to the plasma membrane and, thus, includes the periplasmic space (Arnold, 1991). By analogy to the situation in plants (Kohorn, 2000), the wall compartment of fungi may also be referred to as the extracellular matrix (ECM), which embodies the plasma membrane–wall interface (for examples, see Carpita et al., 1996, 2001; Anderson et al., 2001). This broad definition has been adopted here.

1.1. Current views of wall-associated enzymes (WAEs)

That fungi harbour functionally relevant enzymes in muro, synthetic as well as hydrolytic ones, was postulated very early in the era of fungal cell wall biochemistry (Burnett, 1979). Previous studies on fungal walls had been directed almost exclusively to the chemical and ultrastructural analysis of the extracellular compartment of these organisms and resulted in the elaboration of various wall fractionation procedures (Fleet, 1991), of which the protocol of Hunsley and Burnett (1970) is the most generally applicable one. Progress in the field of WAEs has been sluggish, however, despite its prime importance for understanding fungal morphogenesis and for biotechnological applications (see Peberdy, 1994; Gooday, 1995).

The main reason for the scarcity of data on WAEs in fungi might be the long-lived notion that enzymes detected in the wall fraction are not genuinely located there, but either simply proteins en route for secretion into the milieu, or artefacts of cell disruption (lit. cit's in Ruiz-Herrera, 1992). This assumption has been nurtured by the observation that some chemical and biochemical tests with cell walls for the presence of entities presumed to be exclusively protoplastic (Taylor and Cameron, 1973) are quite regularly positive. Thus, the problem of the occurrence of WAEs in these organisms has been tackled to some extent only during the last decade and the situation has been addressed in a relatively small number of species, the selection of which has been heavily biased towards medically or biotechnologically important representatives of the Deutero- and Ascomycetes. Further, the approaches followed to demonstrate a mural location of enzymes vary greatly (compare, e.g., Williams et al., 1984, with Lam et al., 1994; Vainstein and Peberdy, 1990; Ruiz-Herrera et al., 1995; Lucio et al., 2000; Zhu et al., 2001), frequently rely on indirect methods (see Molina et al., 2000) and—although often based on a variety of quite sophisticated genomic and microscopic techniques (e.g., green fluorescent protein methodology; Cubitt et al., 1995; Cormack et al., 1997)—sometimes do not even encompass confirmatory direct enzyme activity tests with isolated cell walls. Finally, the terminology used to specify the physicochemical relationships between WAEs and other components of the wall varies con-

siderably. Thus, reliable comparisons of data on fungal WAEs provided by different authors are often difficult and satisfactory generalisations are not possible to date.

1.2. Purpose of this article

To advance research on fungal WAEs, facile and standardised extraction protocols are required that yield the proteins sought as non-denatured, catalytically active entities, upon which more extensive biochemical and genomic analyses can be based. With current methods, contamination of wall fragments by protoplastic entities occurs and is, in fact, intrinsically unavoidable (details in Section 3.1). Nevertheless, with the rapidly increasing knowledge of cellular biochemistry, the detection of so-called intracellular markers in the cell wall can no longer be construed as evidence per se for a largely artefactual consortium of enzymes present in the cell wall fraction. Indeed, nucleic acids (Celerin et al., 1994) as well as some enzymes previously held to be exclusively cytosolic, must now be considered to occur as native extraprotoplastic entities (see references in Chaffin et al., 1998).

In view of the difficulties outlined (Section 1.1) and to make full use of earlier investigations into WAEs and the multiple functions they may play, not only should wall fractionation schemes be generally applicable, but also be suitable for primary surveys of the subcompartmentation of WAEs in muro and assessments of their species-, organ-, tissue- and cell-specific expression, as well as for the systematic search for further WAEs. It is the aim of the present article to: (i) compile and critically discuss previous reports on the identity of fungal WAEs as well as to list their species occurrence; (ii) present a facile and rapid protocol for the fractionation and preliminary assessment of the physicochemical relationships of WAEs with other components of the ECM; and (iii) give an account of the physiological significance of some selected examples of mural proteins for which sufficient knowledge is available to make them promising model systems for future broad-based general investigations into the function and biotechnological potentialities of WAEs (Sections 4 and 5).

2. Identity of wall-associated enzymes (WAEs)

2.1. Bona fide WAEs

Based on direct catalytic activity tests with isolated cell walls (either the crude low-speed sediment of the cell homogenate or subfractions thereof) and excluding inducible exoenzymes presumably involved merely in the transformation of nutrients present in the medium, published records of WAEs in fungi relate to the following biochemical classes-subclasses (Table 1): oxidoreductases acting on diphenols as donors and oxygen as the acceptor, aminoacyltransferases, phosphoric monoester hydrolases (also catalysing transphosphorylation), hexosyltransferases and *O*-glycosyltransferases. The latter two groups encompass the large majority of enzymes reported to date as having an extraprotoplastic location. The present authors' recent findings of some enzymes not reported to exist previously in wall-associated forms (see Section 3.2) have also been included in Table 1.

For some of the WAEs listed in Table 1 as well as for some other entities not included, there is complementary, confirmatory or strongly suggestive evidence for a genuine extraprotoplastic occurrence. Frequently, the evidence presented for wall-association of a particular enzyme is indirect, i.e., inferred mainly from simple histochemical tests or immunocytochemical and genomic analyses, without biochemical studies. Other papers report the presence of a particular WAE in just another species than the one in which it had originally been detected. These additional data refer to tyrosinase (in *Agaricus bisporus*: Rast et al., 1981; Hollenstein, 1997), β-(1–3)-glucanosyltransferase (in *Candida albicans*: Hartland et al., 1991), acid phosphatase (in *Botrytis cinerea, Hebeloma* ssp. and *C. albicans*: Weber and Pitt, 1997; Tibbett et al., 1998; further references in Chaffin et al., 1998), phospho- and lysophospholipases (in *C. albicans*: citations in Chaffin et al., 1998), chitinase (in *Saccharomyces cerevisiae, C. albicans* and *Kluyveromyces* ssp.: Rodriguez-Medina et al., 1998; Iranzo et al., 2002; Bahmed et al., 2002), β-glucosidase (in *C. albicans*, *Aspergillus kawachii* and *Acremonium persicinum*: Ram et al., 1984; Iwashita et al., 1999; Pitson et al., 1999), trehalase (in *Neurospora crassa, C. albicans* and *Mucor rouxii*: Hecker and Sussman, 1973; Ram et al., 1984; Molloy et al., 1995; Lucio et al., 2000), (1–3)-β-glucanase (in *C. albicans* and *A. persicinum*: Ram et al., 1984; Pitson et al., 1999), (1–6)-β-glucanase (in *A. persicinum*: Pitson et al., 1999) and *N*-acetylhexosaminidase (in *C. albicans* and *M. rouxii*: Molloy et al., 1995; Rast et al., 1991).

2.2. Cytosolic enzymes as putative WAEs

Not unexpectedly, reports of some glycolytic enzymes as bona fide WAEs, namely, glyceraldehyde 3-phosphate dehydrogenase (Gil-Navarro et al., 1997; Gozalbo et al., 1998; Delgado et al., 2001), phosphoglycerate kinase (Alloush et al., 1997) and enolase (Angiolella et al., 1996; Eroles et al., 1977; Edwards et al., 1999) are bound to encounter considerable reserve and scepticism (see Chaffin et al., 1998). These, of course, are generally accepted to be cytosolic, non-glycosylated marker enzymes. Other major conceptual impediments to a broad acceptance of the authors' conclusions appear to be the following: (a) the observations are at odds with the firmly entrenched view of the fungal wall as a venue

Table 1
A list of cell wall-associated enzymes (WAEs) in fungi[a,b]

Enzyme	EC no.	Species	Reference
Tyrosinase	1.10.3.1	*Agaricus bisporus*	This article (Fig. 3b)
Laccase	1.10.3.2	*Cryptococcus neoformans*	Zhu et al., 2001
Protein-glutamine γ-glutamyltransferase	2.3.2.13	*Candida albicans*	Ruiz-Herrera et al., 1995
Chitin synthase	2.4.1.16	*A. bisporus*	Hänseler et al., 1983b; this article (Fig. 3d)
		Mucor rouxii	Horsch et al., 1996
β-(1–3)-Glucan synthase	2.4.1.34	*A. bisporus*	This article (Fig. 3c)
β-(1–3)-Glucanosyltrans-ferase[c]	2.4.1.x	*Saccharomyces cerevisiae*	Goldman et al., 1995
		C. albicans	
β-(1–3)-Glucanosyltrans-ferase[c]	2.4.1.y	*Aspergillus fumigatus*	Hartland et al.,1996
β-(1,4)-Xylanase	3.2.1.8	*Cryptococcus albidus*	Notario et al., 1979
Acid phosphatase	3.1.2.2	*S. cerevisiae*	See Arnold, 1991;[d] Fleet, 1991d
Chitinase	3.2.1.14	*S. cerevisiae*	Kuranda and Robbins, 1991
		M. rouxii	Mayer, 1997
		Benjaminiella poitrasii	Ghormade et al., 2000
		A. bisporus	This article (Fig. 3d)
β-Glucosidase	3.2.1.21	*C. albicans*	Notario, 1982
		Coccidioides immitis	Kruse and Cole, 1992; Hung et al., 2001
		Aspergillus kawachii	Iwashita et al., 1998
β-Galactosidase	3.2.1.23	*Aspergillus nidulans*	Torralba et al., 1996
Invertase[d]	3.2.1.26	*Neurospora crassa*	Chang and Trevithik, 1972
		Claviceps purpurea	Dickerson and Baker, 1979
		Puccinia graminis	Williams et al., 1984
		Aspergillus nidulans	Vainstein and Peberdy, 1990
		S. cerevisiae	see Arnold, 1991d; Fleet, 1991d
		Acremonium typhinum	Lam et al., 1994
Trehalase	3.2.1.28	*N. crassa*	Chang and Trevithik, 1972
(1–3)-β-Glucanase	3.2.1.39	*C. albicans*	Notario, 1982
		S. cerevisiae	Klebl and Tanner, 1989; Mrsa et al., 1993
		A. bisporus	This article (Fig. 3c)
β-*N*-Acetylhexosaminid-ase	3.2.1.52	*M. rouxii*	Mayer, 1997; this article (Figs. 1 and 2) Ghormade et al., 2000
		B. poitrasii	
		A. bisporus	This article (Fig. 3d)

[a] Reports are not listed where the wall-associated protein has not been identified by its catalytic activity in native isolated cell walls or in endogenous autolysates thereof (Fleet, 1991). Papers on enzymes presumably only transiently present within the cell wall (i.e., induced secreted enzymes en route to the medium) have been included neither.
[b] Some enzymes for which there is good, although largely indirect evidence for wall-association are referred to in the text.
[c] Mechanistically different entities.
[d] These authors cite numerous earlier works, also on other yeast species.

for only a very few enzymic activities other than hydrolases involved in the re-mobilisation of nutrients for uptake into the protoplastic compartment, apart from being a passage way for exoenzymes (Wessels, 1999); (b) the findings require the externalisation of the nucleotides necessary as co-substrates in some of the reactions concerned (for which there is no room in the traditional picture of transport mechanisms); and (c) the claims made are incompatible with the localisation of proteins destined for the cell surface via the constitutive vesicle bound signal-peptide dependent secretory pathway, which requires glycoconjugation of the transportants. But there are also arguments against the critical points raised: (i) the now rapidly expanding knowledge of the cell surface compartment as a biochemically very versatile organelle, also in other organisms than fungi (for plants, see Carpita et al., 2001), has created such a new level of understanding cell wall enzymology and dynamics that the classical view can simply no longer hold; (ii) the ATP binding cassette (Higgins, 2001)—universally present—contains members that provide for the high affinity export of such nucleotides; (iii) there are also ABC transporters for virtually any type of proteins (whether small or large, hydrophobic or highly charged; glycoconjugated or not; Higgins, 2001); and (iv) the present knowledge of fungal protein export systems encompasses alternatives to both the ATP binding cassette and the classical endoplasmic reticulum–Golgi pathway. Thus, there exist export machineries for the secretion of proteins that are distinct from either of these, inasmuch as they apparently effect the externalisation of non-glycosylated proteins (Luna-Arias et al., 1991) and of proteins lacking a classical secretory signal sequence (Cleves et al., 1996).

2.3. The pursuit of new WAEs

Considering the present picture of the chemical and supramolecular structure of the wall of *S. cerevisiae* (Fleet, 1991; Van der Vaart et al., 1996; Kapteyn et al., 1999; Klis et al., 2002) and drawing on biochemical and ultrastructural dissection data from the walls of two filamentous fungi, *Schizophyllum commune* (Sietsma and Wessels, 1977; Sietsma et al., 1977; van der Valk et al., 1977) and *A. bisporus* (Michalenko et al., 1976; Rast and Hollenstein, 1977; Rast et al., 1981; Hollenstein, 1997), the consortium of fungal WAEs that have been identified biochemically to date (Table 1; Section 2.1.) provides for some of the basic reactions required to: (i) create and "mould" major building blocks of the wall (refer to Klis et al., 2002); (ii) assemble these into a dynamically remodelled giant heteropolysaccharide–protein–lipid composite; and (iii) eventually transform the latter into an intrinsically non-hydrolysable material through oxidative coupling with phenolic residues. Two examples concerning these are dealt with in Section 4. As most of the many mannan cell wall proteins (CWPs) are considered to be catalytically active (Mrsa et al., 1999), one can safely assume that many more WAEs remain to be detected. Some WAEs reach the extra-protoplastic compartment in the form of vesicles (formerly called periplasmic bodies; Arnold, 1991; Notario, 1982; Weber and Pitt, 1997); others, among which glucanosyltransferases (Mouyna et al., 2000; Bernard and Latgé, 2001), possibly are directly released from their glycosyl-phosphatidylinositol (GPI)-anchored state in the plasma membrane by hydrolytic/transglycosylating reactions, whence they become attached to a β-1,6-glucan residue of the β–glucan-chitin complex (Kapteyn et al., 1999; Mrsa et al., 1999; Klis et al., 2002).

Covalent bonding of WAEs in the interface region between the plasma membrane/periplasmic reaction medium and the more densely packed part of the wall compartment might be a reason why they are apparently intrinsically more stable than their respective protoplastic forms (Notario et al., 1979; Dickerson and Baker, 1979; Horsch et al., 1996; Iwashita et al., 1998). The latency displayed by some WAEs, e.g., β-glucanase (Notario, 1982; Nombela et al., 1988), invertase (Williams et al., 1984; Arnold, 1991), chitin synthase (references in Merz et al., 1999b) and chitinolytic enzymes (Rast et al., 1991; Molloy et al., 1995) is probably another factor contributing to their longevity in muro. Good stability is a property that WAEs share with wall-associated proteins (WAPs) in general (Ruiz-Herrera et al., 2002) and should facilitate the identification of some of the numerous WAPs in search of a function as WAEs (Mrsa et al., 1999; Montijn et al., 1999). Directly extracting these in their native form from ballistically prepared walls and subfractions thereof (see Section 3.2.) and concomitantly screening for presumptive catalytic activities, the choice guided, e.g., by molecular genetic data, appears to be a promising first stage towards that goal, eventually followed by more extensive biochemical and genomic journeys. The work of Iwashita et al. (1998, 1999) with β-glucosidase represents a good example for such a straightforward integrated approach. Finally, the high stability of naturally immobilized WAEs could also be made use of in the search for inhibitors of WAEs as potential antifungal agents (see Section 5).

3. Fractionation of wall-associated enzymes (WAEs) in situ

3.1. The crude cell wall experimental system

The view is quite common that the crude cell wall fraction (CWF) is heavily contaminated by protoplastic components and, therefore, is unsuitable per se as an experimental system in its own right. Implicit in this view is the assumption that rigorous washing of the CWF with dilute hydrophilic solutions is mandatory for reliable wall studies as it removes these contaminants. However, this contention appears to be ill-founded. Thus, it has been shown by a combination of chemical and electron-microscopical analyses [applying the wall fractionation procedure of Hunsley and Burnett (1970) to *A. bisporus* cells] that the innermost part of the wall compartment is delimited by a thin amorphous zone (ca. 20 nm; Michalenko et al., 1976; Rast and Hollenstein, 1977; Hollenstein, 1997; Fig. 5, layer III). As this can be clearly detected only if washing with buffer is not extensive it could, therefore, prima facie be discounted as a protoplastic contaminant. However, it reveals itself as a genuine and distinct structural mural element by differential staining upon treatment of the wall fragments with lipase or KOH. In other studies, with intact mushroom spores, it was observed that the inner surface of the wall sometimes displays projections connecting it to the plasma membrane (Greuter and Rast, 1975; Rast et al., 1981; L.E. Nyhlén and D.M. Rast, unpublished), which are somewhat reminiscent of Hechtian adhesion sites in plant cells. Indeed, Hechtian strands have been shown now to be a regular feature of fungal hyphae (Bachewich and Heath, 1997). This means that in preparations of buffer-washed cell walls the extracellular compartment is reduced to the more densely packed regions of the wall fabric or, in terms of physical biochemistry, a near-solid-state reaction medium with a severely reduced mobility of reactants and products. The clean wall experimental system may, in fact, be unsuitable per se for a straightforward comprehensive search of WAEs, as some of these can be expected to be located at the plasma membrane–wall interface and to be involved in mediating the communication of the cell

with its environment, e.g., wall-associated receptor protein kinases and co-located polysaccharide synthases. Most of the extensive data on signal transduction in fungi come from phenotypic analyses of mutant strains and related genetic evidence concerning MAP kinase pathways (Navarro-Garcia et al., 2001; Popolo et al., 2001; Klis et al., 2002), whereas biochemical analyses are virtually absent. A promising approach to tackle this problem would be to consider the situation in plants, where wall-associated protein kinases with presumptive function in signalling have been isolated by biochemical means (He et al., 1996; Gens et al., 2000; Kohorn, 2000; Anderson et al., 2001), and then use the crude cell wall experimental system, together with the cell fractionation protocol presented in Section 3.2.2.

By the same token used to discount the crude cell wall system as artefactual, it could be argued that this holds vice versa for the crude cell extract (CCE), since this is likely to be heavily contaminated by hydrophilic entities of the ECM. Such criticism is hardly ever encountered (for a notable exception, concerning ATPase, see Serrano, 1985; and Section 3.2.2). For example, the conspicious presence of quantities of polysaccharide hydrolases (e.g., chitinases and β-glucanases: Rast et al., 1991; Mayer and Rast, 1997; Pitson et al., 1999) in the CCE of fungi cultivated under non-inducing conditions is assumed to represent just a pool of vesicle-bound enzyme en route to the cell surface (Nombela et al., 1988). Tagging the cell surface proteins prior to cell breakage (e.g., by biotinylation; Casanova et al., 1992; Mrsa et al., 1997; Chaffin et al., 1998; Ruiz-Herrera et al., 2002), followed by searching for label in the CCE, could help clarify the problem, although it is highly unlikely that all proteins of the ECM potentially contaminating the CCE become tagged by that procedure. Because some of the periplasmic enzymes intrinsically partition between the CWF and the CCE, cross-contamination of these is unavoidable (Arnold, 1991). The problem just has to be dealt with in separate, individual studies, should the issue of contamination be a critical one.

3.2. A facile and rapid fractionation scheme for WAEs

In principle, the binding of wall-associated proteins (WAPs) to other components of the ECM can be brought about by: (i) hydrogen and (unspecific) weak ionic bonding; (ii) van der Waals forces and hydrophobic interactions; (iii) strong electrostatic forces; and (iv) covalent linkage. As detailed below, a system of operationally defined classes of WAEs (called I–IV, respectively) according to the type of interaction of their respective members with binding partners in muro could be a valuable tool for further, broad-based research into the metabolism of the fungal cell wall. Applying Ockham's Razor, such a classification of WAEs would allow: (a) more objective comparisons between data relating to different species; (b) disclose similarities/dissimilarities of the microenvironment in which WAEs belonging to different operational classes are active; and (c) enhance the establishment of an information retrieval system of WAEs as an heuristic basis upon which to build hypotheses for their functions. This is not possible at present, because the number of species and enzymes studied is relatively small, isolation procedures for them vary greatly, and the terminology used to refer to non-buffer-extractable WAEs are incompatible. Thus, for example, the term "strongly wall-bound" can mean both ionically and covalently attached to a mural partner (for references, see Section 1.1). Attempts to obtain an operationally-based classification system for WAEs, therefore, will aim primarily at selecting suitable chemical discriminators for wall fractionation protocols that yield the isolated WAPs—probably mostly all WAEs (Section 2.3)—as catalytically active entities. Considerations of the general applicability, simplicity and speed of such an extraction protocol will, additionally, be of importance.

3.2.1. Choice of experimental organisms, test enzymes and extractants

3.2.1.1. Experimental organisms. The choice of the main experimental material, *A. bisporus* sporocarp hyphae, was guided by the following conditions and considerations (references given are not comprehensive): (i) it is a filamentous fungus; (ii) easily available in the form of exponentially growing hyphae (refer to Section 6.1.); (iii) its chemical composition and wall ultrastructure are well documented (Michalenko et al., 1976; Rast and Hollenstein, 1977; Rast et al., 1981; Hegnauer et al., 1985; Mol and Wessels, 1990; Pierce and Rast, 1995; Hollenstein, 1997); (iv) it has a more-than-average repertoire of identified enzymes, both ubiquitous (Rast et al., 1976, 1979; Hammond, 1985; Van Gelder et al., 1997; Jolivet et al., 1998) and more specific ones (Häsler-Küng, 1974; Häsler-Küng and Rast, 1974; Ruffner et al., 1978; Thurston, 1994; Volc et al., 1998; Wannet et al., 1998; Hörer et al., 2001); (v) it is well investigated with respect to enzyme systems that are undoubtedly major players in the metabolism of basic wall components (Hänseler et al., 1983a,b; Galan et al., 1999), and (vi) a good record of molecular genetics data are available for this species (Stoop and Mooibroek, 1999; Whiteford and Thurston, 2000).

If only point (v) were considered, *M. rouxii* would be an alternative excellent choice, as it has served over a period of more than 20 years as an experimental object of many investigations into the metabolism of cell wall polysaccharides (Pedraza-Reyes and Lopez-Romero, 1989; Lending et al., 1990, 1991; Rast et al., 1991; Horsch et al., 1996, 1997; Mayer, 1997; Merz et al., 1999a; further references in Merz et al., 1999b). This

species, however, presents some disadvantages in the present context: (a) as a zygomycete, the structure of its wall is different from both yeasts and non-dimorphic filamentous fungi (Bartnicki-Garcia, 1968); (b) it is not so well studied with respect to both primary and secondary metabolism; (c) as a dimorphic fungus, it displays a strong tendency to grow as mixed mycelial/yeast cells; and (d) the submerged liquid cultivation system necessary to obtain quantities of homogeneously growing cells at μ_{max} is exacting (Rast et al., 1991). Nevertheless, some of the experiments reported here have been performed with *M. rouxii*.

Should the data of the composition, catalytic activity and location in muro of the assembly of WAEs eventually be interpreted in relation to their functional significance in growth, it is essential that they should have been collected from log-phase cells (see Rast et al., 1991). This condition is met here with both experimental organisms (Section 6.1).

3.2.1.2. Test enzymes. The array of enzymes tested for their presence in operationally defined fractions of the ECM was selected on the basis of two criteria: (a) well known in the test organisms as components of the crude cell extract; and (b) as a whole, potentially displaying all four types of physicochemical interactions between WAEs and other components of the wall compartment (as defined in the preamble to Section 3.2). On the experimental level, the palette of enzymes thus contained at least one representative of: (i) soluble protoplastic enzymes that are generally considered not to genuinely occur in the ECM, but might, nevertheless, be present in the crude cell wall system (Section 3.1), either as contaminants (by entrapment in interstitial spaces of the CWF pellet or by unspecific weak bonding) or as hitherto unknown authentic mural components; (ii) soluble cytoplasmic enzymes for which extraprotoplastic forms have been suggested; (iii) membrane-associated enzymes which may have corresponding entities in the wall, or enzymes that are both in the plasma membrane and the ECM (for examples, see Section 3.1); and (iv) enzymes whose substrates or products in the wall are insoluble.

3.2.1.3. Extractants. The common procedures to isolate WAEs are based on the insolubility of β-glucan as well as of chitin and, in this, they draw from previous, straightforward chemical methods to extract wall-associated proteins (WAPs) which involve treatment of the wall material with alkali and acid, sometimes followed by digestion of the residue with β-glucanase and chitinase (Hunsley and Burnett, 1970; Fleet, 1991; Ruiz-Herrera et al., 1996). In general, the sample for isolation of WAEs consists of: (i) whole cells (native or surface-labelled) or residues obtained therefrom by treatment with dilute hydrophilic and hydrophobic solvents and/or with glucanase mixtures (e.g., Elango et al., 1982; Kuranda and Robbins, 1991; Kruse and Cole, 1992; Molloy et al., 1995; Gil-Navarro et al., 1997; Pitson et al., 1999); (ii) the low-speed sediment of ballistically prepared cell homogenates (Section 2.1; Table 1); and (iii) autolysates therefrom (Notario, 1982; Fleet, 1991; Hartland et al., 1996; Mouyna et al., 2002). Whereas the number of low-molecular strongly hydrophilic extractants used (mostly simple salts) is small (see Chaffin et al., 1998), the array of lipophilic agents applied is large, encompassing organic solvents and solutes covering an extended range of dielectric constants as well as low/high-molecular, and ionic/non-ionic surfactants (Hänseler et al., 1983b; Vainstein and Peberdy, 1990; Casanova and Chaffin, 1991; Horsch et al., 1996; Ruiz-Herrera et al., 1995; Mrsa et al., 1997; for further rererences, see Chaffin et al., 1998; Molina et al., 2000). However, as with proteins in general, application of lipophilic agents to extract proteins from a membranous environment is conducive to inhibition, denaturation and, thence, abolishment of the catalytic activity of enzymes sought. Systematic investigations do not exist of the chemical features of hydrophobic agents which may have an influence on the array of proteins that they solubilise from the wall. The few comparative studies available are very restricted in scope, relating merely to WAPs unidentified biochemically as to their potential catalytic activity (Casanova and Chaffin, 1991; see also Chaffin et al., 1998), or to just one particular enzyme (e.g., invertase: Vainstein and Peberdy, 1990). Hence, the overwhelming majority of studies on WAEs address only one enzyme, and the choice of the lipophilic extractant is determined less by a general rationale, but more by trial-and-error decisions as well as by empirical and technical considerations, among which especially the compatibility with current electrophoretic techniques and other protein purification methods. This could explain why sodium dodecyl sulfate (SDS) has a key position within the array of extractants with detergent action, although it can be perceived as the prototype of an "aggressive" denaturing surfactant (le Maire et al., 2000).

It is in keeping with these considerations that the most critical item in the search for a generally applicable fractionation procedure for WAEs including hydrophobically bound species and enzymes displaying other types of interaction with components of the ECM, is the availability of a detergent combining solubilising efficacy with reasonable preservation of catalytic activity, at least to a workable extent (see Section 3.3). Should the surfactant play a discriminator role within a protocol suitable for evaluation of data obtained for an operational classification of WAEs (as outlined in the preamble to Section 3.2), it should be rationally positioned within the sequence of extraction steps.

For the arguments (i)–(iv), the steroid glycoside saponin digitonin (DIG; for formulae of its molecular

and supramolecular structures, refer to Merz et al. 1999a) may be the detergent of choice for the extraction of hydrophobically-bound WAEs:

(i) Ionic detergents are in general more inactivating than non-ionic, "mild" ones (le Maire et al., 2000).
(ii) DIG is an established tool for solubilising membrane-associated proteins and has been applied extensively in studies of polysaccharide synthases of fungi (Gooday and de Rousset-Hall, 1975; Ruiz-Herrera et al., 1980; Hänseler et al., 1983a,b; Leal-Morales et al., 1997; Merz et al., 1999a, and references cited therein). Its use as an extractant is far from being restricted to these types of enzymes, however, but extends to membranous systems of other, widely disparate origins, e.g., from plant, epithelial and muscle cells (Mohanty et al., 2002; Simons and Ikonen, 1997; White et al., 2002; Ivanov et al., 2002).
(iii) In contrast to the application of other non-ionic detergents (e.g., Tween-20 and Triton X-100), application of DIG in low concentrations (0.01-ca. 0.25%) generally produces a stimulation of enzyme activity; thereafter, a gradual decline may occur below the reference value of untreated membranes (e.g., Robinson and Wiskich, 1975; Ruiz-Herrera et al., 1980; Hänseler et al., 1983a; Merz et al., 1999a). This inhibition is, however, either relatively low, even when applied in saturated "solution" (see below), or is otherwise reversible by dilution, dialysis, gel filtration or with polystyrene beads (le Maire et al., 1987).
(iv) Clearly, DIG as an extractant for membrane-associated enzymes must have some properties not possessed by other surfactants. Certainly, it would be unreasonable to suppose that DIG should not comply with the general three-stage model of the solubilisation of protein-containing membranes by non-ionic detergents (le Maire et al., 2000), which encompasses uptake of detergent in non-micellar form, predominantly by the lipid phase (stage I), followed by detergent–detergent interactions leading to destabilisation of the bilayer structure and membrane fragmentation (stage II) and, thence, formation of mixed lipid–detergent micelles, exposure of membrane proteins to micellar detergent and, finally, protein solubilisation (stage III).

Against this scheme as background, three properties of DIG can be supposed to contribute significantly to its special ability to extract enzymes as catalytically active entities. Firstly, the avid interaction of DIG with 3β-hydroxysterols, resulting in a very stable steroid/sterol adduct (references in Merz et al., 1999a). This, of course, would prima facie not be expected to further membrane destabilisation as a prelude to solubilisation—unless the proteins to be extracted are strongly attached to a special membrane domain that is inaccessible to other detergents. This holds for GPI-anchored enzymes, which are grafted to sphingolipid–sterol rafts (Simons and Ikonen, 1997; Nosjean and Roux, 1999), from where they can be displaced by other non-ionic detergents only if membranes are pre-extracted with DIG (Cerneus et al., 1993). In any case, the anchoring of DIG with membrane sterol will drive the equilibrium between detergent in the solubilisation medium and detergent in the membrane towards the latter and, thus, allow maximum detergent "loading" of the membrane, while transitorily stabilising it and, therefore, keep the enzyme in a pseudo-natural hydrophobic micro-environment (stage I). With increasing concentration of non-sterol-bound detergent occurring upon saturation of the sterol binding sites with DIG, the system, will, nevertheless, gradually become more perturbed (stage II), and be transformed into stage III, with the protein in the DIG-solubilised state, which might have the character of a proteoliposome embodying some sterol. Secondly, DIG has an extremely low solubility in water and a correspondingly low critical micellar concentration. In other words, the solubilisation process is carried out practically exclusively by pre-formed micelles, which then readily fuse with mixed lipid–detergent structures formed by detergent already taken up into the membrane. This is a situation particularly well suited for co-operative action of supramolecular detergent structures on membrane lipid, which is a "hallmark" of solubilisation by non-ionic detergents (le Maire et al., 2000). Thirdly, due its highly amphiphilic nature, DIG has a strong tendency to self-organise into vesicular micelles, for which the expression 'digitosomes' has been coined (Merz, 1997; Merz et al., 1999a), and fusion products thereof. By calculation, using S- and M_r-values and suitable references, "digitosomes" have a diameter of about 8 nm; in completely different experiments, using electron microscopy, the mean diameter of DIG-micelles present in solubilisation medium was in the 15-nm-range, with a few giant vesicles with diameters roughly about twice this value (Hänseler et al., 1983b). Another method sizes the premicellar and micellar aggregate structures of DIG in H_2O to ca. 6 and 100 nm, respectively (Menger and Keiper, 1998). Through "digitosome"/"digitosome" interaction even larger DIG-vesicles may, thence, be formed additionally–a situation ideally suited for extracting membrane-bound proteins through hydrophobic/amphiphilic transfer, thus reducing interference by water resulting in the release of un-shielded, inactive enzyme. In practical terms, this might represent a rational explanation for the fact that the solubilisation of membrane-protein is more efficient if the detergent is added directly to the

membrane sample (pellet) just prior to suspension in buffer (see Section 6.2; based on unpublished observations of E. Hänseler, M. Horsch and D.M. Rast on chitosomal chitin synthase).

3.2.2. The protocol (including experimental data)

In a number of previous attempts to classify wall-associated proteins (WAPs) operationally, at least in part, two groups have been distinguished, non-covalently vs covalently bound proteins, using as discriminators the extractability with hot SDS or reducing agents (most often β-mercaptoethanol) for WAPs of the first group, and the release of enzyme from the wall only upon treatment with glucanase and/or chitinase for members of the second (Sentandreu et al., 1994; Chaffin et al., 1998). In another system, WAPs are divided into three groups, based in principle on these criteria, but sub-dividing covalently linked WAPs on their solubility upon enzymic digestion of the wall fabric *vs* solubility only in dilute NaOH (Mrsa et al., 1999; Molina et al., 2000). A straightforward and generally applicable extraction protocol suitable for the classification of WAEs is not available. By applying dilute buffer, DIG and NaCl at high ionic strength as extractants (Sections 3.2.1 and 6.2), considering the resulting insoluble residue as a wall sub-fraction in its own right, and using appropriate enzyme assays (for examples, see Section 6.3), four fully operationally defined classes of WAEs, called I–IV (see preamble to Section 3.2), can be probed for in the crude cell wall experimental system (Section 3.1) presented here (Section 3.2.2).

Application of the principles and practical tools outlined in these Sections to a number of test enzymes from *A. bisporus* afforded the results shown in Fig. 3(a–d). Some of the data given have already been published (Sassoon and Mooibroek, 2001). Exploratory experiments designed to set up the protocol have used *M. rouxii*, the fungus most extensively studied with respect to both chitin synthase and chitinolytic enzymes (Section 3.2.1). A full-fledged cellular fractionation scheme for chitinase in this material, encompassing the soluble and a mixed-membrane fraction (54 000 *g* supernatant and sediment, respectively), the buffer, the DIG and the NaCl extracts of the CWF as well as the insoluble residue thereof, located 77, 5, 16, 2, ≈0 and 1% of the total cellular activity to these. The corresponding values for β-*N*-acetylhexosaminidase (HexNAc'ase) amounted to 72, 5, 12, 1, ≈0 and 10% (Mayer, 1997). When aiming for a facile and rapid fractionation procedure suitable for assessing several enzymes in one and the same CWF-isolate, however, such a labour-intensive procedure is neither practicable, nor even necessary, as long as the evaluation of the results is not critically dependent on their being quantitative (Section 3.3.). Based on experiments with HexNAc'ase from *M. rouxii* (Fig. 1) and similar experiments with mannitol dehydrogenase from *A. bisporus* (D. Baumgartner and J. Sassoon, unpublished data), the number of extraction steps to be performed routinely with each, buffer, DIG and NaCl, was set at five, two and two, respectively. There is no evidence that WAEs would be altered in their basic catalytic properties by the extractants used. Thus, with *M. rouxii* HexNAc'ase, the non-extractable enzyme form showed the same ratio of activity with the standard fluorogenic substrate (Section 6.3.11) and the galactose analogue therefrom as observed with the soluble enzyme (Mayer, 1997). Moreover, the HexNAc'ase of subfraction IV could act as a HexNAc-transferase as well (Fig. 2). This is a catalytic potentiality of the enzyme that is only displayed under certain favourable conditions and is, in fact, intrinsic to all β-glycosidases performing hydrolysis with over-all retention of configuration at the anomeric center (see scheme in Horsch et al., 1997).

The results on WAEs of *A. bisporus* are as follows.

3.2.2.1. γ-Glutamyltransferase (GT) and agaritine γ-glutamyltransferase (AGT), [(i)–(ii)]

(i) GT has never been reported other than as a membrane-associated enzyme, either in the plasma membrane, with the active site oriented to the outer cell surface (Tate and Meister, 1981; Chikhi et al., 1999; Dominici et al., 1999), or in the membrane of melanosomes, a special type of lysosomes (Borovansky and Hach, 1999). In bacteria, GT is located in the periplasm (Suzuki et al., 1986). Its cellular function is seen as that of

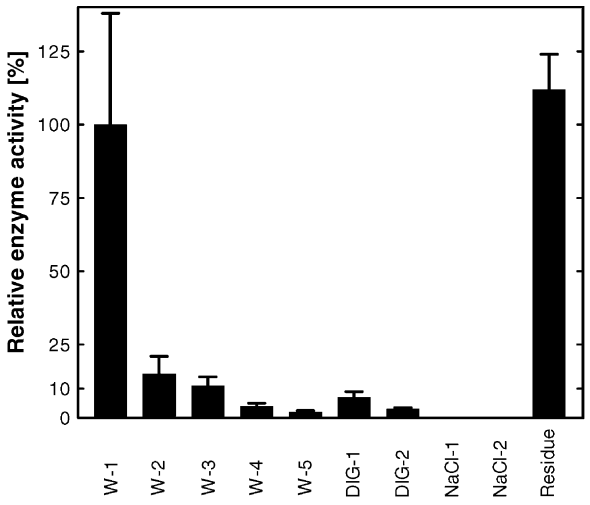


Fig. 1. Activities of β-*N*-acetylhexosaminidase (HexNAc'ase) present in crude cell walls of *M. rouxii* log-phase hyphae upon exhaustive extraction with buffer (×5, fractions W-1–W-5), digitonin [(DIG); ×2; fractions DIG-1 and DIG-2], NaCl (×2; fractions NaCl-1 and NaCl-2) and the activity residing in the insoluble residue (final sediment). The amount of enzyme present in the first buffer extract (217±83 [nkat]; $N=3$; independent experiments) was set as 100% (redrawn from Mayer, 1997).

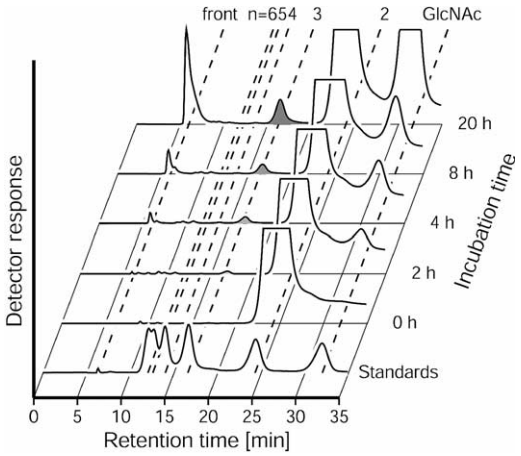


Fig. 2. Hydrolysing/transglycosylating activity, with *N,N*-diacetylchitobiose as the substrate, of genuinely wall-associated HexNAc'ase from *M. rouxii* hyphae (see Fig. 1, fraction residue), as assessed by HPAEC/PAD analysis of the products (for details of the method, refer to Section 6.3.11) (reproduced from Horsch et al., 1997; by permission of Elsevier Science).

a component of the γ-glutamyl cycle, serving: (a) as a mechanism for the uptake of amino acids and (certain) dipeptides (Tate and Meister, 1981) as well as of biologically active analogues therefrom (Furter and Rast, 1985); and (b) as a detoxification system for a variety of xenobiotics by using the γ-glutamyl moiety of glutathione as the conjugating group (Chikhi et al., 1999; Dominici et al., 1999). The γ-glutamyl cycle is operative also in yeast (Mooz and Wigglesworth, 1976), and two of its constituent enzymes, GT and 5-oxoprolinase, have been identified in *A. bisporus* as well (Stüssi, 1979). As shown in Fig. 3a, there is no evidence for GT being a class-IV, -III, or -II enzyme (the occurrence of GT-activity in the DIG-fraction is too low to allow another conclusion). There is, thus, no artefactual transfer of GT, used here as a membrane marker system, on to the cell wall fabric, where, otherwise, it could just mimic the presence of a hydrophobically-bound WAE.

(ii) The natural substrate of AGT is agaritine, γ-glutamyl-4-hydroxymethylphenylhydrazine, which has been found only in *A. bisporus* and some closely related species (for a list, see Baumgartner, 1995). AGT differs from GT inasmuch as it can be isolated without the use of a detergent (Gigliotti and Levenberg, 1964) and is apparently not membrane-bound. Also, it has the ability to utilise aryl hydrazines and their γ-glutamyl derivatives as acceptor and donor substrates, respectively, while not effecting the cleavage of typical γ-glutamyl peptides (as performed by GT), but to accept the desglutamyl moiety of γ-glutaminyl-4-hydroxybenzene, the melanogenic precursor in *A. bisporus* (Rast et al., 1979, 1981; Stüssi and Rast, 1981). Another link of AGT to melanisation is also evident from the fact that agaritine inhibits tyrosinase (Baumgartner, 1995; Espin et al., 1998). Both distinctions between GT and AGT concur with the different biosynthetic pathways of their respective substrates (Baumgartner et al., 1998). It is in keeping with the presumed subcellular location of AGT that it behaves as a typical cytosolic enzyme, having no special affinity to sub-compartments of the wall (Fig. 3a).

3.2.2.2. NAD^+-Malate dehydrogenase (MalDH), $NADP^+$-Mannitol dehydrogenase (MtDH) and tyrosinase, [(i)–(iii)]. A feature common to all three enzymes is that they are generally regarded as cytoplasmic.

(i) The following facts and observations cast doubt on the validity of the general perception of MalDH as an absolutely specific marker for cytoplasmic enzymes. That it exists as isoforms in intracellular organelles is well known, of course (for plant systems, see Reumann et al., 1994), not, however, that it occurs, additionally, as a bona fide WAE of plants, according to its solubility properties either as a class-IV or class-II entity (Gross, 1977). With *A. bisporus*, there was some MalDH activity in the DIG fraction (Fig. 3b), but for reasons given in Section 3.3 it is not assigned here the status of a WAE.

(ii) MtDH has been noticed repeatedly in the DIG extract of the CWF of *A. bisporus* (Pfyffer et al., 1989; D. Baumgartner and D.M. Rast, unpublished data). With the existence of mannitol, a typical constituent of fungi (Pfyffer et al., 1986; Rast and Pfyffer, 1989) occurring also in membrane-compatible forms (Tulloch and Spencer, 1964; Griffin et al., 1970; Byrne and Brennan, 1975; Fex, 1982), and with a 1-mannitol-β-glucosidic partial structure existing in the wall fabric of *A. bisporus* (D. Baumgartner, I. Rodriguez, F. Schär and D.M. Rast, unpublished), the possibility cannot a priori be ruled out, that MtDH in fungi disposes of a plasma membrane-bound form, in addition to the soluble one. Such a situation would not be unprecedented, as polyol dehydrogenases bound to the outer surface of the plasma membrane are known from bacteria (Shiniwaga and Ameyama, 1982; Adachi et al., 2001). Since the catalytic activity displayed by hydrophobically wall-bound MtDH amounted to some 12% of that present in the CWF (Fig. 3b), i.e., was some 5× higher than the activity poten-

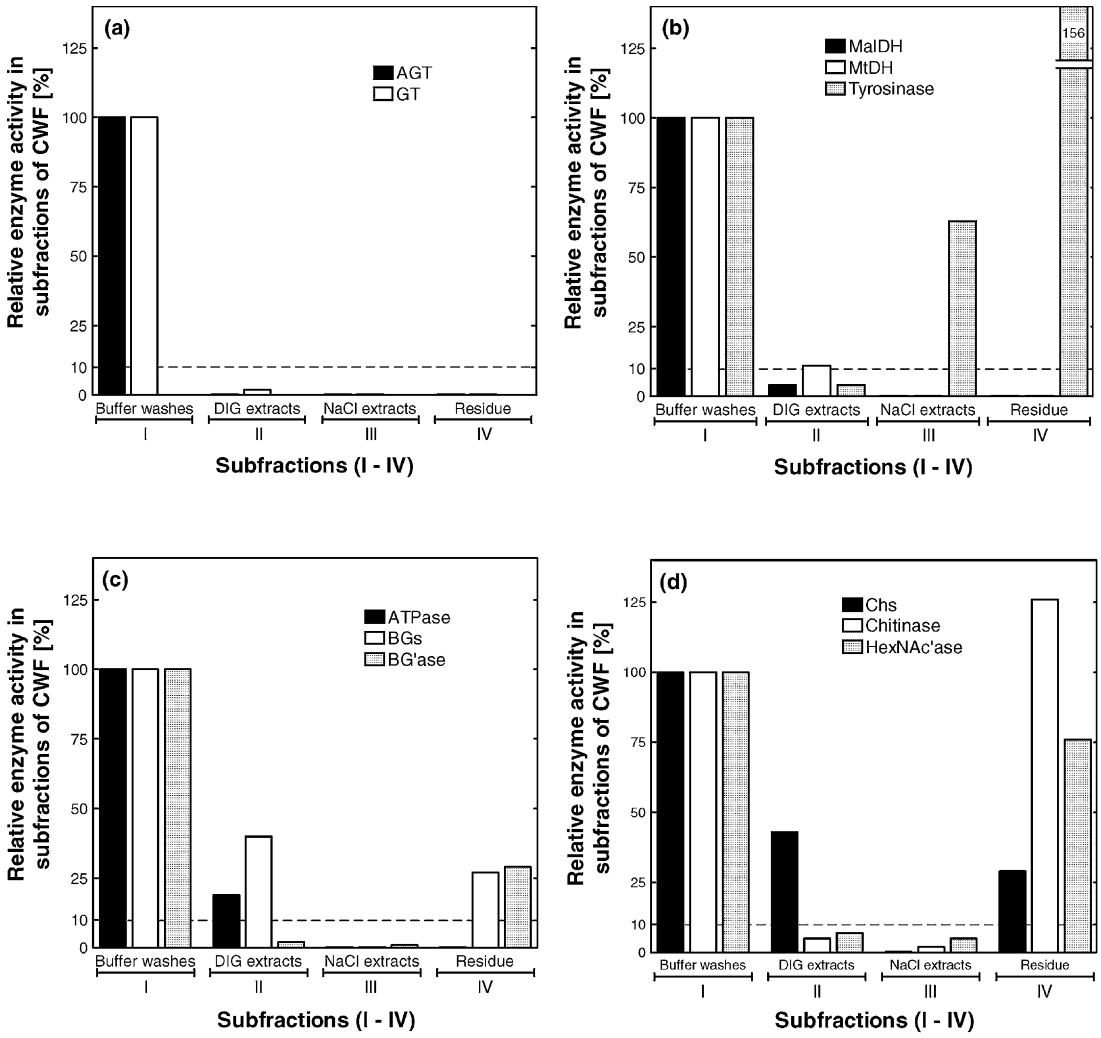


Fig. 3. (a)–(d). A general fractionation protocol for WAEs present in the crude cell wall fraction (CWF) as assessed by their sequential differential extractability with buffer, digitonin (DIG) and NaCl, yielding subfractions I–IV (refer to Section 6.2). The total activity of the combined buffer washes of the crude walls is set as 100% relative activity; the activities of the pooled DIG and NaCl extracts as well as the activity displayed by the insoluble residue are expressed as a proportion of this reference. An example: *A. bisporus* sporocarp hyphae (for details, see Sections 6.1. and 6.2). Numbers in square brackets represent the total activity in nkat displayed by the pool of the buffer washes. For each enzyme representative data are given from one out of three independent isolations/fractionations: (a) γ-glutamyltransferase (GT), [939]; agaritine γ-glutamyltransferase (AGT), [615]; (b) malate dehydrogenase (MalDH), [13476]; mannitol dehydrogenase (MtDH), [20055]; tyrosinase [182]; (c) ATP phosphohydrolase (ATPase), [81]; β-(1–3)-glucan synthase (BGs) [21]; (1–3)-β-glucanase (BG'ase), [927]; (d) chitin synthase (Chs), [60]; chitinase, [3]; β-*N*-acetylhexosaminidase (HexNAc'ase), [19].

tially present as a contaminant (as compared with the membrane marker GT; Fig. 3a), on the operational criteria set up here (see preamble to Sections 3.2 and 3.3), MtDH is a class-II WAE. An argument, which could be raised against the hypothesis of hydrophobically wall-associated MtDH being an enzyme active also in vivo, is the requirement for an extraprotoplastic pool of NADP (similarly as for NAD-MalDH in plants; see above). But translocation mechanisms which could provide for this necessity do exist (see Section 2.2).

(iii) Tyrosinase is the key enzyme of melanogenesis and may be both membrane-bound and soluble (see Section 4.2 for a more detailed account). Using the protocol and the standard substrate for assaying the enzyme, dopa (Robb, 1984; Prota, 1992), two kinds of bona fide wall-associated tyrosinases were found here, a class-III and a class-IV species (Fig. 3b). This is the first report of wall-associated tyrosinase (cf. with Wheeler and Bell, 1988), but the claim made is convincing only if there is not co-occurrence with laccase, a related phenol-oxidising enzyme that also reacts with dopa (Mayer and Staples, 2002) and is sometimes held to be a fungal melanogenic entity having at least the same importance as tyrosinase (Wheeler and Bell, 1988; Zhu et al., 2001).

However, laccase is a constitutive enzyme in white rot fungi only, which secrete it into the medium (Mayer and Staples, 2002), whereas tyrosinase is ubiquitous and not an exoenzyme (for *A. bisporus*, see Häsler-Küng and Rast, 1974; Häsler-Küng, 1974). Moreover, the melanin produced by laccase action differs from that synthesized by most other fungi and animals as a whole, inasmuch as it occurs extracellularly in the medium and is to some extent soluble. In any case, laccase can be excluded from taking part in the oxidation of dopa in the experimental material used here (plectenchyme from the, cap and the upper third of the stipe: see Section 6.1), for the following reasons: (a) *A. bisporus* sporocarp hyphae display laccase activity in the rhizomorphs and the base of the stipe only (Häsler-Küng,1974; Häsler-Küng and Rast, 1974); (b) using *p*-phenylenediamine or syringaldazine as the substrate (Thurston, 1994; Mayer and Staples, 2002), no laccase activity was found, either in the crude cell extract, or in the CWF and subfractions thereof; and (c) product formation with dopa was completely suppressed by salicylhydroxamic acid, a specific tyrosinase inhibitor (Allan and Walker, 1988) in as low a concentration as 10 μM. Since more than 95% of the total potential cellular activity of tyrosinase of *A. bisporus* is in a latent form (see below), the existence of tyrosinase as a class-II enzyme also is quite probable, but the enzyme cannot be assigned this status here, since the relative tyrosinase activity occurring in subfraction-II was only some 5% (Fig. 3b; refer to Section 3.3 for the conditions set for including an enzyme in the operationally defined classification system presented here). The high catalytic activity concentrations of class-IV- and -III tyrosinases must not be mis-interpreted as indicative of particularly high tyrosinase protein contents in the subfractions concerned, since *A. bisporus* tyrosinase diplays zymogenicity, a distinct feature of tyrosinases in general (Van Gelder et al., 1997). Activation of the pro-enzyme in the present situation is probably caused by the extractants used, especially DIG, and/or by self-activation through co-occurring proteinase. Indeed, latent *A. bisporus* tyrosinase becomes activated by treatment with detergents (Moore and Flurkey, 1990; Espin and Wichers, 1999) as well as by endogenous serine protease (Burton et al., 1993; Espin et al., 1999).

3.2.2.3. ATPase, β-glucan synthase (BGs) and β-glucanase (BG'ase), [(i)–(iii)]. Using a standard assay for ATPase (Section 6.3.6.), a typical integral plasma membrane protein (Serrano, 1985; Kasamo and Sakakibara, 1995), enzyme activity was detected in subfraction II (Fig. 3c). The same was observed with BGs, which is employed widely as a biochemical marker for plasma membrane association (Serrano, 1985).

(i) Against this background alone, the ATPase present in the CWF could be discounted as a contaminant. But, even if this were the case, it would represent a conspicious artefact worthy of special attention, since tight association of ATPase in plasma membrane preparations with non-membrane polysaccharide (Nurminen et al., 1970) has been considered, vice versa, an artefact (Serrano, 1985; see also Section 3.1). It is thus difficult comprehensively to rule out a genuine association of plasma membrane ATPase with a component of the ECM, as holds for some GPI-anchored enzymes that occupy a dual location at the cell surface (refer to Sections 2.3 and 3.1). While most surfactants activate fungal plasma membrane ATPase, only a few, however, solubilise it effectively with preservation of reasonable catalytic activity (Serrano, 1985). That the hydrophobically wall-associated ATPase is extractable into DIG is an interesting observation per se, since this is the detergent of choice to solubilise GPI-anchored enzymes clustered in microdomains enriched in cholesterol and glycosphingolipid bases that connect the plasma membrane with the ECM (refer to Section 3.2.1.3). Thus, regardless of whether the wall-associated ATPase observed here is a typical MgATPase or another ATPase, e.g., a protein kinase (Serrano, 1985)—on operational criteria it is a class-II WAE. The existence of wall-associated ATPases has been reported from plant cells as well (Kiba et al., 1995).

(ii) The cell wall fraction of fungi accounts for about half of the total cellular activity of BGs (Fèvre and Dumas, 1977; Fèvre, 1979). While the same critical argument brought forward above against ATPase as a genuine WAE could be raised in the case of class-II BGs, it is clearly at odds with the fact that BGs is present in subfraction IV also (Fig. 3c), where it cannot possibly be a contaminant. The existence of two types of BGs species in two different domains of the ECM, even on simple operational grounds calls for a reconsideration of the general assumption that β-glucan, in contrast to chitin, is synthesised intracellularly in transport vesicles releasing the polysaccharide at the cell surface as a preformed wall component (Bartnicki-Garcia, 1973). Further, it might be tempting to hypothesise on a

precursor-product relationship between class-II and class-IV BGs. Judged from some observations on the stability and dynamics of protein pools from the cell wall of several fungi (Ruiz-Herrera et al., 2002), this appears not to be the case, however. Alternatively, one could speculate that the two BGs species play different functions within the ECM and travel to the cell surface via two different secretory pathways (as discussed below for Chs). Since GPI-anchored cell wall proteins (see Section 2.3), to which BGs is believed to belong (Kapteyn et al., 1999), display environmentally induced changes in expression (Klis et al., 2002), the enzyme suggests itself as an ideal target for novel antifungals (Section 5).

As with ATPase, DIG appears to behave in a special way with BGs, inasmuch as, unlike other detergents, it solubilises the enzyme at low concentrations and stabilises it as well: a property that has been taken advantage of from the very beginning of research into BGs (which was with plants: Flowers et al., 1968; Hayashi et al., 1987). More recently, however, DIG has had to share its exclusive position as a solubilising agent of plant BGs with the structurally related, likewise strongly amphiphilic detergent CHAPS (Lawson et al., 1989). The same has been observed with fungi (Beauvais et al., 1993; Billon-Grand et al., 1997). The apparent failure to solubilise BGs with DIG (see, e.g., San Blas and San Blas, 1982) is probably an experimental artefact, due to inappropriate application of the detergent (Section 3.2.1.3, last paragraph) and mis-interpretation of inactivation with lack of solubilisation, while not attempting re-activation through detergent removal (refer to Section 3.2.2.4).

(iii) As evidenced by Fig. 3c, BG'ase is a genuine WAE. This comes as no surprise, since it is the wall where the enzyme's substrate occurs. Like other wall-associated proteins in general, BG'ase can be assumed to be glycoconjugated (Mrsa et al., 1999; Kapteyn et al., 1999; Ruiz-Herrera et al., 2002). This and the fact that the wall embodies glucanosyltransferases (for examples, refer to Table 1) as well as transglycosylating β-glycosidases (Horsch et al., 1997, Mayer, 1997; Fig. 2), suggests that the covalent attachment of BG'ase in muro is likely to be effected by transglycosylation reactions. In contrast to class-IV BG'ase, class-II BG'ase is probably represented by a periplasmic form of the enzyme (Nombela et al., 1988). β-Glucanases not destined for secretion into the medium and, hence, not subjected to catabolite repression, are supposed to be morphogenetically important, providing for some plasticity of the ECM as necessary for growth (Nombela et al., 1988; Mouyna et al., 2002). Thus, a BGs–BG'ase–"laminaribiase"-system could be operating in muro, analogous to the Chs–chitinase–HexNAc'ase system proposed for controlled metabolism of chitin in the ECM of growing cells (Sections 3.2.2.3 and 4.1).

3.2.2.4. Chitin synthase (Chs), chitinase, and β-N-acetylhexosaminidase (HexNAc'ase), [(i)–(iii)]. The protocol reveals the presence of all of these as WAEs (Fig. 3d). For an interpretation of their co-occurence in the ECM, see Section 4.1.

(i) Just as BGs, Chs is represented by both a class-II and a class-IV entity (Fig. 3d and Table 2). This indicates that the generation of chitin chains could take place at the outer surface of the plasma membrane, an opinion which is at odds with the conventional view that the catalytic site of Chs is located in the cytoplasm (Cabib et al., 1983, 2001). But this need not to be the case, if cellulose synthesis is considered as a paradigm (Brown and Saxena, 2000), where it is envisaged that the catalytic site of plasma membrane-bound cellulose synthase may be oriented towards, and part of, the periplasmic space (models 2 and 4 of the authors). The existence of

Table 2
Operational classification of wall-associated enzymes (WAEs), based on the type of their interaction with binding partners of the extracellular matrix (ECM)[a], as represented by a crude cell wall experimental system[b], and using a new cell wall fractionation procedure:[c] an overview of results obtained with *A. bisporus* as the test organism (Section 3.2.2)

Enzymes	Classes of WAEs[c]			
	I	*II*	III	IV
γ-Glutamyltransferase	•			
Agaritine γ-glutamyltransferase	•			
Malate dehydrogenase	•			
Mannitol dehydrogenase	•	•		
Tyrosinase	•		•	•
Laccase				
ATP phosphohydrolase	•	•		
β-(1–3)-Glucan synthase	•	•		•
(1–3)-β-Glucanase	•			•
Chitin synthase	•	•		•
Chitinase	•			•
β-*N*-Acetylhexosaminidase	•			•

[a] As defined in preamble to Section 1.1.

[b] Described in Section 3.1.

[c] Classes-I, -II, -III and -IV of WAEs, embodying, respectively, entities the interaction of which with other components of the ECM is mediated by hydrogen bonding or (unspecific) weak ionic bonding, van der Waals forces and hydrophobic interaction, strong electrostatic forces, or covalent linkage (for details, see preamble to Sections 3.2. and 3.3).

two types of bona fide Chs WAEs implies, furthermore, that the plasma membrane-bound enzyme is not the only Chs protein catalyzing the generation of chitin chains. Considering the particular features of the two general major pathways for the delivery of enzymes that have the cell surface as the locus operandi, non-regulated and regulated, respectively (Satiat-Jeunemaitre and Hawes, 1993; Satiat-Jeunemaitre et al., 1996), it could be tempting to localise the class-II and -IV Chs entities to these pathways, respectively, although this might appear somewhat speculative. Nevertheless, there are reasons to assume that one of them travels to the cell surface in specific transport vesicles, chitosomes (Leal-Morales et al., 1988), via a complex regulated vesicular protein carrier system intersecting the exocytotic and endocytotic pathways by routing through an endosomal compartment (Chuang and Schekman, 1996; Ziman et al., 1998; Lucero and Robbins, 2002).

Further insight into the function of the class-II and class-IV Chs proteins (for definition of classes, refer to Table 2) as well as into the secretory pathways effecting their transport to the cell surface could be obtained by: (i) rigorous identification of the physical structure of at least one of them (conveniently class-II Chs), applying the sensitive analytical methods of proteomics (Blackstock and Weir, 1999), (ii) using this Chs as a reference probe in protein microarray chips (MacBeath and Schreiber, 2000) containing the proteins of the CWF (Section 3.1), and (iii) matching the data thus obtained with those generated by suitably designed DNA microarrays (see Heller, 2002). Applying the affinity chromatography procedure worked out to isolate a Chs from *M. rouxii* (Merz et al., 1999a; see also Geng et al., 2001), which has yielded the purest Chs preparation described hitherto, and using protein and DNA microarrays from *S. cerevisiae* would appear to be a most promising approach to address the problems raised above, since this is the fungus studied best with respect to the structure, biochemistry, molecular genetics and cell biology of Chs (see reviews of Merz et al., 1999b; Rast et al., 2000; Munro and Gow, 2001; Cabib et al., 2001; Klis et al., 2002; Roncero, 2002).

DIG has remained the only detergent to efficiently solubilise both plasma membrane-bound and chitosomal Chs (100 S), yielding 16 S Chs particles, without irreversibly inactivating the enzyme (Duran and Cabib, 1978; Ruiz-Herrera et al., 1980; Hänseler et al., 1983b; Horsch et al., 1996). This particular solubilisation behaviour of Chs is interesting, inasmuch as it is a common feature of GPI-anchored enzymes (cf. with Section 2.3 and 3.2.1.3). Depending on experimental conditions, the catalytic activity of 16 S ChS particles, whether ex walls or ex chitosomes, can be equal or even higher than that displayed by the chitosomes from which they were released, provided the detergent is partially removed, leaving the solubilised entity in a strongly amphiphilic environment mimicking the situation in vivo (Hänseler et al., 1983b, Merz et al., 1999a; Section 3.2.1.3, last paragraph).

(ii) On operational criteria, the chitinase present in fraction IV has the status of a bona fide WAE (Fig. 3d). The high activity displayed by this chitinase, exceeding that of the chitinase pool present in subfraction I, either as truly periplasmic or a cytoplasmic transport form of the enzyme, a "contaminant" (refer to Section 3.1), is likely to be due to its release from latency. This activation in situ might occur by the preceeding DIG extraction step, similarly to the earlier discussion for latent tyrosinase (Section 3.2.2.2). Considering the existence of several chitinase forms displaying close to 100% latency (Rast et al., 1991), one can safely assume, that the chitinase present in subfraction II is a WAE, too, although it cannot be classified as such on the criterion set for inclusion of a WAE in the operationally-based classification system (see Section 3.3).

(iii) Besides chitinase class-IV, there exists class-IV HexNA'ase. By much the same reasoning as followed in the case of chitinase, the HexNAc'ase present in subfraction II cannot be classified (as yet), since its relative activity is below 10%. For a biochemical description of HexNAc'ase that sets it apart from other, similar enzymes, refer to Horsch et al. (1997).

Table 2 summarises the basics of the new wall fractionation protocol and the results of its application to *A. bisporus*.

3.3. Evaluation and refinement of the basic protocol

The procedure described here for the fractionation of WAEs probes for four classes of WAEs, I–IV, which are operationally defined according to the type of interaction of component members with other constituents of the ECM (see preamble to Section 3.2 and legend of Table 2). As evident from the results obtained with the crude cell wall experimental system (Sections 3.1 and 3.2.2), there is no tapering carry-over to any extent through the extraction run, and WAEs are not randomly distributed in the four wall subfractions. Thus, there are WAEs which do not occur in any other wall subfraction than I, some are additionally

present in another fraction as well, while others display the II/IV combination of wall association, notably the polysaccharide synthases (for the significance of the latter finding in the cellular context, refer to Sections 3.2.2.3, 3.2.2.4 and 4.1). To assess the extent that subfraction I embodies cytoplasmic contaminants, a combination of genomic and proteomic methods could represent a promising approach (see Blackstock and Weir, 1999; for further references and a specific example, refer to Section 3.2.2.4), but because of considerations of facility, speed and the sophistication of instrumentation needed, this lies outside of the scope of the protocol. It is implicit to the concept upon which the basic wall fractionation procedure has been set up that the data it yields cannot be evaluated quantitatively. Nevertheless, in order to address the problem of contaminants and WAEs present in very low catalytic activity concentrations, without harming the high discriminatory value of the classification system, enzymes displaying relative activities below a certain level, arbitrarily set at 10% (as indicated in the ledger lines in Fig. 3), are not assigned the status of bona fide WAEs, i.e., class-II, -III or -IV enzymes. If this is to serve as a general information retrieval system both for basic research in the developmental cell biology of fungi (examples in Section 4) and the search of antifungals for use in agriculture and medicine (Section 5), neglecting potential false negatives seems to be preferable to having many enzymes of doubtful status included in a fully operational classification system for WAEs.

For the purpose of classifying WAEs based on the protocol and the 10% limit set for inclusion of a WAE into the system, it is neither necessary, nor even meaningful, to push the reliability of the method beyond the semi-quantitative level, at the most, for the following reasons: (i) the simultaneous isolation of several WAEs in one and the same run means that there is just one buffer for homogenisation and the same, or another (see Section 6.2) as the solvent for the extractants, disregarding the fact that the enzymes tested for might differ largely in pH optima; (ii) the cocktails used to assay the WAEs are standard ones, having been established with the soluble or the solubilised forms of the enzymes, and are thus not optimised for the conditions prevailing in the wall; (iii) most WAEs appear to display at least some degree of latency; and (iv) the substrate used may not be reacted upon only by the WAE sought, or the reaction product may be transformed by a co-occurring enzyme not accounted for. There are some counter-measures, of course, to any of these items.

(a) Due to the facility and speed by which enzymes can be screened for wall-association using the protocol, to run the homogenisation and the extraction steps with buffers of different pHs is a simple matter.

(b) To follow up item (ii) would be time-consuming, however, and worth the effort only, if the enzyme concerned was of some special interest, additional to the purpose of the protocol alone.

(c) Inclusion of a proteinase in the homogenisation and extraction media to activate the WAEs to full catalytic capacity, might appear the most appropriate means to distinguish enzymes displaying very low activity due to near-total latency from non-zymogenic entities present in traces only. But this suggestion needs special consideration, since it is well known that large differences exist in the susceptibility to limited proteolysis between different zymogenic enzymes, and even of the same latent enzyme species present in different fungi. Thus, the standard proteinase used to activate Chs of *M. rouxii*, Rennilase, has very little effect on the same enzyme preparation from *A. bisporus*, whereas trypsin, routinely applied in studies with *S. cerevisiae*, is a good activator, but fairly quickly destroys the *A. bisporus* enzyme (Hänseler et al., 1983a). Nevertheless, a similar rationale has been successfully followed to detect latent forms of chitinase and HexNAc'ase, applying the proteolytic activator at two largely different concentrations (Rast et al., 1991).

(d) Another caveat against over-interpreting the data obtained with the protocol in quantitative terms addresses the use of chromogenic substrates to assay WAEs, when there is co-occurrence with tyrosinase. Due to its broad substrate tolerance, tyrosinase oxidises the product of WAE action, the free phenol, a situation falsely indicating lesser activities of the WAE concerned than the actual ones, or even producing false negatives. This might well be the reason for some of the peculiar results obtained with chromogenic glycosides used to assay β-glucanases (Nombela et al., 1988; Luna-Arias et al., 1991). This problem is not confined, however, to WAEs, but has to be considered also with the corresponding soluble enzyme forms present in the crude cell extract (for a detailed account of the fallacies inherent in the use of chromogenic substrates for glycosidases in the presence of tyrosinase, refer to Horsch et al., 1997).

Finally, an extension of the basic protocol could involve sub-dividing the consortium of class-IV WAEs by sequential treatment of the insoluble residue with β-glucanase, chitinase and NaOH, as used previously (Mrsa et al., 1999; Molina et al., 2000) to fraction wall-

associated proteins (WAPs). But it remains to be shown, whether the WAPs thus solubilised are still identifiable as WAEs by their catalytic activity.

4. Significance of some wall-associated enzymes (WAEs)

4.1. WAEs of chitin metabolism

In contrast to chitinolytic enzymes induced during growth on chitin, under carbon limitation, or during senescence, growing fungi have a complex chitinolytic system consisting of chitinases and HexNAc'ases that is produced under a severe regime of catabolite repression. These enzymes are thus clearly constitutive (Rast et al., 1991). The simultaneous presence of chitinase (producing *N,N'*-diacetylchitobiose = $(GlcNAc)_2$ = "chitobiose" as the end product) and HexNAc'ase is in keeping with the general concept that this represents an obligate component of any biological system effecting the complete degradation of chitin (Kramer and Koga, 1986). There exists a synergism between the two enzymes which act together as a "tandem binary" system (Fukamizo and Kramer, 1985). Mechanistically, this synergism could involve a stereo-controlled transfer of chitobiosyl units from chitinase to a closely associated HexNAc'ase. Indeed, chitobionolactone oxime (Beer et al., 1990), which mimics nascent $(GlcNAc)_2$, is a reasonably good inhibitor of chitinase, whereas free $(GlcNAc)_2$ is not (Rast et al., 1991). The binary chitinolytic system shows some self-regulation, inasmuch as the hydrolytic action of both enzymes is subjected to end product inhibition by $(GlcNAc)_2$ and GlcNAc, respectively. By itself, this control must be weak, since the EC_{50} values are high, i.e. ca. 20 mM for chitobiose (Rast et al., 1991) and 4–6 mM for GlcNAc (Kappes and Legler, 1989; M. Horsch and D.M. Rast, unpublished data). However, the situation is different if the transglycosylating activity of the enzymes is considered additionally. As implicated by the general reaction mechanism of retaining β-glycosidases (Sinnott, 1987, 1990), to which both enzymic components of the binary chitinolytic system belong (for a special consideration of HexNAc'ase, see Horsch et al., 1997), an increased concentration of the end product of chitinase action favours the transglycosylating activity of either enzyme, $(GlcNAc)_2$ serving as an acceptor for higher oligomers with chitinase, and as a donor/acceptor with HexNAc'ase. The increase in the proportion of transglycosylation vs simple hydrolysis events upon the accumulation of $(GlcNAc)_2$, therefore, must lead to a slowdown of further degradation of chitin to GlcNAc. Nevertheless, the action of the binary tandem system on a given pool of chitin is geared inevitably to its complete breakdown—unless there is a continuous replenishment by chitin synthesised de novo.

There are two types of log-phase chitinases. Representatives of the first type (A) are acidic, display partial latency, show an unusual affinity to polysaccharide gels and act weakly on preformed chitin; their performance with chito-oligomers, however, is excellent. Conversely, chitinases of the second type (B) are basic or neutral and nonzymogenic, do not show strong adsorption to such gels and can degrade preformed chitin easily (Rast et al., 1991). This strongly suggests that the two chitinolytic systems (A,B) perform different functions within the wall. The system A presumably acts in the innermost area of the ECM, and B in the adjoining outer regions. These systems are tentatively assigned to WAE-II and WAE-IV entities.

With chitin representing the fibrillar skeleton of the wall conferring mechanical resistance to the protoplast, the presence of an array of chitinolytic enzymes in log-phase hyphae calls for a controlled lysis and co-ordinately regulated synthesis of chitin to provide for both necessities: expansion of the hypha at the tip and at sites of branch initiation and prevention of bursting of the cell. Although regulation of Chs activity by non-allosteric and allosteric activation is sufficient to allow some control of the enzyme's activity in vitro, additional mechanisms must exist that provide for a tight regulation of chitin synthesis in vivo, at the transcriptional as well as at the post-translational level (Choi et al., 1994). Examples are phosphorylation/dephosphorylation as mediated by the Ca^{2+} calmodulin system (Martinez-Cadena and Ruiz-Herrera, 1987; Suresh and Subramanyam, 1997), interaction of Chs secretory vesicles with components of the cytoskeleton (for references, see Gow, 1995) and controlled action of Chs with respect to co-occurring enzymes at the site of product deposition. Concerning these, the correct balance between the activity of an appropriately regulated chitinolytic system and that of the Chs system appears to be paramount in vivo, not only in filamentous but also in yeast growth of fungi (Rast et al., 1991; Cabib et al., 1992; Horsch et al., 1997; Merz et al., 1999b). A speculative scheme for the controlled synthesis and partial lysis of chitin through the concerted action of Chs, chitinase and HexNAc'ase is depicted in Fig. 4. The hypothesis is based on premises (i)–(vii):

(i) The topology of the sites of action of the chitin synthesizing system at the cell surface, encompassing the plasma membrane, the periplasmic space as well as the wall fabric itself (Horsch et al., 1996; Merz et al., 1999a; Section 3.2.2.4).
(ii) The regulatory properties of Chs, encompassing mechanisms of non-allosteric activation (including partial latency; for a discussion of the phenomenon, see Merz et al., 1999b) and allosteric stimulation (Horsch et al., 1996).
(iii) The co-occurrence in log-phase hyphae of genuinely wall-associated Chs, chitinase and Hex-

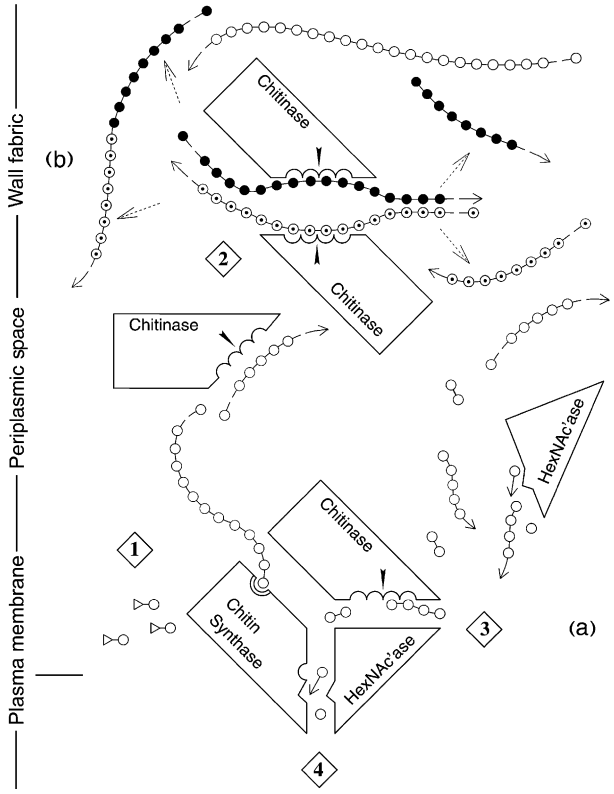


Fig. 4. A biochemical model for the function of WAEs of chitin metabolism in the coordinately controlled synthesis and lysis of chitin in the extracellular matrix (ECM) of growing fungal cells. An indication is given in the graph of the approximate spatial arrangement of the enzymes in the plasma membrane–periplasmic space–wall fabric continuum. An integrated triple enzyme system consisting of chitin synthase (Chs), chitinase and β-*N*-acetylhexosaminidase (HexNAc'ase), and a chitinolytic system effecting hydrolysis of chitin concomitantly with transglycosylation are the two key components of the scheme (adapted from Rast et al., 1991; Horsch et al., 1997; Merz et al., 1999b). Part (a) of the model concerns the synthesis of chitin, and part (b) both the remodelling of chitin through the combined hydrolysing/transglycosylating action of chitinase and the final breakdown of some the polymer chains to the monomer. The chitinolytic enzyme species of parts (a) and (b) differ in substrate chain length preference and some other properties (Rast et al., 1991). The concerted action of (a) and (b) encompasses four steps: (1) de novo synthesis of chitin by activated Chs; (2) remodelling of nascent as well as of preformed chitin chains through the combined transglycosylating/hydrolysing activity of chitinase and HexNAc'ase; (3) progressive lysis of chitin, with increasing amounts of shorter chito-oligomers and *N*,*N*′-diacetylchitobiose ('chitobiose') becoming available for chitinase and HexNAc'ase associated with Chs; and (4) HexNAc'ase-mediated attack of chitobiose resulting in both the generation of GlcNAc available for chitin synthase activation and a GlcNAc residue for transglycosylation to suitable acceptors (for a graphic scheme of the reaction mechanism of HexNAc'ase, refer to Horsch et al., 1997).

NAc'ase, any of which being held in the wall compartment by two types of bonding, namely, hydrophobic interaction in a strongly amphiphilic environment and covalent linkage to a partner of the wall fabric (Horsch et al., 1997; Mayer, 1997; Section 3.2.2.4).

(iv) The presence in Chs species (100 S as well as 16 S size forms) of a chito-oligosaccharide residue, besides an *N*-glycoconjugated partial structure (Merz et al., 1993, 1996, 1999a,b; Merz, 1997), which could conceivably serve as a region of complexation, by carbohydrate recognition, for the non-catalytic high-affinity binding domain of chitinase (Kuranda and Robbins, 1991).

(v) The characteristics of the constitutive chitinolytic system of log-phase hyphae, consisting of several HexNAc'ases and two types of chitinases (A, B; see above).

(vi) The tandem action of the binary chitinolytic system in effecting the breakdown of chitin to chitobiose and GlcNAc (see above).

(vii) The transglycosylating activity of genuinely wall-associated as well as of soluble fungal chitinases and HexNAc'ases (Mayer et al., 1996; Mayer, 1997; Mayer and Rast, 1997; Horsch et al., 1997; Section 3.2.2).

Our near-steady-state model of the synthesis of chitin de novo coupled with partial lysis of chitin—energetically geared by the Chs substrate UDPGlcNAc and a pool of preformed chitin—is depicted in Fig. 4. Part (a) of the scheme concerns the synthesis of chitin, and part (b) the dynamic re-modelling and lysis of chitin, with HexNAc'ase playing a mediator role between the two. For convenience, one turn of the "cycle" may be described as occurring in four steps. (1) Chitin synthesis de novo by activated Chs representing a component of an integrated triple enzyme system encompassing a chitinase preferentially hydrolysing chito-oligomers (chitinase A; see above) and a HexNAc'ase. (2) Restriction as well as extension of the chain length of newly synthesised chitin by the action of chitinase. Because it has substrate length specificity of $n \geqslant 4$—as opposed to HexNAc'ase; which almost exclusively uses $(GlcNAc)_2$ (Horsch et al., 1997)—chitinase can use as alternative acceptors β-1,4-linked residues of the required length that are covalently bound to other polymers, for example, chito-oligosaccharides conjugated with protein or lipid, and thus effect cross-linking. (3) Rearrangement by chitinase (type B) of preformed chitin also, the attack gradually resulting in an increased production of chito-oligomers as the preferred substrates of a proteolytically activatable chitinase (type A) associated with Chs by carbohydrate recognition. (4) Hydrolysis of the end product of chitinase action by HexNAc'ases, one of which is latent and strongly associated with Chs (regardless whether it is present in 100 S Chs and 16 S Chs, or isolated ex walls; presumably the HexNAc'ase entity present in subfraction II of the wall; Section 3.2.2.4), whereby the Chs effector GlcNAc is generated to provide for full capacity binding of UDPGlcNAc and, thence, de novo synthesis of chitin.

There are three major implications inherent in this biochemical scheme for the concerted action of Chs and chitinolytic enzymes in log-phase hyphae:

(i) The β-linked partial structures present in individual chitin chains can be of a dual origin, namely, stretches generated de novo and residues attached by transglycosylating chitinase at the non-reducing, that is the acceptor end of a growing chain.
(ii) Entrapment of Chs into its product (Ruiz-Herrera et al., 1977; Kang et al., 1984) reduces its activity, as undoubtedly the displacement of the enzyme from the source of its substrate at the cell surface to the adjacent area of the developing wall must (for potential mechanisms of externalisation of UDPGlcNAc, see Abeijon et al., 1996; Rini and Sharon, 2000; further references in Merz et al., 1999a). It is this situation, where the mural non-zymogenic chitinolytic system [Fig. 4(b)] has its main function, besides that of providing for a dynamic remodelling of preformed chitin, inasmuch as reduction of the length of Chs-attached chitin to that of a shorter oligomer restores some of the catalytic potential of the Chs system concerned. In addition, the concomitant breakdown of preformed chitin yields an ample supply of the effector GlcNAc to temporarily maintain some further polymer synthesis at this site.
(iii) Considering the mediator function of the triple enzyme system, the transfer of GlcNAc to Chs would be particularly effective if the association between the two enzymes were such that the active site of the former was positioned in close proximity to the allosteric site of the latter. The mediator role of HexNAc'ase could be even more sophisticated, if the enzyme, provided with its substrate by nearby chitinase, was also to modify Chs chemically, and thus, generate a covalently linked acceptor structure. With its generally high leaving group/acceptor tolerance (for reaction mechanism, see Horsch et al., 1997), HexNAc'ase appears intrinsically well befitted for such a function.

The parts (a) and (b) of this biochemical model of the coupled synthesis and partial lysis of chitin in the ECM of growing fungi must not be misinterpreted, at the cellular level, as representing two separate events with, first, complete breakdown of some of the chitin to the monomer and, then, synthesis of chitin to "repair" the weakened chitin skeleton (Wessels, 1999), but to take place simultaneously, in a dynamic manner. For an account of the biochemical model of the controlled chitin metabolism in hyphal growth in the physiological context, refer to Rast et al., 1991, and Horsch et al., 1996, 1997.

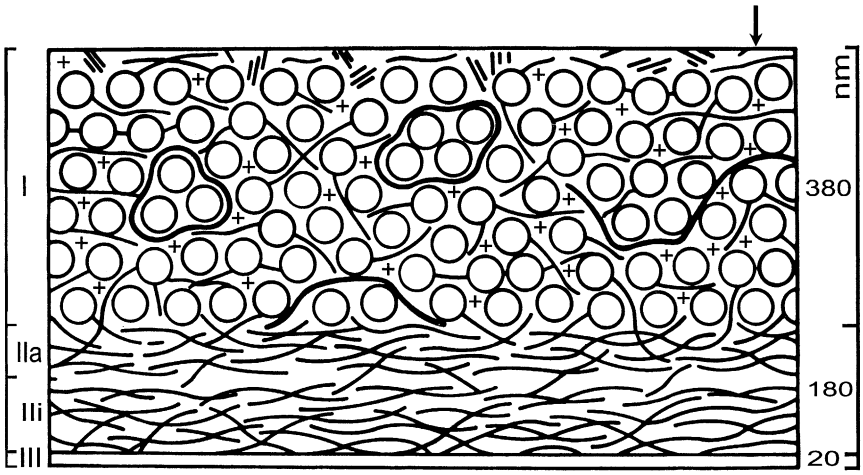
4.2. WAEs oxidising phenols

There can be no doubt that tyrosinase is the only enzyme absolutely essential for the synthesis of "biomelanin" (Robb, 1984; Hearing and Jimenez, 1987, Prota, 1992), although some other enzymes functionally or subcellularly associated with it may play a modulatory role (Orlow et al., 1994; Del Marmol and Beermann, 1996). Melanin can be prepared in vitro, either chemosynthetically or by using tyrosinase from *A. bisporus* as a catalyst, but the resulting product differs from the biogenic material, both physicochemically and ultrastructurally (Pierce and Rast, 1995; Hollenstein, 1997). Tyrosinase displays a broad substrate acceptance, for which reason it is often referred to as polyphenol oxidase in organisms disposing of a variety of potential melanogenic substrates, whereas animals use almost exclusively tyrosine. In animals, tyrosinase is an integral membrane protein located in melanosomes (Hearing and Jimenez, 1987; Prota, 1992), which are specialised lysosomes (Orlow, 1995; Donatien and Orlow, 1995). An analogous situation holds for the intracellular deposition of melanin in vacuoles of *A. bisporus* (Hollenstein, 1997). Melanisation catalysed by soluble tyrosinase is considered either an artefact of cell disruption, which results in the production of melanin-like, fairly soluble brown pigments, or a wound/defense reaction occurring as a response to a variety of stressors, rather than a component of cellular differentiation (Robb, 1984; Prota, 1992). For a comprehensive comparison of the animal and fungal melanogenic systems, refer to Hollenstein (1997).

A fundamental distinction between the melanogenic systems of animals and fungi is that, in the latter, pigment deposition takes place also extracellularly, in a distinct region of the ECM, namely, the part oriented towards the environment (Fig. 5). Since the tyrosinase-catalysed oxidative polymerisation of phenols encompasses all other organic components of the wall as co-reactants and, thus, renders the wall composite intrinsically non-hydrolysable, melanin deposition in muro represents the final step of differentiation in fungal cells. Isolated melanin from *A. bisporus* spores occurs in two modifications of the pigment, granular and amorphous (Rast et al., 1981; Hegnauer et al., 1985; Hollenstein, 1997). This is in keeping with the presence of two types of tyrosinase in the ECM, soluble and particle-bound, respectively (Fig. 3b). A schematic representation of the compartmentation of these *in muro* is shown in Fig. 5.

5. Outlook

Considering the easiness and speed with which WAEs can be fractionated with the protocol presented here (Sections 3.2.2 and 3.3), its application to a large num-



Areas of the wall occupied by class-IV tyrosinase that is bound to the membrane of a 50-60 nm vesicle (termed tyrosinase vesicle). The enzyme becomes covalently linked to and then buried within a complex heteropolymeric supramolecular product, melanin, generated by oxidative polymerisation of phenolic radicals, created through the action of tyrosinase, with co-located protein, lipid and carbohydrate. The resulting product is absolutely inert for the organism synthesizing it and also recalcitrant to most organic solvents

Regions of the wall where deposition of amorphous melanin through the action of class-III tyrosinase occurs, *in statu nascendi* of which all components of the material present in the interstitial spaces between melanin granules become attached to each other through multiple cross-links, the product thus formed being a complex space-filling heteropolymer that is intrinsically non-hydrolysable

Melanin granules with chitin fibrils emanating from them

Sub-regions of the wall loosely delimited by membrane material reminiscent of lomasomal configurations generated upon fusion with the plasma membrane of large vacuoles (ca. 200 nm) that contain tyrosinase vesicles

Net of non-melanised chitin fibrils

Hydrophobin rodlets (Wessels, 1999) embedded in β-1,3- and α-1,3-glucans

The arrow points to the outer face of the wall

Fig. 5. A scheme of the location of wall-associated tyrosinase in the extracellular matrix (ECM) of melanising fungal spores (adapted from Hollenstein, 1997). The model is based on the intrinsic co-location in situ of tyrosinase and its insoluble product, melanin, as well as on a combination of chemical, enzymatic and histochemical studies, by electron microscopy, of spore and subhymenial cells of *A. bisporus* (Rast and Hollenstein, 1977; Rast et al., 1981; Hegnauer et al., 1985; Pierce and Rast, 1995; Hollenstein, 1997). For explanation of symbols, refer to the legend, for the classification of WAEs, see Section 3.2). Class-IV tyrosinase is contained in the membrane of periplasmic bodies, which—upon apposition to the thin wall of the spore initial and activation of the enzyme—serve as the bases for the generation of melanin granules. Class-III tyrosinase is responsible for the deposition of amorphous melanin. For details, see text.

ber of species and cell types could potentially yield valuable data for building-up an operationally-based general information retrieval system of fungal WAEs. This would be useful both as an heuristic basis for investigations into their physiological significance (refer to Section 4) and for biotechnological applications, such as the development of novel antifungal agents.

Interference with fungal parasite–host interactions at the cell-surface level through the application of exogenous inhibitory compounds evidently represents a most appropriate means to prevent or disrupt antagonistic inter-organismic relationships. In order to specifically target the pathogen, such agents must affect a metabolic site of the pathogen that is not present in the host. Disregarding sheer serendipity and setting apart other strategies for the management of fungal plant diseases or mycoses in animals, there are basically two major approaches to the discovery of novel low-molecular weight antifungals: (i) drug design relying on "lead" substances detected by biochemical high-throughput screening (HTS) with the target enzymes as the "screen", followed by chemical modification of the inhibitory compounds detected to increase their potency according to quantitative structure–activity relationships; and (ii) drug design based on the elucidation of target enzyme–ligand interactions, ideally with a three-dimensional model. While the second method may appear intellectually more satisfying, for practical purposes it is clearly not suitable as a tool in the early stages of the search for antifungals (see Horsch et al., 1997). Whether HTS is conducted with enzyme-based screens using libraries of chemosynthetic or natural products, is not critical—inhibitors can be found with either, for example, reaction-based GlcNAc analogues for HexNAc'ase (Horsch et al., 1991, 1993; for a compilation of HexNAc'ase inhibitors, see Rast et al., 2000), and nucleoside-peptides for Chs (Furter and Rast, 1985; for further Chs inhibitors, refer to Rast et al., 2000). Setting aside the type of test compounds and screens used, which might even consist of living cells (Evans et al., 2002), WAEs appear to be most promising targets in antifungal drug research, since antifungals targeted at them need no special mechanisms to overcome the plasma membrane barrier.

Solubilisation of the target enzyme is not a pre-requisite for a meaningful primary screening. On the contrary, the crude cell wall system (Section 3.1) appears to be a better approximation of the situation in vivo than solubilised and partially purified enzymes, and yet does not have the complexity of whole cell screens, results obtained with which are difficult to interpret in terms of structure–activity relationships of inhibitors. The conceptual superiority of the crude cell wall screen over the soluble enzyme screen for predicting inhibition in vitro to reflect antifungal activity in vivo probably lies in the fact that the former intrinsically encompasses some physicochemical parameters that have an influence on the activity of WAEs, but which are not accounted for in the latter. Thus, designed to perform in a semi-solid medium, reactions catalysed by naturally immobilised WAEs have some special features, such as attachment to the matrix in an oriented position, which, though restricting the degree of freedom, stabilises them (refer to Section 2.3), a lowered diffusibility of leaving groups, spatially co-ordinated interaction with co-located enzymes, and restricted accessibility by xenobiotics and substrates—unless the latter ones are generated in situ by closely associated enzymes (for an example, see Section 4.1). More sophisticated biochemical and mechanistic analyses of the enzymes concerned are not required at an early stage of the lead discovery process, except in cases where the inhibitory potency of the test compound is particularly high or the enzyme is known to exist as isoforms with significantly different catalytic performance, as known for some enzymes of β-glucan and chitin metabolism, for example, β-(1–3)-glucanosyltransferases (refer to Table 1) and chitinolytic enzymes (Mayer, 1997; C. Mayer and D.M. Rast, unpublished data).

With the exception of BGs and BG'ase, quite a few good reference compounds are available for the search of new inhibitors of any of the bona fide WAEs referred to in Section 3.2.2 (Rast et al., 2000; Rescigno et al., 2002; D. Baumgartner, C. Unger, A. Jeanguenat, F. Schaub, E. Mösinger and D.M. Rast, unpublished data). With respect to enzymes of β-glucan metabolism, however, the choice of good leads seems to be restricted to certain papulacandins and lipopeptides (see Georgopapadakou and Tkacz, 1995; Georgopapadakou, 2001; Douglas, 2001) and possibly also bisvertinolone (Trifonov et al., 1986; Kontani et al., 1994).

6. Experimental

6.1. Fungus material

Stage-2-sporocarps (Hammond and Nichols, 1976) of *A. bisporus*, produced under conditions of commercial mushroom cultivation as described previously (Baumgartner et al., 1998), served as the main experimental material. *Mucor rouxii* was cultivated as described by Rast et al. (1991).

6.2. Isolation and fractionation of cell walls

All manipulations were carried out in the cold room.

The lower part (two thirds of the stipe) of freshly collected mushrooms (50 g) was removed and the upper part sliced into 50 ml ice-cold 0.25 M Tris–HCl buffer (pH 8.0). The material was collected on a glass filter,

transferred into 70 ml bottles containing 10 ml of glass beads (0.5 mm diam.) and disintegrated in a Braun MSK homogenizer for 4×15 s at 4000 rpm. Phase-contrast microscopy showed that ⩾99 of the cells were broken. The homogenate was centrifuged (4000 *g*, 15 min, 4 °C), to yield the crude cell extract (CCE) and the cell wall fraction (CWF, unwashed sediment). After suspension in 50 ml buffer (50 mM KH_2PO_4/NaOH, pH 7.0; hereafter referred to as P-buffer), the wall fragments were re-pelleted. The washing/extracting procedure was repeated until no more enzyme activity could be detected in the supernatant (typically ×5; sum of buffer washes = subfraction I). The sediment was suspended in P-buffer (50 ml), the wall suspension supplemented with solid digitonin (DIG) to give a final concentration of 0.3% (w/v) and stirred slowly for 3 h at 4 °C (essentially as described previously for the isolation of wall-associated chitin synthase: Hänseler et al., 1983b; Horsch et al., 1996) and chitinolytic enzymes (Mayer, 1997). Following centrifugation (as above), the supernatant was assayed for the presence of released enzyme, and the pellet re-extracted with DIG (sum of the two DIG extracts = subfraction II). The same extraction/centrifugation procedure as with DIG was followed for the subsequent incubation of the wall fragments with NaCl (2 M, 3 h; sum of the two NaCl extracts = subfraction III). The final pellet (subfraction IV) was taken up in buffer (10 ml) and an aliquot of the suspension assayed directly for the respective enzyme activity.

6.3. Enzyme assays

The activities of most of the enzymes tested were sufficiently high to require only small aliquots of the pools in the assay mixtures.

6.3.1. γ-Glutamyltransferase (EC 2.3.2.2; GT)

The assay medium consisted of 1 ml Tris–HCl (0.1 M, pH 8.0) containing γ-L-glutamyl-4-nitroanilide (1 mM), glycylglycine (20 mM) and $MgCl_2$ (11 mM). After equilibration at 37 °C, the reaction was started by adding enzyme extract (20 µl) and the released 4-nitroaniline measured at A_{410} (Bergmeyer, 1974; modified).

6.3.2. Agaritine γ-glutamyltransferase (EC 2.3.2.9; AGT)

The reaction medium contained, in 1 ml Tris–HCl (0.1 M, pH 8.0), $MgCl_2$ (11 mM), agaritine (1 mM; isolated and purified as described by Baumgartner, 1995) and sodium glyoxylate. To this 20 µl of enzyme extract was added after equilibration at 37 °C. The formation of the product, glyoxylic acid-4-hydroxymethylphenylhydrazone, was measured at A_{325} (Levenberg, 1970; modified).

6.3.3. Malate dehydrogenase (EC 1.1.1.37; MalDH)

The cuvette contained 1.3 ml 25 mM HEPES buffer (pH 7.4), 1.4 ml H_2O, 100 µl NADH (3 mg ml^{-1}) and 20–100 µl enzyme preparation. The reaction was started by adding 100 µl oxaloacetic acid (2 mg ml^{-1}) and measured as the oxidation of NADH at A_{340} (Bergmeyer, 1974).

6.3.4. Mannitol dehydrogenase (EC 1.1.1.138; MtDH)

The incubation mixture consisted of 2.4 ml 0.1 mM Tris–HCl buffer (pH 8.0) containing 0.64 M fructose, 200 µM NADPH and 10–40 µl enzyme extract. Other parameters were as described previously (Ruffner et al., 1978).

6.3.5. Tyrosinase (= polyphenol oxidase; EC 1.10.3.1)

The cuvette contained 2.8 ml 5 mM dihydroxyphenylalanine (DOPA) in 50 mM P-buffer at 30 °C. The reaction was started by adding enzyme preparation (200 µl) and the formation of product (Dopa quinone and derivatives thereof: Robb, 1984) measured at A_{490}.

6.3.6. ATPase

The reaction mixture consisted of 0.5 ml 3 mM Tris–ATP and 3 mM $MgSO_4$ in 30 mM Tris–MES (pH 6.5) and 100 µl enzyme extract. Incubation of the samples was in a water bath at 37 °C for 15 min. To terminate the reaction, the vials were placed on ice and 1 ml of stopping/developing reagent was added (freshly prepared by mixing 10 ml of 10% (w/v) of sodium ascorbate with 50 ml of 0.42% (w/v) of ammonium molybdate tetrahydrate in 0.5 M H_2SO_4. The colour was developed at room temperature for 30 min and A_{620} measured (Perlin and Spanswick, 1981). Control tests to assess the suitability of the method also with the *A. bisporus* experimental material contained 30 µg of membrane protein (Bradford microprocedure) from a mixed-membrane fraction (MMF), which was obtained by centrifugation of the CCE at 54000 *g* (R_{av}, Beckman T-50 rotor; 10 min) and washing the pellet with buffer (as described by Hänseler et al. 1983a).

6.3.7. β-(1–3)-Glucan synthase (EC 2.4.1.34)

Considering the dissimilar enzymological properties of β-glucan synthases of different origins, particularly with respect to activating/inhibitory components (Ruiz-Herrera and Sentandreu, 1989), only a minimal incubation medium has been used. The assay mixture consisted of 50 µl of a stock solution of HEPES buffer (pH 7.2) containing (final concentrations of) DIG (0.1%), $CaCl_2$ (50 mM), cellobiose (0.1 M), UDP[^{14}C]Glc (2.0 mM, 3.7 mBq) and 100 µl of enzyme preparation. The mixture was incubated at 30 °C for 30 min, the reaction stopped by addition of EtOH (200 µl) and the product processed as in the standard assay for chitin synthase (see below).

6.3.8. (1–3)-β-Glucanase (presumably EC 3.2.1.39)/ β-glucosidase (EC 3.2.1.21)

The glucanase activity was assayed using 2 ml of wall suspension in sodium citrate buffer (25 mM, pH 4.5) containing 50 mg laminarin (Sigma), and incubating for 45 min at 30 °C. The increase in free glucose was assessed in microtiter plates using 20 μl of the reaction mixture and 200 μl of ABTS [2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid)] cocktail, measuring at A_{450} (Bergmeyer, 1974).

6.3.9. Chitin synthase (EC 2.4.1.16)

The assay was according to a standard method (Hänseler et al., 1983b): the incubation mixture contained, in a total volume of 225 μl, UDP[^{14}C]GlcNAc (0.6 mM; 0.74 Bq), GlcNAc (20 mM), pronase (7.5 μg) and enzyme preparation (125 μl). All solutions were prepared in P-buffer with 10 mM $MgCl_2$. After a reaction time of 30 min, the radioactive chitin formed was determined by a filtration method (Bartnicki-Garcia et al., 1978).

6.3.10. Chitinase

The test used is based on the liberation of 4-methylumbelliferone (MU) from 4-methylumbelliferyl (MUF)-β-*N,N′,N″*-triacetylchitotrioside (MUF3) through the action of the enzyme, as originally described by Leaback and Walker (1961) for the use of the MUF1 analogue as a fluorogenic substrate for HexNAc'ase (see below). Due to the very high sensitivity of the test, i.e. <0.1 pkat ml^{-1} (Mayer, 1997; Horsch et al., 1997), the original WAE preparations had to be diluted prior to use. Assay conditions were: 50 μl enzyme preparation (with catalytic activity conc. of 0.5–5 pkat ml^{-1}) and 50 μl phosphate buffer (pH 6.5, 100 mM) placed in microtiter plates; start of the reaction by addition of 50 μl of MUF3 (50 μM in H_2O; assessment of the formation of MU at 390 nm excitation and 460 nm emission wave length, using a Millipore Cytofluor 2350 fluorescence measurement system (further details in Mayer, 1997).

6.3.11. β-N-Acetylhexosaminidase (HexNAc'ase)

The fluorogenic test based on the model substrate MUF-β-*N*-acetylglucosaminide was as described for chitinase; its principles and potential fallacies upon applying it with crude enzyme preparations have been discussed elsewhere (Horsch et al., 1997). For HexNAc'ase product assessment with its natural substrate, a combination of high performance anion exchange chromatography (HPAEC)/pulsed amperometric detection (PAD) has been used (Horsch et al., 1996). Besides its high sensitivity, this method allows the separation of the monomer and higher β-1,4-linked GlcNAc oligomers, hence, the simultaneous assessment of the products of both hydrolysis and transglycosylation (Mayer et al., 1996; Mayer, 1997; Mayer and Rast, 1997).

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