EXTRACELLULAR POLYSACCHARIDES AS TARGET COMPOUNDS FOR THE IMMUNOLOGICAL DETECTION OF *ASPERGILLUS* AND *PENICILLIUM* IN FOOD

H. J. Kamphuis

Promotor: Dr. ir. F. M. Rombouts,
Hoogleraar levensmiddelenhygiëne en -microbiologie,
Co-promotor: Dr. ir. S. H. W. Notermans,
Plaatsvervangend hoofd van het Laboratorium voor Water- en Levensmiddelenmicrobiologie.
Rijksinstituut voor Volksgezondheid en Milieuhygiëne,
Bilthoven.

H. J. Kamphuis

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STELLINGEN

1. Het produceren van antilichamen tegen enzymatisch en/of chemisch gemodificeerde schimmelimmunogenen is een mogelijkheid voor de ontwikkeling van detectiemethoden voor specifieke, pathogene schimmels in levensmiddelen.
(Dit proefschrift)

2. In verband met de sterke heterogeniteit van polysachariden geproduceerd door schimmels zou (immuno)chemisch onderzoek aan deze polysachariden gebaat zijn bij standaardisatie m.b.t. produktie en isolatie.
(Dit proefschrift)

3. De opvatting dat monoklonale antilichamen de voorkeur verdienen boven polyklonale antilichamen is niet gerechtvaardigd en is mede afhankelijk van het doel waarvoor men de antilichamen gebruikt.
(Dit proefschrift)

4. Gelet op de grote aantallen bekende mycotoxinen en hun toxicologische effecten beperkt de Warenwet zich ten onrechte tot alléén aflatoxinen.
(Warenwet, Algemeen besluit, Uitvoeringsvoorschrift C 1-7)

5. Het detecteren van biogene aminen in levensmiddelen met behulp van isotachoforese is ongeschikt en leidt ten onrechte tot het veelvuldig afkeuren van partijen levensmiddelen.
(K. Rubach, P. Offizorz en C. Breyer, Z. Lebens. Unters. Forsch., **172**, 351-354, 1981)

6. Opname van *Listeria monocytogenes* bv. met voedsel, zou bij de bevolking, met uitzondering van bepaalde groepen, kunnen leiden tot een langdurige bescherming tegen listeriose. (S. Notermans en I. Chakraborty, Proc. 11th Int. Symp. on problems of Listeriosis, Copenhagen, 11-14 may, 1992)

7. De door Hahn en Bransch beschreven immuno-dipstick test voor het aantonen van staphylococcen enterotoxinen werkt niet.
(G. Hahn en B. Bransch, Zbl. Bakt. Hyg. A, **267**, 519-527, 1988)

8. Het door Reyes *et al.* gezuiverde polysacharide splitsende enzym wordt ten onrechte aangeduid als een endo-ß(1,5)-galactofuranase.
(F. Reyes, C. Alfonso, M.J. Martinez, A. Prieto, F. Santamaria en J.A. Leal, Biochem. J., **281**, 657-660, 1992)

9. Cacaoboter heeft geen effect op het cholesterolgehalte in het bloed.
(I.S. Chen, S. Subramaniam, G.V. Vahouny, M.M. Cassidy, I. Ikeda en D. Kritchevsky, J. Nutr., **119**, 1569-1573, 1989)

10. Gezien de afwachtende houding met betrekking tot de applicatie van de schimmel immunoassay zou het op dit moment onmogelijk zijn om de nu wereldwijd geaccepteerde schimmelkiemgetalbepaling te introduceren.

11. Goed Laboratorium Personeel (GLP) is de belangrijkste voorwaarde die Good Laboratory Practice (GLP) doet welslagen.

12. Het verdraaien van feiten geeft meestal een mooiere voorstelling van zaken dan de werkelijkheid, zo ook "rechtsdraaiend" zuivel.

13. Televisiebeelden van voetbalwedstrijden worden ten onrechte gebruikt als bewijslast in arbitragezaken.

14. Nederlands drama wordt door enkele televisieomroepen wel erg letterlijk opgevat.

H.J. Kamphuis
Extracellular polysaccharides as target compounds for the immunological detection of *Aspergillus* and *Penicillium* in food. 29 september 1992, Wageningen.

Aan mijn ouders

The work presented in this thesis was performed at the Department of Food Science, Wageningen Agricultural University, Wageningen, The Netherlands, and at the Laboratory of Water- and Food Microbiology, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands.

CONTENTS

CHAPTER 1

INTRODUCTION

Food mycology may be defined as the study of the interaction of fungi with food, in both beneficial (fermentation) and deleterious (spoilage and mycotoxin production) ways. Fungi are a large group of eukaryotic organisms which can be divided in moulds and yeasts. Yeasts are important micro-organisms because of the application of their alcoholic fermentation and the interest in metabolic products from yeasts, e.g. enzymes. Also, conditions favourable for their growth can be a major factor in deterioration of food, and may cause great economic losses.

Moulds have also been exploitated in biotechnology for the production of metabolic products (enzymes) and for the production of various types of fermented foods including some types of cheeses (Roquefort and Camembert cheese). Tempeh is another well-known product obtained by fermentation of soya beans, using *Rhizopus oligosporus*. Moulds are also used as protein source. In 1985 the Ministry of Agriculture, Fishery and Food in the United Kingdom officially approved the sale of a mycoprotein, called Quorn. This food, produced by continuous fermentation of *Fusarium graminearum*, is a fibrous and protein rich food with a texture close to meat.

The presence of moulds in raw materials, feed and food, however, can also lead to spoilage and the production of mycotoxins. Food losses resulting from the occurrence of moulds are a worldwide economic problem. The Food and Agriculture Organization have estimated that due to moulding approximately 25 % of all food production worldwide is lost after harvesting (O'Neill *et al.*, 1991). Moulds present on raw materials, like cereals, have traditionally been divided in "field moulds", (amongst others *Cladosporium, Alternaria, Verticillium* and *Fusarium*) and "storage moulds" (such as *Aspergillus* and *Penicillium* species). Especially "storage moulds" cause spoilage. It has been shown, however, that also "field moulds" can induce spoilage during storage. The production of mycotoxins can result in mycotoxicoses both in animals and humans (Moreau and Moss, 1979). The toxicology of only some mycotoxins is well established (Moreau and Moss, 1979; Wyllie and Morehouse, 1978). The major targets of some naturally occurring mycotoxins are liver (amongst others aflatoxins),

kidneys (ochratoxin A), uro-genital tract (zearalenone) and the immune system (trichothecenes). Some mycotoxins such as aflatoxin and fumonisins possess carcinogenic activity (Gelderblom *et al.*, 1988).

Because of economic losses as a result of spoilage of food and feed and the public health risk due to mycotoxin production it is essential to possess reliable detection methods for moulds and/or mycotoxins. Techniques for the detection of mycotoxins have been developed and some are commercially available. However, due to the high number of mycotoxins known (>400) and the different isolation methods it is almost impracticable to determine all mycotoxins potentially present in food. To guarantee safe food, absence of moulds may be the most appropriate approach. In course of time several assays have been developed for the detection of moulds in foods. A comprehensive overview of the existing methods for the detection and classification of moulds is presented in **Chapter 2.**

Aim of this thesis

This thesis is devoted to the immunological detection of *Aspergillus* and *Penicillium* in food products. More specifically, the extracellular polysaccharides of these moulds are studied as target compounds for their immunological detection in foods.

As pointed out in **Chapter 3**, some studies give strong evidence of the presence of immunodominant ß-D-galactofuranosyl residues in mycelial galactomannans of *A.fumigatus* (Bennett *et al.*, 1985; Suzuki and Takeda, 1975) and extracellular glycopeptides produced by *Penicillium* (Gander *et al.*, 1974; Preston *et al.*, 1970). The galactofuranosyl residues have also been determined in extracellular polysaccharides of *Aspergillus* and *Penicillium* (Notermans *et al.*, 1988). The immunogenicity and antigenicity of these EPS were used to develop an immunoassay for the detection of these EPS in food and feed (Kamphuis *et al.*, 1989).

The aim of this study is to obtain additional knowledge about the (immuno)chemical characteristics of *Aspergillus* and *Penicillium* EPS and their use for the detection of these genera in food and feed.

An overview of the mould detection methods is given in **Chapter 2** and the immunogenicity and antigenicity of polysaccharides produced by moulds is described in **Chapter 3**.

Conjugates of tetanus toxoid and ß(1,5)-linked D-galactofuranosyl oligomers were synthesised for the production of specific antibodies against the ß(1,5)-linked D-galactofuranosyl residues. The antigenic specificity of these antibodies was compared with the antibodies raised against *P.digitatum* EPS (**Chapter 4**).

In **Chapter 5** the purification and characterisation of an exo-ß-D-galactofuranosidase are described. Enzymatic and chemical studies were carried out with *Aspergillus* and *Penicillium* EPS. A reductive cleavage method has been used for the reliable determination of ß(1,5) and ß(1,6)-linked galactofuranose residues in these EPS. This study resulted in a new structural model for the immunodominant galactofuranose side chains of *Penicillium digitatum* galactomannans.

Applied research has shown, that *Penicillium* EPS could not be detected in mould ripened cheeses like Camembert and Roquefort. Since no reasons were available for the absence of EPS in these cheeses, the influence of medium components on the production of EPS by two *Penicillium* species, namely *P.digitatum* and *P.aurantiogriseum* was studied. The results are described in **Chapter 6**.

Antibodies raised against *Penicillium* EPS are directed to the immunopotent ß(1,5)-linked galactofuranosyl residues of these EPS. However, EPS of *Penicillium* may possess immunosilent structural features. These may also be present in EPS of other genera. Production of antibodies against these immunosilent residues and the possible use of these antibodies for the detection of different food-borne mould genera in one assay is described in **Chapter 7**. For this the immunopotent ß(1,5)-linked galactofuranosyl residues of *Aspergillus* and *Penicillium* EPS were hydrolysed and antibodies against these acid-hydrolysed EPS were raised in rabbits.

In **Chapter 8** a description of a rapid and reliable latex-agglutination assay for the detection of *Aspergillus* and *Penicillium* EPS in food and feed is presented. The reliability could be enhanced by the introduction of a synthetic ß(1,5)-linked galactofuranose tetramer for the determination of false-positive results.

Finally, in **Chapter 9** the importance and the (immuno)chemistry of extracellular mould polysaccharides and the use of these EPS for the detection of *Aspergillus* and *Penicillium* is discussed. Results of comparative and collaborative studies using the latex-agglutination assay in combination with other mould detection methods are described.

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CHAPTER 2

METHODS OF DETECTION AND CLASSIFICATION OF MOULDS

Several methods have been developed for the enumeration of moulds. They can be divided in direct, indirect and culturing methods. A direct method is amongst others the Howard Mould Count. With indirect methods metabolic products of moulds such as ergosterol and enzymes are assessed. Culturing methods include mould count, electrical impedance measurement and adenosine triphosphate assays. The advantages and disadvantages of these enumeration methods for the detection of moulds have been described amongst others by Jarvis (1978) and Jarvis *et al.* (1983). Relatively new are DNA techniques, determination of electrophoretic protein patterns and secondary metabolite profiles (chemotaxonomy). These methods are used for taxonomic purposes, mainly.

The immunogenic and antigenic properties of the cell wall (components) and extracellular substances of moulds has recently resulted in the development of immunoassays for the detection of moulds in food (See Chapter 3).

Howard Mould Count

The Howard Mould Count (HMC) is one of the most widely used direct microscopic methods for detection of moulds. Originally, the method was used for quality control of tomato products (Howard, 1911). Using the HMC, a number of standardised microscopic fields are examined for the presence of mycelium pieces. However, a positive field may contain one single mould hyphae or large clumps of mycelium, which results in a coefficient of variation of 15% or more. In general, homogenisation of samples results in an increase of the HMC, indicating that homogenisation is a major variable in the HMC technique. Since mould identification with the use of HMC often leads to errors in the final results, specific mould staining techniques are of benefit. However, some stains such as Congo red are not sufficiently specific. The use of specific labelled antibodies, towards mould mycelium has proven to be more successful. This technique, referred to as immunofluorescence can reduce errors in

the HMC technique. Initially it was very difficult to produce genus specific antibodies against mycelial fragments (Robertson *et al.*, 1988). However, for quantification of total mould count genus specific antibodies are not always necessary (Robertson and Patel, 1989).

Mould count

The principle of enumeration of viable moulds in food consists of preparation of a homogenate followed by producing serial 10-fold dilutions in an appropiate diluent and plating the different dilutions on suitable solid culture media (Jarvis *et al.*, 1983). Viable moulds present will produce colonies after timely incubation. These colonies are counted and determine the mould count. The results of the mould count depend on many factors e.g. the sample size, which should be as large as can be handled. The results are also influenced by the preparation of the homogenates, for example blending or stomaching. Even the type of the blendor may influence the results (Jarvis *et al.*, 1983). Effective blending depends on the nature of the food, the type of blendor and the blending time. In general, the use of wetting agents such as Polysorbitan 80 and Tween 80 is beneficial in the analysis of dried products. However, these agents may influence the viability of moulds (Richard-Molard, 1986). For the detection of sublethally damaged moulds, resuscitation or pre-soaking prior to plating have been shown to be benifical, especially from dried, intermediate moisture and low pH foods (Jarvis *et al.*, 1983; Seiler, 1986). Aliquots for subsequent dilution and plating of food homogenates should be removed within 2 min after mixing because longer settling times will result in underestimation of the viable moulds. For the enumeration of moulds different plating techniques, incubation times, -temperatures and culture media are used (Zipkes *et al.*, 1981; Jarvis, 1973; Jarvis *et al.*, 1977; Koburger and Norden, 1975). It has been found that the spread plate technique results in significantly higher mould counts than the pour plate technique. The spiral count technique in turn gives higher counts than the spread plate or pour plate technique. Frequently, mould propagules present in the homogenate do not follow the strict decimal progres-

sion. Possible explanations are 1) fragmentation of mycelium and breaking of spore clumps during prolonged shaking/agitation or dilution and 2) competitive inhibition on plates with large numbers of colonies (Jarvis *et al.*, 1983).

Generally used counting media are oxytetracycline glucose yeast agar (OGY), malt extract agar (MEA), potato dextrose agar (PDA), wort agar, oatmeal agar (OA) and dichloran rose bengal chloroamphenicol agar (DRBC). It may be clear that the results obtained by different methods cannot be compared easily. This has lead to recommendations concerning the enumeration of moulds in foods (ISO/DIS 6611.2; King *et al.*, 1986; Samson *et al.*, 1992).

Selective media are available for specific detection of some species or genera. Pitt *et al.* (1983) developed a selective medium for the detection of *Aspergillus flavus* and *A.parasiticus*, based on the specific reaction of these species with ferric ammonium citrate. Within 42 hours (after plating) results can be obtained with a very low percentage of false-positive or false-negative results. Dichloran (2,6-chloro-4-nitro-aniline) chloroamphenicol peptone agar (DCPA), described by Andrews and Pitt (1986), inhibits many common storage moulds allowing growth of other genera such as *Fusarium* and the dermatiaceous hyphomycetes. For the detection of colonies of some *Penicillium* species, Frisvad (1983) developed the pentachloronitrobenzene rose bengal yeast extract sucrose agar (PRYES). Groups of *Penicillium viridicatum*, producing different mycotoxins, e.g. ochratoxin A, can be recognised on this agar by specific colour formation. Creatine sucrose agar was helpful as an additional tool in the identification of *Penicillium* subgenus *biverticillium* species (Frisvad, 1985). Agar, containing dichloran and 18% (w/w) glycerol (DG 18) is a medium with a low water activity and has been developed for the enumeration of xerophylic fungi like *Eurotium* and *Wallemia* species from low-moisture foods (Hocking and Pitt, 1980). Nash and Snyder (1962) described a selective medium containing pentachloronitrobenzene (PCNB) as selective agent. The medium is claimed to be selective for *Fusarium* species. PCNB has also been used in a modified potato-dextrose agar for the isolation of *Fusarium* on barley (N.N., 1981). However, due to the potential carcinogenic properties of PCNB it is less desirable as media component. Czapek-Dox agar inclu-

ding the fungicides iprodione and dichloran (CZID) has also been described as a selective medium for isolation of *Fusarium* species (Abildgren *et al.*, 1987). However, the selectivity for *Fusarium* is subject of discussion (Thrane *et al.*, 1992). Growth and pigmentation on media containing different sugar alcohols, such as sorbitol and xylitol has been used for selective enumeration of *F.oxysporum* and *F.solani* (Brayford and Bridge, 1989).

After enumeration of the moulds it is possible to identify the mould isolate to species level. For this, colonies have to be transferred to specific identification media. These media enable identification based on growth characteristics and morphology. For example, oatmeal agar in combination with Synthetischer Nährstoffarmer Agar are used for identification of *Fusarium*. Malt Extract Agar in combination with Czapek Agar are used for identification of *Aspergillus* and *Penicillium* (Samson and van Reenen-Hoekstra, 1988).

For detecting low numbers of organisms the so-called Most Probable Number (MPN) technique has been used. The MPN technique for moulds is rarely used because for this technique a Poisson distribution and linear dilution factors are required; criteria which are not met for moulds.

Ergosterol assay

Ergosterol is the major fungal sterol and is suitable for a relatively straightforward assay (Seitz *et al.*, 1979). The assay consists of blending the sample, methanol extraction, alkaline saponification and petroleum ether extraction, followed by HPLC detection using UV absorption. Seitz *et al.* (1979) showed that in case of growth of important grain fungi on milled rice, the ergosterol assay was very sensitive to early growth of these fungi in comparison with the chitin assay. Zill *et al.* (1988) showed, that zearalenone production by *Fusarium graminearum* 480 started at a level of 50 mg/kg ergosterol and increased rapidly in the stationary phase of growth.

The ergosterol content can also be used as an indicator of fungal invasion in grains (Seitz *et al.*, 1977). Müller and Lehn (1988) determined the ergosterol content in

different cereals. They found, that the ergosterol content in corn was significantly lower than in oats, barley and wheat. Furthermore, no relation was found between the ergosterol content and the quality of the cereals. Nout *et al.* (1987) showed, that in case of *Rhizopus oligosporus* the ergosterol content can vary considerably when grown in laboratory media. The ergosterol content was influenced by the extent of aeration and incubation time. The ergosterol content was higher in soya beans (60-90 μg/mg biomass dry matter versus 2-24 μg/mg biomass dry matter in laboratory media). It was also demonstrated by Johnson and McGill (1990) that the media composition affects ergosterol contents.

The disadvantages of the ergosterol assay are 1) the use of caustic reagents, 2) the variation in ergosterol content and 3) ergosterol is not specific for mould growth only. Additionally, there are some reports concerning the instability of ergosterol if exposed to oxygen or UV light (Fieser and Fieser, 1959; Roberts and Caserio, 1964). Schwadorf and Müller (1989) could not confirm the instability of ergosterol if exposed to oxygen.

Chitin assay

Unlike cell walls from bacteria and plants, those of moulds contain chitin (poly ß-(1,4)-N-acetyl-glucosamine) in association with other structural polysaccharides such as mannan and glucan (Bartnicki-Garcia, 1968). Glucosamine, released from chitin by acid (Lin *et al.*, 1985; Stahmann *et al.*, 1975), alkali (Cousin *et al.*, 1984; Bishop *et al.*, 1982; Ride and Drysdale, 1972) or enzymatic hydrolysis (Ride and Drysdale, 1971), can be assayed chemically. Usually colorimetric methods have been used to detect glucosamine (Tsuji *et al.*, 1969) for example in corn and soya bean seeds (Donald and Mirocha, 1977) and processed tomato products (Jarvis, 1977; Bishop *et al.*, 1982). Hubbard *et al.* (1979) and Stahmann *et al.* (1975) used ion-exchange chromatography to detect specifically glucosamine in grains and vegetables. Lin and Cousin (1985) used this technique for the detection of glucosamine in processed fruit and vegetable products. Jarvis (1977) showed that the presence of chitin in non-homogenised juices

and purees was highly correlated with the Howard Mould Count. However, a low correlation was observed when homogenised juices were analysed. Addition of various levels of fungal mycelium or glucosamine before hydrolysis gave recoveries ranging from 92 to 104% of the expected levels. A significant correlation between the HMC and the fungal glucosamine content (chitin) was also confirmed in a study of Bishop *et al.* (1982). Cousin *et al.* (1984) evaluated the chitin assay using several tomato pathogenic fungi. Inoculation of tomatoes, fruit purees and nectars with these moulds showed variation in the glucosamine levels depending on the product, quantity of mycelium, hydrolysis time, potassium hydroxide concentration, age and species of mould. It was shown that *Rhizopus stolonifer* mycelium contains substantially higher chitin levels than the other species investigated e.g. *Geotrichum candidum* and *Alternaria alternata*. When foods where spiked with moulds and analysed, the mould biomass added was linearly correlated to glucosamine, but not to the Howard Mould Count. Chen and Johnson (1983) showed, that the chitin-assay may be suitable for rapid quantitative determination of chitin in wood decay fungi.

The disadvantages for the estimation of moulds by the chitin assay are 1) the variation in intrinsic food glucosamine levels 2) contamination by invertebrates which contain chitin in their exoskeletons and 3) the assay is laborious and involves the use of caustic reagents.

Enzymatic methods

Enzymatic methods are based on the detection of fungal enzymes. However, some of these enzymes are food-borne, which is a great disadvantage of this method. Wood (1979) determined the mycelial growth of the edible fungus *Agaricus bisporus* on composted wheat straw by measuring the extracellular laccase activity. The mycelium growth was directly proportional to the quantity of extracellular laccase. Fungal laccases are normally copper-containing proteins capable of oxidizing *o*- and *p*-phenols and aromatic amines. In a subsequent study, Wood (1980) estimated that the laccase production was medium dependent. Therefore quantification of the enzyme is

not suitable for accurate assaying unknown quantities of *A.bisporus* mycelium in solid substrates. Matcham *et al.* (1985) showed that the dry matter content of mycelium of *A.bisporus* in liquid cultures during 56 days growth period was directly proportional to quantities of fungal derived chitin, ergosterol and extracellular laccase. However, after 28 days a decline in laccase activity was observed probably due protease activity.

The production of laccase is one of the rare studies done to use enzyme production for fungal detection. Recently however, Johnson and McGill (1990) studied the use of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) activities as a measurement of fungal mass. In defined conditions these activities could be used as an analogue of fungal mass. However, further research is needed to investigate the enzyme activities under other physiological conditions and the (possible) presence of these enzymes in complex biological systems from non-fungal sources (Cohen, 1971).

Electrical Impedance Measurement

Growth of micro-organisms results in changes in the electrical conductivity of culture media as a consequence of metabolism. A study of more than 30 species of "storage moulds" showed, that changes in the electrical properties of the medium can be related to the level of conidia inoculated (Jarvis *et al.*, 1983). When used for examination of food samples it is also necessary to use appropriate selective culture media containing amongst others antibiotics to inhibit bacterial growth. Even so, the method lacks sensitivity, many constituents cause interference and therefore it is not suitable as detection method.

Adenosine triphosphate (ATP) assay

Detection of ATP is based on the photochemical reaction of luciferin-luciferase. Estimation of mould ATP can only be applied if the intrinsic levels of ATP in foods are low and/or when mycelium and conidia can be separated from food materials (Sharpe *et al.*, 1970). It is, however, impossible to separate fungal ATP from bacterial

ATP in mixed populations, as normally present in food.

Immunoassays

Immunoassays are worldwide used as detection methods in different fields, for example microbiology and medical science. As already mentioned Robertson and Patel (1989) used antibodies in the Howard Mould Count. The immunogenic properties of polysaccharides has resulted in an extensive research to use cell wall or extracellular polysaccharides of moulds for the development of an immunoassay for the detection of moulds. Lin and Cousin (1987), Notermans and Heuvelman (1985) and Notermans and Soentoro (1986) used the immunogenicity of extracellular polysaccharides for the production of antibodies, which were very suitable for specific detection of moulds. Since that time much research has been carried out to investigate the applicability of immunoassays for the detection of moulds. An overview is given in Chapter 3.

CLASSIFICATION METHODS OF MOULDS

DNA techniques

Molecular biological techniques have been introduced for the detection of fungi. Henson (1989) used a DNA probe for the identification of the soilborne ascomycete *Gaeumannomyces graminis* var. *tritici*, an important etiologic agent of root diseases of wheat and barley. MspI digested total (nuclear and mitochondrial) DNA was cloned into a multicopy vector, pUC18. One plasmid, pMSU315 containing a 4.3-kilobase mitochondrial DNA fragments was used for hydridisation experiments.

The so-called polymerase chain reaction (PCR) is based on *in vitro* amplification of a specific DNA fragment. Oligonucleotide primers are annealed in positions flanking the DNA of interest. Then, by successive heating and cooling, a heat-stable DNA polymerase elongates these primers. Since the newly synthesised DNA can serve as a

template in the next amplification step, the number of copies increases exponentially. The fragments of interest which will be produced after amplification can be visualised on stained agarose gels (Schesser *et al.*, 1991). For the detection of *Gaeumannomyces graminis* primers were used which amplify two fragments (287 and 188 basepairs in size). The PCR technique has also been used to detect *Phoma tracheiphila*, causing so-called "mal secco', a serious wilt disease of lemon orchards (Rollo *et al.*, 1990). At the moment, however, in food mycology molecular biological techniques are almost exclusively used to determine the genetic relatedness of fungi.

Manicom *et al.* (1987) screened a range of *Fusarium* species for DNA restriction fragment length polymorphisms (RFLP). For this, random probes were generated from total DNA of an isolate of *F.oxysporum* f.sp. *dianthi*. Four probes within a range of 760 to 3400 basepairs were used for hybridisation experiments after DNA digestion with Hind III and EcoR1. Combination of probes and restriction enzymes enabled differentiation at species, formae speciales and isolate level. Because of the complexicity of bands after total DNA digestion the use of less complex mitochondrial DNA is advantageous.

Using mitochondrial DNA Mochizuki *et al.* (1990) showed close relationship between the human dermatophyte *Trichophyton interdigitale (T.mentagrophytes* var. *interdigitale*) and the teleomorphic members of the *T.mentagrophytes* for example *Arthroderma benhamiae, A.simii* and *A.vanbreuseghemii*.

Moody and Taylor (1990) used chromosomal DNA for RFLP analysis of the *Aspergillus flavus* group, including the species *A.flavus, A.parasiticus* and *A.nomius*. The results indicate that *A.flavus, A.parasiticus* and *A.nomius* can be distinguished by nuclear and mitochondrial DNA polymorphisms. However, the variation in rDNA spacer regions and the mitochondrial DNA's of these is relatively small, indicating that the three species are closely related. The same results were observed by Kurtzman *et al.* (1987). Kurtzman and co-workers (1986) determined that the aflatoxin-producing fungi *A.flavus* and *A.parasiticus* showed many phenotypic similarities with the non-aflatoxinogenic species *A.oryzae* and *A.sojae*. The latter two species are widely used throughout the Orient as koji moulds for fermentation of sake, miso and soya

sauce. In the West *A.oryzae* has been additionally used as a source for amylolytic enzymes. The genome size was similar and all the four species have high (69-100%) nuclear DNA complementarity, indicating a close genetic relation. It has been suggested that *A.oryzae* may have been derived from a natural variant of *A.flavus* through longterm successive cultivation on rice. Klich and Mullaney (1987) digested total DNA (chromosomal and mitochondrial DNA) of *A.flavus* and *A.oryzae* with the restriction enzyme SmaI. The electrophoretical pattern of the digested DNA had a consistent intraspecific pattern for the *A.flavus* and *A.oryzae* isolates.

Recently, Meyer (1991) suggested that it may be possible to use mitochondrial DNA (mtDNA) restriction patterns alone to identify *Trichoderma* strains. mtDNA characteristics have been shown to be promising in identifying species (Bruns *et al.*, 1988; Martin, 1990[a]; Martin and Kistler, 1990[b]; Smith and Anderson, 1989; Taylor and Natvig, 1989).

Protein patterns

Techniques using the differences in protein and enzyme patterns are in general only used for taxonomy and not for the detection of moulds. These techniques are based on the electrophoretical separation of crude extract of proteins/enzymes on for example polyacrylamide gels. However, the interpretation of enzyme patterns, exhibiting many bands, may be very complex, if not impossible. It is also necessary to have the disposal of optimal buffer systems and the optimal conditions for extraction of each enzyme. The intraspecific variation may be low and some isoenzymes are developmentally regulated and show tissue specificity. Glynn and Reid (1969) investigated the electrophoretic patterns of soluble fungal proteins produced by *Fusarium* species and they concluded that their concept is not useful as a taxonomic tool, because soluble protein patterns depend amongst others on the growth state of the moulds. It is important to determine whether the age and the culture conditions may influence the protein patterns before this technique will be used for taxonomic purposes. Nevertheless, work on *Neurospora, Septoria, Phytophthora* suggested that protein

patterns could be useful as taxonomic criterion (Chang *et al.*, 1962; Durbin, 1966; Clare, 1963). Recently, de Hoog and Amberger (1990) compared the electrophoretic patterns of water-soluble proteins of 35 strains (7 species) of *Galactomyces* and *Dipodascus* with their respective *Geotrichum* anamorphs. With iso-electricfocusing quantitative differences among some species were found. Qualitative differences were found between the teleomorph genera.

Scala *et al.* (1981) determined the endopolygalacturonase zymograms of 6 *Fusarium* species, using iso-electricfocusing in polyacrylamide gels. All isolates were surface cultured on Czapek-Dox with 1% pectin. After 5 days at 23°C the culture filtrate was concentrated and iso-electricfocusing was carried out. With exception of *F.moniliforme* and *F.solani* the patterns of isolates of each species were identical. In case of *F.moniliforme* and *F.solani* the intensity of the enzyme activity varied. The endopolygalacturonase pattern of *F.oxysporum* formae speciales were simpler than those of the other *Fusarium* species. The endopolygalacturonase zymograms may be useful for identification of *Fusarium* at species level. Abd-el-rehim and Fadel (1980) showed in a comprohensive study that immuno-electrophoretical differences exist between phytopathogenic *Fusarium* species.

Correlations of pectinase and amylase isoenzymes were observed between groups of *Penicillium* subgenus *biverticillium* species (Paterson *et al.*, 1989). Electrophoretic patterns of cellulase and pectin lyase of *A.sojae* and *A.oryzae* showed that these two species were distinct (Nasuno, 1974).

Chemotaxonomy

Chemotaxonomy is based on the specific production of both intra and extracellular secondary metabolites, giving a profile which may be unique for species (Andersen, 1991). This means that the metabolite profiles may be useful in taxonomy. Frisvad and Filtenborg (1989) determined secondary metabolite profiles of the terverticillate penicillia, which were specific for each taxon. However, relatedness between some species was very high according to the metabolite profiles. Frisvad *et al.* (1990) also

used chemotaxonomy for the identification of species of the ascomyceteous genus *Talaromyces*. Andersen (1991) showed that *Penicillium brevicompactum* produced a consistent group of phenolic compounds which could be used as taxonomic tool.

Conclusion

A large variety of methods exists for the detection and classification of moulds. Many of the detection methods are based on entirely different principles and they cannot serve the same purpose, neither can results obtained with different methods be compared. In view of the importance of moulds in foods, and because changes in food manufacture and storage practice, increase the problems of mould spoilage, there is an urgent need for new, convenient and reliable methods in food mycology. To quote Williams (1989):"There is no single "mould method", neither should there be. The choice of method depends entirely on the problem to be solved".

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CHAPTER 3

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IMMUNOGENICITY AND ANTIGENICITY OF EXTRACELLULAR POLYSACCHARIDES PRODUCED BY MOULDS

Immunogenicity of polysaccharides

The interest in polysaccharide research has been enhanced since the observation that polysaccharides possess immunogenic and antigenic properties (Heidelberger and Avery, 1923). Immunogenicity is defined as the ability to provoke an immune response and antigenicity will be circumscribed as the capability to react with specific antibodies.

Many investigators have worked on the immunogenicity of bacterial polysaccharides (Jann and Westphal, 1975; Sutherland, 1977). Structurally simple polymers with repeating determinants, like pneumococcal polysaccharide type III are as a rule poor immunogens (Humphrey *et al.*, 1964; Möller and Michael, 1971; Richter, 1970). In contrast to proteins, the typical oligosaccharide pattern of polysaccharides results in only one or a few immunological determinants (epitopes), each of which occur in relatively large numbers (high epitope density). The immunogenicity of polysaccharides has been shown to be molecular weight dependent (Kabat and Bezer, 1958; Howard *et al.*, 1971). Polysaccharides are capable of direct induction of B-cells to produce specific antibodies. This thymus-independent (TI) antibody response of many polysaccharides, however, does not result in a strong secondary IgG response, but elicits mainly an IgM response. On the other hand, the immunogenic memory can be maintained for several years (Richter, 1981). The differences with protein immunogens may be explained by the hypothesis that antibody production requires a high epitope density delivered to B-cells for elicitation. The low natural epitope density of proteins makes processing, concentration and presentation of determinants by macrophages and T-cells necessary for the elicitation of B cells. Thus, proteins mostly elicit thymus dependent, and polysaccharides mostly thymus-independent (TI) antibody responses (Richter, 1981). TI-antigens are sub-divided in two groups, namely T-cell independent type 1 and type 2 (TI-1 and TI-2). TI-1 antigens are able to induce

an immune response in CBA/N mice, this in contrast to TI-2 antigens (Mosier *et al.*, 1977). However, conjugation of structural features of oligosaccharides as haptenic group to protein carriers results in a thymus-dependent immune response, which elicit antibodies against the haptenic part of the protein-conjugate (Rüde *et al.*, 1966). These antibodies are in general capable to react with polysaccharides exhibiting the same structural features as the haptenic group (Avery and Goebel, 1931; Goebel and Avery, 1931; Richter and Kagedal, 1972; Richter and Akerblom, 1983; Richter and de Belder, 1976; Richter and Eby, 1985). The specificity of the antibodies thus induced is dependent upon the chemical constitution of the carbohydrate oligomer irrespective of the protein to which it is bound. This observation has been used for making vaccines (Mäkelä *et al.*, 1977). In general, there is a great heterogeneity of antibody combining sites to polysaccharide antigens. (Cisar *et al.*, 1974; 1975; Lai and Kabat, 1985).

The immunogenic properties of polysaccharides resulted in many studies concerning the structural analysis of polysaccharides produced by moulds and the development of immunoassays for the detection of these polysaccharides in food and feed. However, the designation of these immunogenic and antigenic polysaccharides is not univocal and therefore misleading. Despite the presence of a minor part of protein still the term polysaccharide is often used. In this chapter the name, as mentioned in the references will be used. In Chapters 4-9, however, the term extracellular polysaccharide will be used.

Production of extracellular polysaccharides by moulds

Haworth *et al.* (1935[a], 1937) published the structure of galactocarolose, a 5-O-ß-D-galactofuranosyl containing decasaccharide and mannocarolose, an α-D-mannopyranosyl containing nonasaccharide isolated from a 28-days culture filtrate of *P.charlesii*. Preston and Gander (1968) and Preston *et al.* (1969[a,b]) determined that the major extracellular polysaccharides produced during a 30-days stationary culture period by *Penicillium charlesii*, contained primarily mannose, galactose in the furanose configu-

ration, glucose and phosphorus. Approximately 50% of the carbohydrate content of the intact polymer existed of galactose. The molecular weight range of these polymers was estimated between 10 and 50 kD and the polymers were heterogeneous in composition. Investigations under more controlled conditions of shaked cultures revealed that the produced glycopeptide contained no glucosyl residues and was reasonably homogeneous in composition (Gander *et al.*, 1974). This glycopeptide, called a peptidophosphogalactomannan (pPGM; Fig. 1) contained a peptide part to which mono- and oligosaccharides were attached through O-glycosidic linkage of the terminal mannosyl residues to the hydroxyl groups of seryl and threonyl residues (Rick *et al.*, 1974). The mannose residues were primarily linked throught α(1-2)- and α(1-6)-O-glycosidic linkages. The galactose residues occurred in the furanosyl configuration ß(1-5)-linked in chains of variable lenght (8-10 residues) which were attached to the C-3 of a mannopyranosyl residue (Gander *et al.*, 1974). It contained also ethanol-amine and choline and both were linked through phosphodiester linkages to C-6 of mannopyranosyl residues of the pPGM (Unkefer and Gander, 1979; Unkefer *et al.*, 1982; Rick *et al.*, 1974). Variation in phosphoryl substituents in pPGM of *P.charlesii*, grown upon a wide variety of media, has been observed (Salt and Gander, 1985). The polysaccharides characterised by Haworth *et al.* (1935[a], 1937)

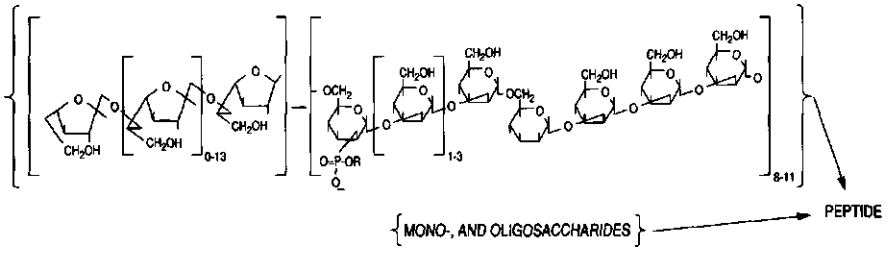

Figure 1. Model of the exocellular glycopeptide (pPGM) from *Penicillium charlesii* (Gander *et al.*, 1974)

were derived from this pPGM.

The interest in these fungal extracellular glycopeptides was enhanced due to the immunogenicity of these glycopeptides. The galactofuranosyl residues play an important role in the antigenicity as will be shown later in this paragraph. Extracellular polysaccharides from *P.charlesii, P.chrysogenum, P.raistrickii, P.claviforme* and *P.patulum* contain also galactofuranosyl residues and cross-react with anti-serum raised against *P.charlesii* mycelium (Preston *et al.*, 1970). However, the exocellular polysaccharide of *P.varians* showed no cross-reactivity. In this polysaccharide no acid-labile galactofuranosyl residues were determined. These findings are in agreement with the results of Haworth *et al.* (1935[b]). In contrast, Jansson and Lindberg (1980) determined the presence of both α- and ß-galactofuranosyl residues in the same *P.varians* polysaccharide (varianose) as investigated by Haworth *et al.* (1935[b]). The structures of the above-mentioned extracellular polysaccharides show similarities, especially the presence of galactofuranosyl residues, with the polymers produced by the dermatophytes *Trichophyton* and *Microsporum*. Grappel *et al.* (1968[a]) raised antibodies in rabbits against autoclaved mycelium of *Microsporum quinckneanum* and these antibodies reacted with a neutral galactomannan isolated from this species and four *Trichophyton* species. The terminal galactofuranosyl residues contributed to the antigenicity (Grappel *et al.*, 1968[b]). All galactomannans produced by *Trichophyton* and *Microsporum* in shaked cultures contained numerous non-reducing terminal galactofuranosyl residues. However, without aeration only glucomannans were produced. Suzuki and Takeda (1975) describes that anti-*Hormodendrum pedrosoi* galactomannan serum cross-reacted with galactomannans of *A.fumigatus* and *A.niger*, which was due to the presence of ß(1,5)-linked galactofuranosyl residues in the galactomannans.

Extracellular galactomannans with nonreducing terminal galactofuranosyl residues have been derived from many fungi, for example *Gibberella fujikuroi* (Siddiqui and Adams, 1961), *A.niger* and *P.chrysogenum* (Sakaguchi *et al.*, 1969), *P.citrinum* (Kohama *et al.*, 1974), *Cladosporium herbarum* and *Cladosporium tricoides* (Miyazaki and Naoi, 1974; 1975[a]), *Alternaria solani* and *Fusarium solani* (Miyazaki and Naoi, 1975[b]), *Hormodendrum* species (Suzuki and Takeda, 1977), *Cordyceps cicadae* (Ukai *et al.*,

1982), *Sporothrix schenckii* and *Ceratocystis stenoceras* (Mendonca-Previato *et al.*, 1980), *Trypanosoma cruzi* (Schnaidman *et al.*, 1986) and *Penicillium allahabadense* (Rupe´rez *et al.*, 1984).

Cell wall polysaccharides

The carbohydrate composition of the cell wall is considered to be useful in delineating taxa at various levels and has been used for the correlation or rearrangement of species into the same genus or in different genera (Bartnicki-Garcia, 1968; Rosenberger, 1976; Bobbitt and Nordin, 1978; Leal *et al.*, 1984). The chemical composition of the cell wall of many *Aspergillus* and *Penicillium* species has been determined (Johnston, 1965; Grisaro *et al.*, 1968; Miyazaki and Yadomae, 1968; Sakaguchi *et al.*, 1969; Rizza and Kornfeld, 1969; Troy and Koffler, 1969; Martin *et al.*, 1973; Unger and Hayes, 1975; Bulman and Chittenden, 1976; Hearn *et al.*, 1979; Gomez-Miranda and Leal, 1981; Rupe´rez and Leal, 1986). Gomez-Miranda *et al.* (1986) studied the hyphal polysaccharides of *Eupenicillium* species, a teleomorfic state of the genus *Penicillium*. The investigated *Eupenicillium* species, taxonomically divided in 2 groups, showed differences in especially the mild-alkali hydrolysed fraction (F1). For group 1 species this fraction (20%) contained an α-glucan. In contrast, F1 of group 2 (less than 5%) contained only ß-linked polysaccharides (galactose and glucose). Recently, Leal *et al.* (1992) determined the most characteristic fractions of the cell wall of *Aspergillus*, *Penicillium* and *Eupenicillium*, namely an α(1,3)-glucan and a ß(1,5)-galactan. Weijman and Rodriques de Miranda (1988) determined within the genus *Candida* three distinct groups based on carbohydrate patterns of whole cell hydrolysates.

Aspergillus fumigatus, an airborne mould and responsible for various forms of infections in man, such as invasive aspergillosis, produces (peptido)galactomannans, which contain also galactofuranosyl residues (Azuma *et al.*, 1971; Sakaguchi *et al.*, 1969). Antiserum specific for circulating antigens in invasive aspergillosis patients reacted also with galactomannans derived from mycelia of *A.fumigatus*. This galacto-

mannan with a molecular weight between 25 and 75 kD consists of a (1,6)-linked mannose backbone, with short side chains and terminal galactofuranosyl residues. The immunodominance of the galactofuranosyl residues of the galactomannan has been demonstrated (Reiss and Lehmann, 1979).

Circulating antigens reacting with polyclonal antibodies, raised against extracellular polysaccharides of *P.digitatum* and specific for ß(1,5)-linked galactofuranosyl residues were also detected in sera of both healthy and aspergillose patients (Notermans *et al.*, 1988[a]). Galactomannans are not always detected in body fluids of patients with invasive aspergillosis (Rogers *et al.*, 1990). The galactomannan levels may be too low to be detected or the antibodies raised against the galactomannans produced under laboratory conditions, are not suitable for the detection of galactomannans produced in man. However, the presence of protein antigens of *A.fumigatus* cannot be excluded (Hearn and Mackenzie, 1979). Protein antigens have been detected in sera and/or urine of humans and animals infected with *A.fumigatus* (Latgé *et al.*, 1991). Both the sugar and the proteinous antigen, detected as circulating antigen, were located in the mycelium cell wall.

Structural analysis of the fungal cell wall has revealed that cell walls of *A.fumigatus*, *A.niger*, *A.terreus* and *A.flavus* contain structural features which are also present in the extracellular glycoproteins, like the α(1,2)-linked D-mannopyranosyl backbone with α(1,6) branching points and the ß(1,5)-linked D-galactofuranosyl side chains (Barreto-Bergter *et al.*, 1980; 1981). The presence of galactofuranosyl residues in cell wall fragments of *Aspergillus* and *Penicillium* has been suggested (indirectly) by more authors (Bardalaye and Nordin, 1977; Gómez-Miranda *et al.*, 1984; Ruperez and Leal, 1987; Matsunaga *et al.*, 1981; Hearn *et al.*, 1989). In contrast, no galactofuranosyl residues were determined in the cell wall of *P.charlesii*, which is known to produce an extracellular polymer containing ß(1,5)-linked galactofuranosyl residues (Bulman and Chittenden, 1976). The presence of ß(1,5)-linked D-galactofuranosyl residues has also been determined in the cell wall of *Talaromyces helicus* (Prieto *et al.*, 1988).

This leads to the suggestion that galactomannans are common extracellular constituents of *Aspergillus* and *Penicillium*. The question of the origin and the function

Table 1. Immunoassays for the detection of moulds.

Fungal isolate	Immunogen[a]	Immunoassay[b]	Specificity[c]	Reference
Cladosporium carrionnii/C.bantianum	M	IF	species	Al-doory *et al.*, 1963
Alternaria alternata	HF	IF	"genus"	Warnock, 1973
Penicillium marneffei	CFP	ID	species	Sekhon *et al.*, 1982
F.oxysporum f.sp. *dianthi*	HF	DD/RDD	f.sp./race	Ianelli *et al.*, 1982
F.oxysporum f.sp. *lycopersici*	HF	DD/RDD	f.sp./race	Ianelli *et al.*, 1982
F.moniliforme	HF	DD/RDD	race	Ianelli *et al.*, 1982
F.oxysporum f.sp. *lycopersici*	Con	i-ELISA	species	Ianelli *et al.*, 1983
P.digitatum/P.cyclopium	CFP	s-ELISA	genus	Notermans *et al.*, 1985
M.racemosus	CFP	s-ELISA	genus	Notermans *et al.*, 1985
Geotrichum candidum	CFP	s-ELISA	genus	Notermans *et al.*, 1985
Botrytis tulipae	CFP	s-ELISA	genus	Notermans *et al.*, 1985
Cladosporium cladosporioides	CFP	s-ELISA	genus	Notermans *et al.*, 1985
Fusarium oxysporum	CFP	s-ELISA	genus	Notermans *et al.*, 1985
Alternaria alternata	CFP	s-ELISA	genus	Lin *et al.*, 1987
Rhizopus stolonifer	CFP	s-ELISA	genus	Lin *et al.*, 1987
Geotrichum candidum	CFP	s-ELISA	genus	Lin *et al.*, 1987
F.oxysporum f.sp. *cubense* (Foc) race 4	HF	IF	race	Wong *et al.*, 1988
F.oxysporum f.sp. *cucumerinum* F 504	Con	CIM	f.sp.	Kitagawa *et al.*, 1989[a]
F.oxysporum f.sp. *cucumerinum* F 504	HF	CIM	genus	Kitagawa *et al.*, 1989[b]
P.verrucosum var. *verrucosum*	M	i-ELISA	species	Fuhrmann *et al.*, 1989
P.islandicum	SF	i-ELISA	species	Dewey *et al.*, 1990
B.tulipae	CFP	s-ELISA	genus	Cousin *et al.*, 1990
Monascus pilosus	CFP	s-ELISA	genus	Cousin *et al.*, 1990

[a] M: mycelium; HF: hyphal fragments; CFP: culture filtrate precipitate; Con: conidia; SF: spore fragments. [b] IF: immunofluorescence; ID: Immunodiffusion; i-ELISA: indirect ELISA; s-ELISA: sandwich ELISA; CIM: competitive immunoassay, using antigen coated Amino-Dark Balls; DD: double diffusion; RDD: radio double diffusion. [c] determination level.; f.sp: formae speciales.

of these galactomannans has not yet been clarified.

A number of investigators have used successfully the immunogenic and antigenic properties of extracellular glycoproteins derived from the cell wall or culture filtrate for the development of immunoassays for the detection of moulds (Table 1). The specificity of the assays are given according the authors opinion. Detection of extracellular polysaccharides (EPS) render superfluous extraction procedures necessary for detection of cell wall polysaccharides. The use of EPS, produced by moulds (especially *Aspergillus* and *Penicillium*) for the detection of moulds in food will be described in the next paragraphs.

Production and purification of extracellular polysaccharides (EPS)

Notermans and Heuvelman (1985) published a simple method for the production and purification of EPS. Moulds were grown in shaked cultures of dialysed malt extract at 24°C for 14 days. The freeze-dried culture filtrate was dissolved in 80 % ammonium sulphate. The precipitate was removed by centrifugation. Size-exclusion

Table 2. Monosaccharide composition, protein- and sugar content of extracellular polysaccharides of moulds (Notermans *et al.* 1987; De Ruiter *et al.*, 1991; Kamphuis *et al.*, 1992).

Strain	Sugar	Protein	Monosaccharides[a] (mol %)			
	(w/w %)		Man	Gal	Glc	Fuc
Penicillium cyclopium	70	NT[b]	76	17	7	0
P.digitatum	40	40	37	55	8	0
Aspergillus niger	46	18	50	45	4	0
Mucor hiemalis	56	21	16	13	4	18
Rhizopus stolonifer	57	19	9	11	23	12
Cladosporium cladosporioides	61	15	40	46	12	0
Fusarium oxysporum	37	25	24	35	41	0
Geotrichum candidum	84	13	41	18	40	0

[a] Man: mannose; Gal: galactose; Glc: glucose; Fuc: fucose. [b] NT: not tested.

chromatography of the supernatant of different mould species showed that one main antigenic fraction was present (Notermans and Heuvelman, 1985), primarily containing glucose, mannose and galactose (Table 2). Despite the presence of protein these antigens were indicated as extracellular polysaccharides (EPS). In case of *Mucor hiemalis* EPS and *Rhizopus stolonifer* EPS fucose and uronic acids were also present (De Ruiter *et al.*, 1991). Glucuronic acid was also determined in EPS produced by *Fusarium* species (De Ruiter *et al.*, 1992[a]).

Immunogenicity and genus specific antigenicity of extracellular polysaccharides

Polysaccharide fractions isolated after size-exclusion chromatography of *Penicillium digitatum, P.cyclopium, Cladosporium cladosporioides, Mucor racemosus, Fusarium oxysporum and Geotrichum candidum* culture filtrates were used to immunise rabbits and high serum titres against the extracellular polysaccharides were obtained (Notermans and Heuvelman, 1985). With the purified antibodies highly specific sandwich ELISA's were developed for the detection of mould EPS (Table 3).

The ELISA was almost genus specific (Notermans and Soentoro, 1986). Closely related genera, e.g. *Aspergillus* and *Penicillium* were detectable by the ELISA using antibodies raised against EPS of *P.digitatum* or *P.cyclopium*. However, species belonging to the *Penicillium* subgenus *biverticillium*, such as *P.islandicum*, *P.funiculosum*, *P.tardum* and *P.rubrum* did not react in the sandwich ELISA. Antibodies raised against *M.racemosus* were reactive with all tested species of *Mucor* and *Rhizopus*. Moreover, cross reactions were observed with species belonging to the genera *Aspergillus*, *Penicillium* and *Trichotecium*. In further experiments, carried out with antibodies obtained from other immunised rabbits no cross reactions were observed. Species of the closely related genera *Rhizomucor*, *Thamnidium*, *Absidia* and *Syncephalastrum* gave also a positive reaction (De Ruiter *et al.*, 1991 and 1992[b]). In case of antibodies raised against *F.oxysporum* EPS, cross-reactivity was frequently observed with some species of the genera *Aspergillus* and *Penicillium*, especially the *Penicillium* subgenus *biverticillium* species.

Table 3. Production of extracellular polysaccharides by mould species and their immunological relationship (Notermans and Soentoro, 1986).

Moulds species belonging to the genera	Sandwich ELISA reaction carried out with antibodies raised against EPS of					
	P.dig.	*P.cycl.*	*M.rac.*	*Clad.clad.*	*F.oxysp.*	*G.cand.*
Aspergillus	12/12[a]	12/12	8/12	0/12	3/12	0/12
Penicillium	41/44	41/44	11/44	0/44	7/44	0/44
Mucor	0/6	0/6	6/6	0/6	0/6	0/6
Rhizopus	0/4	0/4	4/4	0/4	0/4	0/4
Botrytis	0/3	0/3	0/3	0/3	0/3	0/3
Geotrichum	0/6	0/6	0/6	0/6	0/6	6/6
Cladosporium	0/4	0/4	0/4	4/4	0/4	0/4
Fusarium	0/6	0/6	0/6	0/6	6/6	0/6
Trichotecium	0/4	0/4	3/4	0/4	3/4	0/4

[a] number of species giving a positive ELISA reaction/total number of species tested.

Relation between EPS production and growth

For an immunological detection method of mould EPS in food it is of great importance that EPS production is related to mould growth. It was shown by Notermans *et al.* (1986) that the minimal detectable quantity mould mycelium expressed as mycelium dry weight/reciprocal of highest dilution with positive ELISA result, varied from 6-108 ng/ml with an average of 32 ng/ml under several different culture conditions, such as different media, surface and shaked culturing, incubation temperature and water activity.

Elucidation of the immunodominant part of the extracellular polysaccharides produced by Aspergillus *and* Penicillium *species*

EPS produced by species of *Aspergillus* and *Penicillium* consists mainly of glucose, mannose and galactose. Investigations by Suzuki and Takeda (1975) showed indirectly that immunologically active mycelial galactomannans of *A.fumigatus* possess galacto-

furanosyl residues which are immunodominant. Later, Bennett *et al.* (1985) showed that methyl-ß-O-galactofuranose was an efficient inhibitor in hapten inhibition studies using the galactomannans produced by *A.fumigatus*.

In order to elucidate the immunodominant part of the EPS of *Aspergillus* and *Penicillium* Notermans *et al.* (1987) carried out inhibition experiments with methyl-monosaccharides and oligosaccharides in a sandwich ELISA using antibodies raised against *P.digitatum* EPS. It was demonstrated that of all monosaccharides tested only ß-methyl-D-galactofuranoside was able to inhibit antibody binding to EPS of *P.digitatum* and *P.cyclopium*.

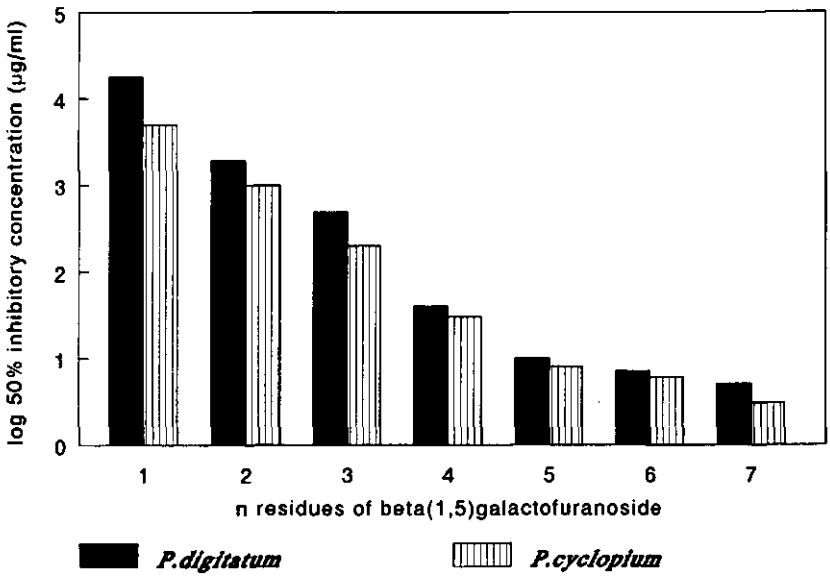

Figure 2. Inhibition of rabbit antibody binding to extracellular polysaccharides (EPS) of *P.digitatum* and *P.cyclopium* by methyl-ß-D-galactofuranoside and dimer up to the heptamer of ß(1,5)-linked D-galactofuranosides (Notermans *et al.*, 1988[b]).

Using differently linked dimers of ß-methyl-D-galactofuranosides it could be concluded that the ß(1,5)-linked D-galactofuranosides possesses the greatest inhibitory effect. The inhibitory effect of antibody binding to EPS of *P.digitatum* and *P.cyclopium*, by ß-methyl-D-galactofuranoside and dimer up to heptamer of ß(1,5)-linked D-galactofuranosides are summarised in Figure 2.

The 50 % inhibitory concentration of the inhibitors decreased with increasing chain length of the galactofuranosides. The pentamer up to the heptamer of ß(1,5)-linked D-galactofuranosides interfered also but the 50 % inhibitory concentration did not decrease as quickly as it was observed with the monomer up to the tetramer. Complete inhibition of the reaction between EPS of *P.digitatum* or *P.cyclopium* and IgG anti-*P.digitatum* EPS was observed with the trimer up to the heptamer of ß(1,5)-linked D-galactofuranosides.

Rietschel-Berst *et al.* (1977) reported the isolation of an exo-ß-D-galactofuranosidase from the culture filtrate of *P.charlesii*. This enzyme was able to degrade the galactofuranosyl residues of the extracellular peptidophosphogalactomannan (pPGM) of *P.charlesii*. Enhanced production of the enzyme was noted after depletion of glucose in the medium (Pletcher *et al.*, 1981). Because of the immunodominance of ß-D-galactofuranosyl residues in *Aspergillus* and *Penicillium* EPS, Cousin *et al.* (1989) investigated the ability of several *Aspergillus* and *Penicillium* species to produce ß-D-galactofuranosidase and found that this enzyme was produced in very small quantities. However, *Penicillium* subgenus *biverticillium* species (*P.funiculosum, P.rubrum, P.islandicum* and *P.tardum*) produced substantial ß-D-galactofuranosidase activity. Probably, this enzyme digests the immunodominant galactofuranosyl residues present in the EPS of these moulds and therefore no reaction will occur with the antibodies raised against *P.digitatum* EPS. This may explain why the *Penicillium* subgenus *biverticillium* species did not show a positive reaction in the immunoassay for the detection of EPS produced by the species of the genera *Aspergillus* and *Penicillium*.

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CHAPTER 4

H.J. Kamphuis, G.H. Veeneman, F.M. Rombouts, J.H.van Boom and S.Notermans (1989) Antibodies against synthetic oligosaccharide antigens reactive with extracellular polysaccharides produced by moulds. Food Agric. Immunol., 1, 235-242.

ANTIBODIES AGAINST SYNTHETIC OLIGOSACCHARIDE IMMUNOGENS REACTIVE WITH EXTRACELLULAR POLYSACCHARIDES PRODUCED BY MOULDS

ABSTRACT

Aspergillus and *Penicillium* species produce extracellular polysaccharides (EPS) of which ß(1,5)-linked D-galactofuranosides are immunodominant. Synthetic tetra- and heptamers of ß(1,5)-linked D-galactofuranosides conjugated to tetanus toxoid were used to produce antibodies in rabbits. The antibodies obtained with the tetramer conjugate reacted only with EPS of a few *Aspergillus* and *Penicillium* species. Antibodies obtained with the heptamer conjugate were reactive with EPS of all *Aspergillus* and *Penicillium* species tested, with exception of the *Penicillium* subgenus *biverticillium* species. The reaction with *P.digitatum* EPS could be inhibited by the synthetic heptamer of ß(1,5)-linked D-galactofuranosides. No reactions were observed with EPS of moulds belonging to any other genus tested.

INTRODUCTION

Raw food materials can be contaminated with mould spores and/or mycelium fragments. During further processing food-stuffs can be infected additionally with moulds. Moulding may result in economic loss due to spoilage and may create a public health hazard due to the production of mycotoxins. Therefore, adequate mould-detection methods are necessary. Jarvis *et al.* (1983) summarised techniques for the enumeration of viable moulds. However viable moulds may be absent due to, for example, heat treatment. Notermans and Heuvelman (1985) and Lin *et al.* (1986) developed a immunological method using the enzyme-linked immunosorbent assay (ELISA) for the detection of heat stable extracellular polysaccharides (EPS) produced by various moulds. It has revealed that the developed immunoassay can be used for testing both heated and unheated products. EPS is a galactomannan and it is known that galacto-

mannans are the most widely distributed polysaccharides among fungi (Gorin and Barreto-Bergter, 1983). They are an essential part of the mycelial cell wall (Johnston, 1965) and during growth of moulds these polysaccharides are released in the surrounding environments (Preston *et al.*, 1969). These extracellular polysaccharides are immunogenic and antigenic (Notermans and Soentoro, 1986; Suzuki and Takeda, 1975) and their presence in food is highly indicative for the moulding stage of the food (Notermans *et al.*, 1986; 1988[a]).

Interest in mould EPS has been enhanced by the observation that *Aspergillus fumigatus* galactomannan can be detected in body fluids of *Aspergillus* infected animals and patients (Reiss and Lehman, 1979). Antibodies against mould EPS were also detected in human serum (Notermans *et al.*, 1988[b]). Suzuki and Takeda (1975) demonstrated an immunological relationship between the galactomannans produced by species of moulds belonging to different genera. They supposed that galactofuranosyl residues, which they observed to be present in the galactomannans, were immunodominant. However, it was shown by Notermans and Soentoro (1986), that the EPS produced by moulds were immunologically almost genus-specific and cross-reactivity occurs only between the closely related genera for example *Aspergillus* and *Penicillium*. Bennett *et al.* (1985) found that methyl-ß-D-galactofuranoside interferes with the immunoassay for detecting the polysaccharides of *A.fumigatus*. In addition, Notermans *et al.* (1987) showed that methyl-ß-D-galactofuranoside interferes with the immunoassay for detecting EPS of other *Aspergillus* and *Penicillium* species. Using these findings as point of departure, differently linked dimers of ß-D-galactofuranosides were synthesised and of them the ß(1,5)-linked D-galactofuranoside gave the highest inhibition. An increasing inhibitory effect of di-, tri-, tetra-, penta-, hexa-, and heptamers of ß(1,5)-linked D-galactofuranosides was observed (Notermans *et al.*, 1988[c]). In the present study, synthetic tetra- and heptamers of ß(1,5)-linked D-galactofuranosides conjugated to tetanus toxoid were used as immunogens to produce antibodies in rabbits. The specificity of the antibodies obtained was determined.

MATERIALS AND METHODS

Chemical synthesis of ß(1,5)-linked D-galactofuranosides. Methyl ß-D-galactofuranoside was obtained by chromatography of a mixture of methyl-D-galactosides over Whatman cc 31 microgranular cellulose (Augestad and Berner, 1954). Tetra- and heptamers of ß(1,5)-linked D-galactofuranosides were synthesised by a solid phase approach (Veeneman *et al.*, 1987). The homogeneity and identity of the produced synthetic oligosaccharides were ascertained by NMR [^{1}H]- and [^{13}C]-spectroscopy and FAB mass spectrometry.

Conjugation. The tetra- and heptamers of ß(1,5)-linked D-galactofuranosides were conjugated to tetanus toxoid ($(GalFur)_4$-TT and $(GalFur)_7$-TT, respectively) by the glutaraldehyde-method in a ratio of 0.75 mg oligosaccharides and 4 mg tetanus toxoid. The $(GalFur)_4$-TT contained 0.2 mg oligosaccharide/ml, while the $(GalFur)_7$-TT contained 0.4 mg oligosaccharide/ml.

Preparation of extracellular polysaccharides (EPS). P.digitatum M 58, *P.dierckxii* M 90, *P.verrucosum* var. *cyclopium* M 60, *A.fischeri* M 85, *A.repens* M 5 and *A.niger* M23/ CBS 553.65 spores were inoculated into dialysed malt extract broth as described earlier (Notermans *et al.*, 1987). After incubation for 6 days at 24°C with shaking (100 rpm), culture fluid was separated from the mycelium by filtration through a Buchner funnel, using Whatman no. 2 filter paper. The filtrate was then freeze-dried and the polysaccharide isolated from it as described by Notermans *et al.*, (1987). For this purpose, the freeze-dried culture fluid was dissolved in 80% saturated $(NH_4)_2SO_4$ solution. After vigorous shaking for 3 hrs, the mixture was centrifuged for 30 min at 15,000 x g at 4°C and subsequently filtered as described above to remove insoluble materials. The filtered fluid was applied to a Sepharose CL-6B column (2.5 x 105 cm), equilibrated with distilled water. Elution was also performed with distilled water. The antigenic EPS peak was freeze-dried.

Antibody preparation. Antibodies against the synthetic oligosaccharide immunogens were prepared by immunising rabbits as follows: 0.5 ml quantities of the synthesised oligosaccharides conjugated to tetanus toxoid were injected subcutaneously on days 0 and 30. For the first injection the immunogens were mixed with Freund's complete adjuvant. For the last injection the immunogens were mixed with Freund's incomplete adjuvant.

Antibodies against EPS of *P.digitatum* were prepared by subcutaneous injection in rabbits of 0.5 mg amounts on day 0 and 30, respectively. For the first injection, EPS was mixed with Freund's complete adjuvant. For the last injection EPS was mixed with Freund's incomplete adjuvant. On day 40 serum was collected and the IgG fraction was isolated by the method of Steinbuch and Audran (1969) and freeze-dried.

Determination of antibody titres. Antibody titres to the synthesised immunogens and EPS of *P.digitatum* were determined with the ELISA. For this 0.1 ml of 10 μg/ml of EPS and tetanus toxoid, respectively in 0.07 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) were added to each well of the polyvinyl trays (Cooke, Dynatech). After incubation overnight with shaking at 20°C, the trays were washed by a continuous flow of tap water containing 0.05% (v/v) Tween 20 for 0.5 min, and 0.1 ml IgG samples diluted in PBS containing 0.05% (v/v) Tween 20 and 2% (w/w) bovine serum albumin were added to each well. After 90 min of incubation the trays were washed as described above. The amount of antibodies adsorbed to the coated EPS and tetanus toxoid, respectively was measured with sheep anti-rabbit immunoglobulins conjugated to peroxidase. For this, 0.1 ml of conjugate diluted in PBS containing 0.05% (v/v) Tween 20 was added to each well. The optimal dilution was determined by checker-board titration. Incubation and washing were carried out as described for the sample. The enzyme activity was determined spectrophotometrically at 450 nm after addition of the substrate solution (0.1 ml of 0.07% 5-amino-salicylic acid (pH 6.0) containing 0.005% (v/v) H_2O_2) to each well. After incubation for 30 min at 20°C, the light absorption was measured.

The amount of specific antibody present in IgG samples is expressed as the

quantity of IgG in μg/ml giving an extinction equal to the average extinction value from 10 blank samples $\pm$ 2.81 x standard deviation of these blank values (confidence limit 99%). Blank samples consisted of PBS containing 0.05% (v/v) Tween 20 and 2% (w/w) bovine serum albumin.

Detection of EPS in culture filtrates of moulds. Culture filtrates of moulds were produced by inoculating spores of moulds into dialysed malt extract broth, as described earlier (Notermans *et al.*, 1987). After incubation for 6 days at 24°C with shaking (100 rpm), culture fluid was separated from the mycelium by filtration using Whatman no. 2 filter paper. The filtrate was tested for reaction with the IgG's obtained after immunisation of rabbits with the synthetic immunogens and EPS of *P.digitatum*, respectively. For this a sandwich ELISA was used. The ELISA was carried out in wells of polyvinyl trays (Cooke, Dynatech). The wells were coated with IgG anti-EPS of *P.digitatum*, IgG anti-$(GalFur)_4$-TT and IgG anti-$(GalFur)_7$-TT, respectively. For this portions of 0.1 ml containing 10 μg/ml of IgG diluted in PBS were added to each well of the trays. After incubation overnight with shaking at 20°C, the trays were washed by a continuous flow of tap water containing 0.05% (v/v) Tween 20 for 0.5 min, and 0.1 ml of culture filtrates diluted in PBS containing 0.05% (v/v) Tween 20 (PBST) and 1% (w/w) bovine serum albumin were added to each well. After 90 min of incubation the trays were washed as described above. The amount of EPS adsorbed to the coated IgG was measured with rabbit IgG anti-EPS of *P.digitatum* conjugated to horseradish peroxidase. For this 0.1 ml of conjugate diluted in PBST containing 1% (w/w) bovine serum albumin was added to each well. The optimal dilution was determined by checkerboard titration. Incubation for 90 min with shaking at room temperature was followed by a washing procedure and subsequent addition of 0.1 ml of the substrate solution. For this 1 ml of 42 mM of 3,5,3',5'-tetramethylbenzidine in dimethylsulfoxyde was added under vigorous shaking to 100 ml 0.1 M sodium acetate buffer pH 5.6. Just before adding the substrate solution to the wells 7 μl 30% H_2O_2 was added to the solution. After incubation for 30 min at 20°C, 0.05 ml 2M H_2SO_4 was added to each well. The enzyme activity was determi-

ned spectrophotometrically at 450 nm.

Inhibition experiments. Inhibition experiments were carried out as follows; 0.1 ml of 5 μg/ml EPS of different mould species in PBS were added to each well of polyvinyl trays. After incubation overnight with shaking at 20°C the trays were washed as described above. A concentration range of the inhibitor was made in PBST. 25 μl of the inhibitor together with 25 μl of the optimal dilution of the immunoglobulin (tested by checkerboard titration) in PBST was added. After incubation with shaking for 90 min at room temperature the plates were washed and 0.1 ml of sheep anti-rabbit immunoglobulin conjugated to horseradish peroxidase, in PBST containing 1% (w/w) bovine serum albumin was added. The optimal dilution of the conjugate was tested by checkerboard titration. The light absorption was measured spectrophotometrically at 450 nm. Incubation, washing and enzyme reaction was carried out as described above. Percent inhibition was calculated as 100-100 (absorbance with inhibitor ÷ absorbance without inhibitor).

RESULTS AND DISCUSSION

Antibodies against EPS of *P.digitatum* and tetra- and heptamers of ß(1,5)-linked D-galactofuranosides conjugated to tetanus toxoid were raised in rabbits. Reactions of these antibodies with purified EPS of different moulds and tetanus toxoid are presented in Table 1. Antibodies raised against EPS of *P.digitatum* were reactive with all EPS tested. No reaction was observed with tetanus toxoid. Antibodies raised against the tetramer of ß(1,5)-linked D-galactofuranoside conjugated to tetanus toxoid ($(GalFur)_4$-TT) showed only reaction with the EPS of *P.digitatum* and *A.fischeri* and with tetanus toxoid. Antibodies raised against the heptamer of ß(1,5)-linked D-galactofuranoside conjugated to tetanus toxoid ($(GalFur)_7$-TT) reacted with all EPS tested and with tetanus toxoid. Reactions of culture filtrates of different moulds with antibodies raised against EPS of *P.digitatum*, $(GalFur)_4$-TT and $(GalFur)_7$-TT are presented in Table 2. IgG anti-EPS of *P.digitatum* was reactive with all culture

Table 1. Reactions of different IgG's with purified EPS and tetanus toxoid in the ELISA.

Origin of EPS	Source of IgG			
	anti-EPS of *P.digitatum*		anti-$(GalFur)_4$-TT	anti-$(GalFur)_7$-TT
	IgG 334	IgG 897	IgG 46	IgG 380
P.digitatum	0.3[a]	0.1	0.3	1.6
P.dierckxii	8.0	1.6	>25.0	2.0
P.cyclopium	0.5	0.3	>25.0	8.0
A.repens	8.0	1.6	>25.0	8.0
A.niger	2.0	1.6	>25.0	5.0
A.fischeri	0.3	0.3	2.0	10.0
Tetanus toxoid	>25.0	>25.0	0.3	0.01

[a] quantity of IgG in μg/ml giving just a positive reaction in the ELISA.

filtrates of *Aspergillus* and *Penicillium* species tested, with exception of *P.funiculosum* and *P.rubrum*. No reactions were observed with culture filtrates of moulds belonging to other genera. The IgG anti-$(GalFur)_4$-TT was only reactive with culture fluids of a few strains. Reactions were observed with 4 out of 13 *Aspergillus* species tested and with 2 out of 11 *Penicillium* species. Antibodies raised against the $(GalFur)_7$-TT showed a reactivity comparable with the antibodies raised against EPS of *P.digitatum*. Using a synthetic tetramer and heptamer of ß(1,5)-linked D-galactofuranosides, inhibition of antibody binding, was carried out. For this IgG anti-EPS of *P.digitatum*, IgG anti-$(GalFur)_4$-TT and IgG-anti $(GalFur)_7$-TT were used as test antibodies and EPS of *P.digitatum* as test antigen. With IgG anti-*P.digitatum* EPS clear inhibition with the tetra and heptamer of ß(1,5)-linked D-galactofuranosides were observed (Table 3). However, with IgG anti-$(GalFur)_4$-TT no inhibition was observed. Only with the heptamer of ß(1,5)-linked D-galactofuranoside inhibition could be established (Table 3). In an earlier study it was demonstrated that antibodies raised against the EPS of *P.digitatum* and *P.cyclopium* were reactive with culture filtrates of *Aspergillus* and *Penicillium* species (Notermans and Soentoro, 1986). Additionally it was observed that the antibody reactions with the EPS of *P.digitatum* and *P.cyclopium*

Table 2. Reaction of culture filtrates of *Aspergillus* and *Penicillium* spp. (and some other moulds) in the sandwich ELISA.

Mould species	Sandwich ELISA[a] carried out with:		
	IgG 897[b]	IgG 46	IgG 380
Aspergillus versicolor M 11	++[c]	-	+
A.nidulans M 22	++	-	++
A.niger M 23/CBS 553.65	++	-	++
A.ostianus M 8	++	-	++
A.parasiticus M 9	++	-	++
A.fischeri M 85	++	++	++
A.candidus M 1	++	++	++
A.flavus M 2	++	++	++
A.fumigatus M 3	++	++	++
A.ochraceus M 7	++	-	++
A.clavatus M 10/CBS 114.48	++	-	++
A.tamari M 6	++	-	++
A.repens M 5	++	-	++
Penicillium digitatum M 58	++	++	++
P.dierckxii M 91	++	-	++
P.verrucosum var. *cyclopium* M 60	++	-	++
P.simplissininum M 49	++	-	++
P.funiculosum M 50	-	-	-
P.implicatum M 52	++	-	++
P.roqueforti M 56	++	-	++
P.viridicatum M 62	++	-	++
P.rubrum M 57	-	-	-
P.diversum M 47	++	++	++
P.velutinum M 92/CBS 318.59	++	-	++
Cladosporium cladosporioides M 20	-	-	-
Alternaria alternata M 13	-	-	-
Geotrichum candidum M 32	-	-	-
Mucor racemosus M 45	-	-	-
Fusarium dimerum M 25	-	-	-

[a] IgG ∞ horseradish peroxidase conjugate consisted of IgG anti-EPS of P.digitatum.

[b] IgG 897: raised against EPS of *P.digitatum*; IgG 46: raised against $(GalFur)_4$-TT; IgG 380: raised against $(GalFur)_7$-TT.

[c] - No ELISA reaction in 1/100 dilution of culture filtrate. + ELISA reaction observed in 1/100 dilution not in 1/1000 dilution of culture filtrate. ++ ELISA reaction observed in 1/1000 dilution of culture filtrate.

Table 3. Inhibition of antibody binding to extracellular polysaccharides (EPS) of *P.digitatum* by tetra and heptamer of ß(1,5)-linked D-galactofuranoside.

Origin of antibodies	50% Inhibitory concentration (μg/ml)	
	Tetramer	Heptamer
anti-EPS of *P.digitatum*		
IgG 334	16	5
IgG 897	25	6
anti-$(GalFur)_4$ -TT IgG 46	>100	>100
anti-$(GalFur)_7$ -TT IgG 380	>100	15

were inhibited by fragments of ß(1,5)-linked D-galactofuranosyl units (Notermans *et al.*, 1988c).

These results indicate that ß(1,5)-linked D-galactofuranosides are immunodominant in EPS of *Aspergillus* and *Penicillium* species. If this is true, antibodies raised against synthesised ß(1,5)-linked D-galactofuranoside fragments have to be reactive with all species of *Aspergillus* and *Penicillium*. The fragments chosen for immunisation consisted of a tetramer and a heptamer conjugated to tetanus toxoid.

The results obtained in this research confirmed that ß(1,5)-linked D-galactofuranosides are indeed immunodominant and that antibodies obtained with the heptamer conjugate were reactive with all *Aspergillus* and *Penicillium* species investigated except *P.funiculosum* and *P.rubrum*. From inhibition experiments carried out by Notermans *et al.* (1988c) using different ß(1,5)-linked D-galactofuranosyl units it was estimated that four galactofuranosyl units was the size of the paratope present on the IgG which is reactive with EPS of *P.digitatum*. This estimation was confirmed by the observation that penta-, hexa, and heptamer of the ß(1,5)-linked D-galactofuranosides were able to link antibodies at an increasing rate. However, the antibodies obtained after

immunisation with the tetramer conjugate reacted only with EPS of a few *Aspergillus* and *Penicillium* species. This is in contrast with the findings that antibodies obtained with the heptamer conjugate were reactive with EPS of (nearly) all *Aspergillus* and *Penicillium* species. These findings are difficult to explain and the following reasons may play a role in the production of antibodies in rabbits against synthetic immunogens. Firstly, the antibody titres of the IgG obtained after immunisation with the synthetic immunogens were much lower than the antibody titres raised against EPS of *P.digitatum* (Table 1), which was especially the case for the antibody titres against $(GalFur)_4$-TT. This may be due to "immunogenic competition", the immune response to one immunogen can be inhibited as a result of a second immunogen (Sela, 1969). It is known that the carrier, in our work tetanus toxoid, is important in raising antibodies against synthetic immunogens (Richter and Kagedal, 1972). In this study a high titre against tetanus toxoid was produced. Another reason may be the role of the terminal monosaccharide of a sugar glycoside. It is known that, when coupled to protein it can play a dominant role in specificity (Kabat, 1966). In relation with the three-dimensional structure of the two synthetic immunogens, it can result in an increase of the number of heterogeneous populations of antibody molecules that can be formed to a single antigenic determinant. As a consequence of the non-specific conjugation procedure applied in this study, the tetramer may be coupled in a different way as the heptamer. As shown by Richter and Eby (1985) the kind of conjugation method is indeed of influence on antibody production. To avoid such problems synthetic high molecular weight ß(1,5)-linked D-galactofuranoside fragments with molecular weights exceeding 10 kD, should be used for immunisation. However, such fragments are until now not available.

As observed earlier, *P.rubrum* and *P.funiculosum* produce EPS which are not reactive with IgG anti-EPS of *P.digitatum* (Notermans and Soentoro, 1986). Also antibodies raised against $(GalFur)_7$-TT were not reactive with culture fluids of these moulds. It was demonstrated by Cousin *et al.* (1989) that these moulds produce considerable amounts of galactofuranosidase. Production of this enzyme may be responsible for the negative reaction of culture filtrates in the ELISA using IgG anti-

EPS of *P.digitatum*. The results described in this paper underlines the findings, earlier observed by Notermans *et al.* (1988[c]), that ß(1,5)-linked D-galactofuranosides are immunodominant in EPS produced by *Aspergillus* and *Penicillium* species. Immunoglobulins for detection of these EPS can be produced by immunisation of animals with synthetic heptamers coupled to a protein carrier. However, for high quality immunoglobulins different protein carriers and different conjugation methods have to be evaluated.

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CHAPTER 5

A.W. van Bruggen- van der Lugt, H.J. Kamphuis, G.A. de Ruiter, P. Mischnick, J.H. van Boom, F.M. Rombouts. New structural features of the antigenic extracellular polysaccharides of Penicillium *and* Aspergillus species *revealed with exo-β-D-galactofuranosidase, J. Bacteriol. (in press).*

NEW STRUCTURAL FEATURES OF THE ANTIGENIC EXTRACELLULAR POLYSACCHARIDES OF *ASPERGILLUS* AND *PENICILLIUM* SPECIES REVEALED WITH EXO-ß-D-GALACTOFURANOSIDASE

ABSTRACT

To study the structure of the epitopes of the extracellular polysaccharides from *Aspergillus* and *Penicillium* species, an exo-ß-D-galactofuranosidase was purified from a commercial crude enzyme preparation from *Trichoderma harzianum*. Analysis of ring size and linkage position of the galactose residues of the EPS of *Penicillium digitatum*, before and after enzymatic treatment, was determined by the reductive-cleavage technique. All the galactose residues occur in the furanose form. Beside terminal and ß(1,5)-linked galactofuranosides, also ß(1,6)-linked and ß(1,5,6)-linked branched galactofuranose residues could be identified. After degradation with the purified exo-ß-D-galactofuranosidase all initial linkages of the galactofuranose residues were still present, but the amount of ß(1,5)-linked galactofuranose had decreased considerably. Treatment of the extracellular polysaccharides of *Aspergillus* and *Penicillium* species with the purified exo-ß-D-galactofuranosidase resulted in the complete disappearance of the antigenicity of these polysaccharides using antibodies raised against *P.digitatum* EPS. Therefore, with the use of this enzyme it was proved that the ß(1,5)-linked galactofuranosyl residues only are responsible for the antigenicity of the extracellular polysaccharides of *Aspergillus* and *Penicillium* species. A new structural model for the immunodominant galactofuranose side chains of the galactomannan of *Penicillium digitatum* is proposed.

INTRODUCTION

Moulds are able to excrete a large variety of polysaccharides, some of them with antigenic properties (Notermans and Soentoro, 1986; Suzuki *et al.*, 1975). Several authors studied the chemical structure of polysaccharides isolated from mycelium of

mould species belonging to the genera *Aspergillus* and *Penicillium* (Bardalaye and Nordin, 1977; Barreto-Bergter *et al.*, 1980; 1981). *Aspergillus* and *Penicillium* are important fungi which are distributed world-wide and are the cause of many cases of food spoilage.

Medically some members are significant because of their ability to cause aspergillosis in man and for the release of mycotoxins (Rodricks, 1976). The polysaccharides are constituted mainly of mannose, galactose, glucose and minor amounts of protein (Bardalaye and Nordin, 1977; Barreto-Bergter *et al.*, 1980; 1981). Galactosamine has been found in the polysaccharides of several *Aspergillus* species (Ruperez and Leal, 1981; Gomez-Miranda and Leal, 1981; Bardalaye and Nordin, 1976). Extracellular polysaccharides (EPS) produced by these moulds appeared to have similar structures (Gander *et al.*, 1974; Preston *et al.*, 1969).

They are characterised as galactomannans, mainly composed of an α(1,6) and α(1,2)-linked D-mannopyranosyl backbone and side chains of galactose residues some of which are ß(1,5)-linked D-galactofuranosyl sequences (Bardalaye and Nordin, 1977; Barreto-Bergter *et al.*, 1980; 1981; Gander *et al.*, 1974). The presence of (1,4)-linked galactopyranosyl residues has also been demonstrated (Bardalaye and Nordin, 1977; Latgé *et al.*, 1991). No detailed information is available about the glycosidic linkage of glucose.

The antigenicity of the extracellular polysaccharides can be used for detection of these moulds in e.g. food by using immunoassays (Notermans and Heuvelman, 1985; Kamphuis *et al.*, 1989). Polyclonal antibodies raised against EPS derived from *P.digitatum* were found to be specific for species of the closely related genera *Aspergillus* and *Penicillium* (Notermans and Soentoro, 1986). Hapten-inhibition studies revealed strong inhibition in both the ELISA and latex-agglutination assay using these antibodies with galactofuranose monomers and ß(1,5)-linked oligomers (Bennett *et al.*, 1985; Kamphuis *et al.*, 1989; Notermans *et al.*, 1988). It was found that the tetramer and higher oligomers of ß(1,5)-linked galactofuranose possess the highest inhibitory effect. As a result of this, it was concluded that ß(1,5)-linked galactofuranosides are immunodominant in EPS of *Aspergillus* and *Penicillium* species.

Enzymatic hydrolysis of ß(1,5)-linked galactofuranosyl residues of *P. charlesii* by an exo-ß-D-galactofuranosidase produced by this fungus was reported (Rietschel-Berst *et al.*, 1977). Also an endo-ß(1,5)-D-galactofuranosidase from *P.oxalicum* and the enzymatic hydrolysis of ß(1,5)-linked galactofuranosyl residues from polysaccharides obtained from *Eupenicillium crustaceum* and *Talaromyces flavus* has been reported (Reyes *et al.*, 1992). However, the influence of the exo-ß-D-galactofuranosidase on the antigenic properties of extracellular polysaccharides has not been studied. Antigenicity of the extracellular polysaccharides was lost by acid hydrolysis of the acid-labile galactofuranosyl residues (Kamphuis *et al.*, 1992). The question remains if the ß(1,5)-linked galactofuranose residues are the epitopes of *Aspergillus* and *Penicillium*.

In the present study, we describe the purification and characterisation of an exo-ß-D-galactofuranosidase isolated from a commercially available crude enzyme preparation of *Trichoderma harzianum* (Dubourdieu *et al.*, 1985). Also, we report on the degradation of EPS preparations derived from *Aspergillus* and *Penicillium* species by this purified enzyme in relation to their antigenicity. Reductive cleavage (Jun and Gray, 1987) was used for the chemical characterisation of the extracellular polysaccharide before and after enzymatic degradation. The results made it possible to prove that ß(1,5)-linked galactofuranose sequences are uniquely responsible for the antigenicity of these EPS and to propose a new structural model for the immunodominant galactofuranose side chains of EPS from *Penicillium digitatum*.

MATERIALS AND METHODS

Enzyme preparation. The exo-ß-D-galactofuranosidase was purified from a crude enzyme preparation of fungal origin (*Trichoderma harzianum*), commercially available as Glucanex (Novo Ferment AG, Basel, Switzerland).

Isolation of extracellular polysaccharides (EPS). Mould strains used in this study were *Penicillium digitatum* M 58, *P.aurantiogriseum* CBS 342.51, *P.dierckxii* M 90, *P.citrinum* CBS 117.64, *Aspergillus fumigatus* M 3 and *A.niger* CBS 553.65. They were grown at

23°C in shaking cultures with yeast-nitrogen base (YNB, Difco Labs. Detroit U.S.A.) as basal synthetic culture medium (6.7 g/l) supplemented with 30 g/l glucose as carbon source. Inoculation was performed with a spore suspension. The extracellular polysaccharides were isolated from the culture fluid after removing the mycelial pellets by filtration and purified by ethanol precipitation as described by De Ruiter *et al.* (1991).

Determination of the neutral sugar composition and protein content. The neutral sugar composition of the EPS preparations was determined as alditol acetates using gas-liquid chromatography. Neutral sugars were released by pre-treatment with 12 M sulphuric acid for 1 hr at 30°C, followed by hydrolysis with 1 M sulphuric acid for 3 hrs at 100°C. Next, sugars were converted to their alditol acetates according to Englyst and Cummings (1984) and analysed by GLC using inositol as the internal standard.

Protein was determined according to the methods of Sedmak (1977) and Lowry (1951), with Bovine Serum Albumin (BSA) as the standard.

Structural analysis of EPS by methylation analysis and reductive cleavage. Native and enzyme treated EPS of *P.digitatum* were permethylated by a modified Hakomori procedure using lithium dimsyl-anion and methyliodide in DMSO (D'Ambra *et al.*, 1988). The permethylated EPS was isolated by extraction with dichloromethane and purified on a LH-20 column. The polymeric fraction was investigated by methylation analysis (Albersheim *et al.*, 1967) and reductive cleavage (Jun and Gray, 1987). The enzyme treated sample was purified on a Sep-Pak C_{18} cartridge.

Reductive cleavage of the permethylated samples was performed by dissolving 1 mg in 100 μl of dry dichloromethane in a silylated screw-cap glass vial. To this solution, 25 equiv. per glycosidic bond of triethylsilane and trimethylsilyl methanesulfonate and 5 equiv. of borontrifluoride etherate were added and stirred for 17 hrs at room temperature. The reaction was quenched with methanol, treated with mixed-bed ion-exchange resin and filtered. After evaporation of methanol the residue was acetylated

with 1-methyl imidazole and acetic anhydride. To prove that all galactose residues occur in the furanoside form, the acetyl groups of the reductive cleavage samples were exchanged for methyl groups (Mischnick, 1991).

Quantitative chromatography was performed on a Carlo Erba Fractovap 4160 GLC equipped with an on-column injector using a DB-5 capillary column (40 m x 0.25 mm). Two-dimensional GLC was performed on a Siemens Sicromat 2 instrument equipped with a CP Sil 5 CB column (25 m x 0.25 mm) and an octakis(2-O-methyl-3,6-di-O-pentyl)-γ-cyclodextrin capillary column (25 m x 0.25 mm). GLC-MS was performed with a Hewlett-Packard 5840 A-5985 A instrument (c.i. ammonia, e.i. 70 eV).

Sandwich ELISA. Polyclonal antibodies were obtained by immunisation of rabbits with EPS from *P.digitatum* as described by Notermans and Heuvelman (1985). The sandwich ELISA was carried out using polyvinyl trays as described (Kamphuis *et al.*, 1992). The ELISA reactivity was expressed as the titre of the EPS which is the reciprocal dilution of a solution of 100 μg/ml EPS in distilled water just giving a positive reaction, i.e. an extinction at 450 nm $\geq$0.1 above that of a blank, containing no antigenic EPS.

Purification of exo-β-D-galactofuranosidase. Exo-ß-D-galactofuranosidase was isolated and purified from the Glucanex preparation according to the scheme as shown in Fig. 1. The purification was carried out at 4°C, all buffers contained 0.01 % (w/v) sodium azide to prevent microbial growth. An amount of 5 g Glucanex was dissolved in 10 ml 10 mM sodium acetate (pH 5.0) and centrifuged to remove solids. Column chromatography was carried out on a Bio-Gel P10 (100-200 mesh) column, DEAE Bio-Gel A column and a Bio-Gel Hydroxyapatite (HTP) column (Bio-Rad Laboratories, Richmond, USA). Further purification was performed on a FPLC system (Pharmacia LKB Biotechnology, Uppsala, Sweden) equipped with a Mono Q HR 5/5 column. During gradient elution peak control was used to elute protein peaks with a minimum amount of contamination by maintaining the composition of the eluent at a fixed

value during elution of the peaks. Protein was determined with the Sedmak assay. The purity of the fractions containing the exo-ß-D-galactofuranosidase was checked by sodium dodecyl sulphate-electrophoresis on a 10-15 % polyacrylamide gradient gel.

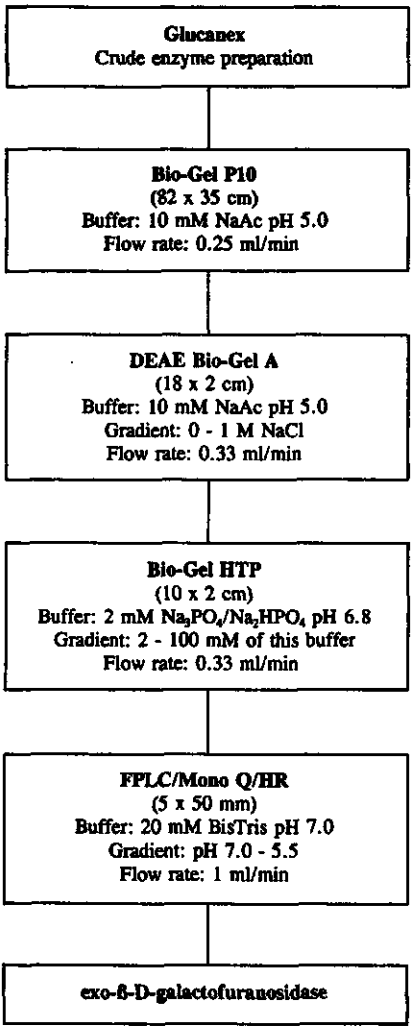

Figure 1. Isolation procedure for the exo-ß-galactofuranosidase from the crude enzyme preparation of *Trichoderma harzianum* (Glucanex).

Determination of exo-ß-D-galactofuranosidase activity. The exo-ß-D-galactofuranosidase activity present in enzyme fractions was determined both by high-performance anion-exchange chromatography and with the sandwich ELISA. The amount of liberated galactose residues was estimated with the use of high-performance anion-exchange chromatography (HPAEC) using a Dionex Bio-LC HPLC system (Sunnyvale, CA U.S.A.) equipped with a CarboPac PA1 column (4 x 250 mm) and pulsed amperometric detection (PAD) using a gold working electrode and an Ag/AgCl reference electrode as described (De Ruiter *et al.*, 1992). The epitope-degrading activity was measured by incubation of EPS from *P.digitatum* and respective enzyme fractions followed by a sandwich ELISA. Incubation was performed in a reaction mixture containing 175 µl of EPS (1 µg/ml) in 50 mM sodium acetate (pH 5.5) and 25 µl of an enzyme fraction. After incubation for 16 hrs at 35°C, the enzyme was inactivated by heat treatment (5 min, 100°C).

Determination of side activities. The APIZYM (BIO Merieux SA, Lyon, France) was used for screening several side activities following the instructions of the supplier.

Glucanase activities were measured after incubation of 100 µl of the solutions of the respective glucan substrates (1 g/l) in 90 µl 50 mM sodium acetate (pH 5.0) together with 10 µl of the enzyme fraction at 35°C for 4 hrs. Laminaran (Nutrional Biochemicals Co., Cleveland, OH, U.S.A.) a ß(1-3,1-6)-glucan and a ß(1-3,1-6)-glucan produced by *Botrytis cinerea* and isolated as described for the EPS of *Aspergillus* and *Penicillium* species, were used as glucan substrates. After inactivation of the enzyme (5 min, 100°C) the release of glucose was measured by high-performance anion-exchange chromatography as described.

The activity towards various *p*-nitrophenyl (PNP)-glycosides (Sigma, St. Louis, USA) was measured spectrophotometrically at 405 nm using the molar extinction coefficient 13700 $M^{-1}.cm^{-1}$. One unit (U) was defined as the amount of enzyme which was able to release 1 µmol *p*-nitro-phenol per minute.

Sodium dodecyl sulphate-gelelectrophoresis, isoelectric focusing and titration curve. For the determination of the molecular weight of the exo-ß-D-galactofuranosidase, sodium dodecyl sulphate (SDS)-gelelectrophoresis was performed on a 10-15 % polyacrylamide gradient gel using the PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to the instructions of the supplier. Standards in the range of 10 to 100 kD were used. For the determination of the isoelectric point of the exo-ß-D-galactofuranosidase, isoelectric focusing and titration curves were performed on homogeneous polyacrylamide gels containing Pharmalyte carrier ampholytes, which generate a linear pH gradient from 3 to 9 in the gel. Standards with an isoelectric point in the range of 3 to 9 were used. Gels were stained for protein with the silver staining.

Temperature and pH optimum, K_m and V_{max}. Optimum pH and temperature were determined with 400 µl of a solution of EPS (0.5 mg/ml) of *P.digitatum* in 100 mM sodium acetate with the pH in the range of pH 3 to 6 incubated with 100 µl of a partly purified enzyme fraction containing 25 µg/ml protein (Sedmak assay) for 2 hrs at a temperature interval between 20 and 50°C. K_m and V_{max} were determined with 400 µl of a solution of *P.digitatum* EPS in 100 mM sodium acetate (pH 5.0) with a concentration in the range of 7.5 - 750 µg/ml incubated with 100 µl of a partly purified enzyme fraction containing 25 µg/ml protein (Sedmak assay) for 2 hrs at 35°C. After inactivation of the enzyme (5 min, 100°C) the release of galactose was measured by high-performance anion-exchange chromatography as described.

Substrate specificity of the exo-ß-D-galactofuranosidase. Dimers of ß(1,2)-, ß(1,3)-, ß(1,5)- and ß(1,6)-linked D-galactofuranose (Veeneman *et al.*, 1989) and a tetramer of ß(1,5)-linked D-galactofuranose (Veeneman *et al.*, 1987) were used to test the specificity of the exo-ß-D-galactofuranosidase. The release of galactose after incubation with the enzyme from the differently linked galactofuranosyl oligomers was determined by incubation of 100 µl (25 µg/ml) oligomer in 50 mM sodium acetate buffer pH 5.0 with 5 µl of the purified enzyme solution for 16 hrs at 30°C. The

amount of galactose released was determined by high-performance anion-exchange chromatography as described.

Degradation of the epitopes of EPS from Aspergillus *and* Penicillium *by the exo-ß-D-galactofuranosidase.* The epitope-degrading activity was studied by analysis of the reaction products after enzyme treatment of EPS from *P.digitatum*, *P.aurantiogriseum*, *P.citrinum*, *A.fumigatus* and *A.niger*. An aliquot of 750 µl of EPS solution (1 mg/ml) and 100 µl 1 M sodium acetate buffer pH 5.0 with 0.01 % sodium azide were incubated with 150 µl of the purified enzyme fraction (protein content < 5 µg/ml (Sedmak)) at 35°C for 16 hrs. After inactivation (5 min, 100°C) the reaction products were analysed using high-performance anion-exchange chromatography. The antigenicity of the EPS was measured using the sandwich ELISA.

RESULTS

Several crude enzyme preparations were screened for the presence of exo-ß-D-galactofuranosidase activity. Glucanex, a crude enzyme preparation isolated from *Trichoderma harzianum* did contain the highest activity. The exo-ß-D-galactofuranosidase was isolated from this preparation according to the procedure as summarised in Fig. 1.

Most of the exo-ß-D-galactofuranosidase activity appeared in the void volume of the DEAE Bio-Gel A anion-exchange column. However, some activity also eluted from the column with the gradient at 70 mM NaCl. Only the exo-ß-D-galactofuranosidase present in the void volume was purified further ona HTP-column. The major part (55 %) of the protein, which did not bind to this column, contained the exo-ß-D-galactofuranosidase. This void fraction also contained most of the side activities present in the crude enzyme preparation as shown in Table 1. Finally, the exo-ß-D-galactofuranosidase was purified from the void fraction of the HTP column on a Mono Q/HR anion-exchange column (Fig. 2). To point out the enzyme fraction able

Table 1. Enzyme activities towards synthetic *p*-nitrophenyl glycosides and 2-naphtyl-derivates, present in fractions obtained in the purification of the exo-ß-D-galactofuranosidase from a crude enzyme preparation of *Trichoderma harzianum* (Glucanex)[a].

Activity towards:	Crude enzyme[b]	HTP void fraction	Purified ß-D-galacto-furanosidase
p-nitrophenyl derivatives of:			
ß-D-galactofuranoside	++	++	-
α-D-galactopyranoside	-	n.d.	n.d.
ß-D-galactopyranoside	++	-	n.d.
α-L-arabinopyranoside	-	n.d.	n.d.
α-L-arabinofuranoside	-	+	-
α-D-glucopyranoside	-	n.d.	n.d.
ß-D-glucopyranoside	+++	++	-
α-D-mannopyranoside	-	n.d.	-
ß-D-mannopyranoside	n.d.	n.d.	-
α-D-xylopyranoside	-	-	n.d.
ß-D-xylopyranoside	-	n.d.	n.d.
ß-D-fucopyranoside	-	n.d.	n.d.
ß-L-fucopyranoside	-	n.d.	n.d.
α-L-fucopyranoside	-	n.d.	n.d.
2-naphtyl-phosphate (pH 8.5)	-	-	n.d.
2-naphtyl-phosphate (pH 5.4)	+	-	-
2-naphtyl-butyrate	+	+	-
2-naphtyl-caprylate	+	-	-
Naphtol-AS-BI-phosphate	+	-	n.d.

[a] The protein content of the crude enzyme preparation (0.1 %), the void fraction of the Hydroxyapatite column and the purified exo-ß-D-galactofuranosidase fraction is 65 μg/ml, 30 μg/ml and < 5 μg/ml, respectively, all determined according to the Sedmak assay.

[b] Activity expressed as order of magnitude of Units; one Unit is defined as the amount of enzyme which is able to release 1 μmol *p*-nitro-phenol, naphtol or glucose per minute. Symbols: -, < 1 mU/ml; +, ≥ 1 - < 10 mU/ml; ++, ≥ 10 - < 100 mU/ml; +++, ≥100 mU/ml; n.d., not determined.

to degrade the epitopes of *P.digitatum* EPS, each fraction eluting from this column was incubated with EPS and the ELISA reactivity thereof was measured subsequently as shown in Fig. 2. The fraction eluting at pH 6.3 (arrow) from this column contained the EPS-degrading enzyme and its purity was checked by sodium dodecyl sulphate-electrophoresis. One single protein band at 35 kD was visualised after staining with

Coomassie Brilliant Blue R-250. Additional silver staining revealed some additional minor protein bands. The titration curve of the purified exo-ß-D-galactofuranosidase revealed a isoelectric point of pH 4 (results not shown).

Incubation with *p*-nitrophenyl derivates, naphtyl derivates and several glucans was performed to detect any side activity that might be present in the purified enzyme fractions. Most of the side activities which were initially present in the crude enzyme preparation were removed with the purification. No activity towards *p*-nitrophenyl-ß-D-galactofuranoside could be detected after 1 hr incubation, but after 24 hrs some

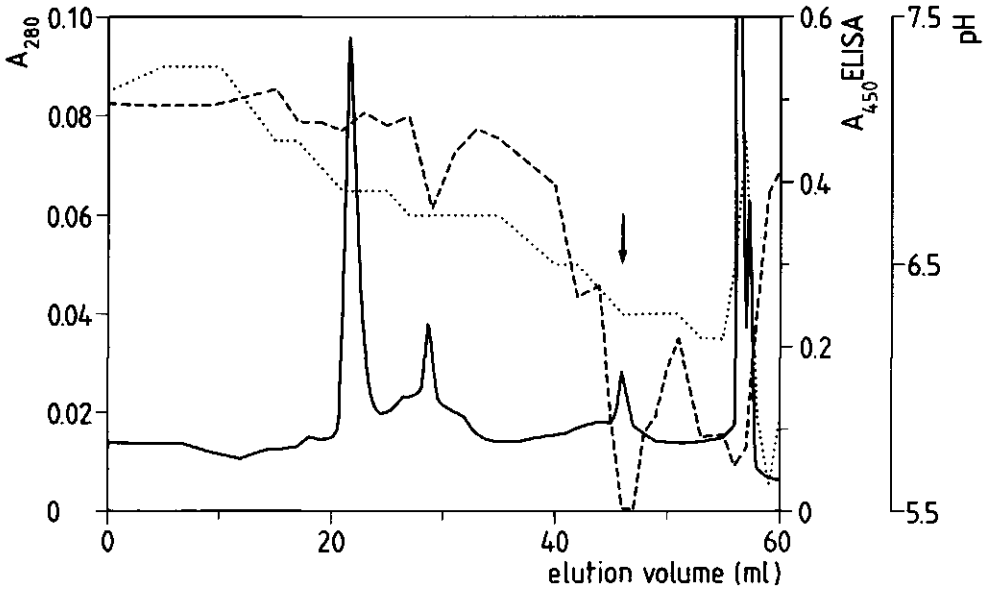

Figure 2. Final purification of the exo-ß-D-galactofuranosidase on a Mono Q/HR anion-exchange column using FPLC with a pH gradient from 7.0 to 5.5 in BisTris buffer (see Materials and Methods). _____, A_{280}; ·····, pH of the effluent; – – – –, ELISA reactivity of EPS incubated with each fraction.

Table 2. Characteristics and kinetic parameters for the exo-ß-D-galactofuranosidase purified from *Trichoderma harzianum*.

pH optimum	4-4.5
Temperature optimum	35-40°C
V_{max} (for EPS of *P.digitatum*)	1 U/mg
K_m (for EPS of *P.digitatum*)	0.2 mg/ml
Isoelectric point	4
Molecular weight	35 kD

release of *p*-nitro-phenol could be observed. The purified enzyme contained traces of glucanase activity. However, no release of glucose was detected after incubation of this purified exo-ß-D-galactofuranosidase fraction with the EPS from *Aspergillus* and *Penicillium* species. Therefore, no further attempts have been made to remove this glucanase activity.

The purified exo-ß-D-galactofuranosidase fraction was partly characterised and the properties are given in Table 2. The enzyme has an optimal activity on EPS of *P.digitatum* between pH 4 and pH 4.5. The enzyme activity dropped above pH 5 and at a pH of 7 or higher, no activity could be detected (results not shown). No galactose was released as a result of chemical hydrolysis due to low pH during incubation as established in a separate experiment (results not shown). Maximum enzyme activity was obtained between 35 and 40°C.

Galactose was released from ß(1,5)-linked D-galactofuranoside tetramer, but not from the ß(1,5)-linked dimer and the corresponding ß(1,2), ß(1,3) and ß(1,6)-linked dimers. The enzyme did not liberate any sugars from EPS of *Fusarium* species or species belonging to the order of Mucorales.

The degradation of ß(1,5)-linked galactofuranosyl residues of *Aspergillus* and *Penicillium* EPS in relation to their antigenicity was studied by incubation of EPS from a number of *Aspergillus* and *Penicillium* species with the purified exo-ß-D-galactofuranosidase. The antigenicity of these extracellular polysaccharides was

determined by measuring their ELISA reactivity before and after enzyme treatment (Table 3). The antigenicity of the enzyme-treated EPS disappeared completely.

The sugar composition of the initial extracellular polysaccharides, and the relative amount of galactose released as a result of enzymatic hydrolysis are listed in Table 4. The extracellular polysaccharides contained mainly mannose, galactose, glucose and a minor amount of xylose. The amount of galactose initially present decreased after treatment with the exo-ß-D-galactofuranosidase. In spite of the fact that the antigeni-

Table 3. Minimal detectable quantity of various extracellular polysaccharide preparations of *Aspergillus* and *Penicillium* species before and after treatment with exo-ß-D-galactofuranosidase[a] in a sandwich ELISA with antibodies raised against *P.digitatum* EPS.

Origin of EPS	Minimal detectable quantity (ng/ml)	
	Native	Enzyme treated
P.digitatum	45	$> 1 \times 10^5$
P.aurantiogriseum	< 2	$> 1 \times 10^5$
P.dierckxii	400	$> 1 \times 10^5$
P.citrinum	10	$> 1 \times 10^5$
A.fumigatus	70	$> 1 \times 10^5$
A.niger	5	$> 1 \times 10^5$

[a] Incubation was performed for 16 hrs at 35°C.

city of EPS disappeared completely upon enzyme treatment, only 26 to 33 % of galactose residues were released by the enzyme. Analysis of the products after enzyme treatment using high-performance anion-exchange chromatography with pulsed amperometric detection showed that only monomers of galactose were liberated (results not shown).

Structural characterisation of the initial EPS from *P.digitatum* by reductive cleavage followed by acetyl-methyl exchange showed that galactose occurs only in the furanoside form and none as pyranosides. The glucose and mannose residues are exclusively linked as pyranosides. All products identified by reductive cleavage were confirmed by

Table 4. Neutral sugar composition of native extracellular polysaccharides (EPS) of several *Aspergillus* and *Penicillium* species and the percentage of galactose released by exo-ß-D-galactofuranosidase (16 hrs, 35°C).

Origin EPS	Sugar composition[a]				% Galactose released[b]
	Xyl	Man	Gal	Glc	
P.digitatum	4	35	46	15	26
P.aurantiogriseum	2	22	30	46	33
P.dierckxii	16	33	18	33	30
P.citrinum	6	30	37	27	30
A.fumigatus	4	18	20	58	32
A.niger	6	46	34	14	32

[a] Expressed in mole percentages. The sugar content of the EPS preparations varied between 40 and 70 % (w/w).
[b] Amount of galactose released from the EPS preparations after enzyme treatment expressed as percentage of the initial galactose content.

standard methylation analysis and the molar ratios of the galactose derivatives before and after enzymic degradation are summarised in Table 5. As shown in this table, both ß(1,5)-linked, ß(1,6)-linked and ß(1,5,6)-linked galactofuranose residues could be identified in the native polysaccharides. After enzymatic degradation with the purified exo-ß-D-galactofuranosidase the relative amount of ß(1,5)-linked galactofuranosides decreased considerably, whereas the amounts of ß(1,6)-linked and ß(1,5,6)-linked

Table 5. Relative amounts of different linked galactose residues in extracellular polysaccharides of *Penicillium digitatum* before and after treatment with exo-ß-D-galactofuranosidase.

Galactose linkage	Native	Enzyme treated
t-galactofuranose[a]	0.3	0.5
(1,5)-galactofuranose	2.1	1.0
(1,6)-galactofuranose	1.0	1.0
(1,5,6)-galactofuranose	0.1	0.1

[a] Non-reducing terminal galactofuranose residue.

galactofuranose did not change. The apparent increase of the terminal galactofuranose residues after enzymatic treatment was not confirmed by standard methylation analysis, therefore this can be considered as not significant.

DISCUSSION

Exo-ß-D-galactofuranosidase-catalysed degradation of extracellular polysaccharides from *Aspergillus* and *Penicillium* was studied in relation to the antigenic properties of these polysaccharides. The exo-ß-D-galactofuranosidase was purified using anion-exchange and adsorption columns combined with FPLC. The enzyme was active on the tetramer of ß(1-5)-linked galactofuranose from which it released galactose. The enzyme acted as an exo-enzyme on EPS as only monomers of galactose were released. A low activity was found on synthetic PNP-ß-galactofuranoside indicating that the enzyme needs more than one galactofuranose residue for binding. The purified enzyme was checked for a large number of side activities, and it appeared to show some glucanase activity only. The influence of the pH on the activity of the exo-ß-D-galactofuranosidase appeared to be similar as described by Rietschel-Berst *et al.* (1977) for the exo-ß-D-galactofuranosidase from *P.charlesii*; its temperature optimum was slightly lower.

Structural characterisation of the EPS preparation of *P.digitatum* revealed that the galactose residues occur in the furanose form only. This was previously established for galactomannans derived from *A.niger* (Barreto-Bergter *et al.*, 1980). Galactopyranose residues as previously found in the cell wall of *A.niger* (Bardalaye and Nordin, 1977) and in the extracellular slime of *A.fumigatus* (Latgé *et al.*, 1991) were clearly absent in EPS of *P.digitatum* as shown by the reductive cleavage procedure. In addition to the earlier reported ß(1,5)-linked galactofuranose residues (Barreto-Bergter *et al.*, 1981; Gander *et al.*, 1974), the presence of ß(1,5,6)-linked and ß(1,6)-linked residues was also demonstrated. The latter compound was previously found in a cell wall arabino-galactan derived from *Mycobacterium smegmatis* (Gruber and Gray, 1990). To our knowledge, this is the first time that ß(1,6)-linked and ß(1,5,6)-linked galactofuranoses

have been identified in fungal polysacccharides.

The antigenicity of the extracellular polysaccharides disappeared completely after treatment with the purified exo-ß-D-galactofuranosidase, indicating that all the epitopes were degraded. After incubation of EPS of *Aspergillus* and *Penicillium* species with the purified exo-ß-D-galactofuranosidase monomers of galactose were liberated only. However, no complete hydrolysis of the galactofuranoses, initially present in the extracellular polysaccharides, could be obtained with the exo-ß-D-galactofuranosidase. In fact, after enzymatic degradation of EPS of *P.digitatum* the molar ratios of ß(1,5)-linked and ß(1,6)-linked galactofuranose residues decreased from 2:1 to 1:1. With these results we can propose a new structural model for the antigenic galactofuranose side chains of the extracellular galactomannans from *P.digitatum* and possibly for all *Aspergillus* and *Penicillium* species (Fig. 3). This model replaces the older model of Gander and coworkers (1974) which constituted of unbranched ß(1,5)-linked galactofuranose chains attached to a mannan backbone. Our model is composed of a branched side chain attached to the mannan core. The side chain is a ß(1,5)-linked galactofuranose sequence which, in turn carries a ß(1,6)-linked galactofuranose sequence, attached to a ß(1,5,6) galactofuranose branching

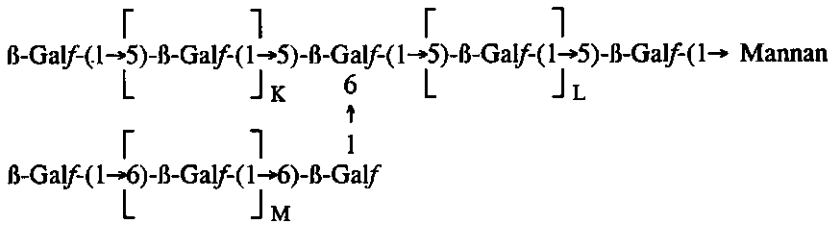

Figure 3. Proposed structural model for the immunodominant galactofuranose side chains of the extracellular galactomannans of *P.digitatum*. The values for K,L and M can vary from 2 to 8 residues.

point. Based on the amount of galactose liberated and of the ß(1,5)-linked galactofuranose residues still present in EPS after enzyme treatment, the value of K, L and M as indicated in Fig. 3 can vary from 2 to 8 residues.

The exo-ß-D-galactofuranosidase attacks the ß(1,5)-linked sequence from the non-reducing end and is stopped near the branching point. It can neither hydrolyse the ß(1,6)-linked galactofuranose sequence, nor the interior ß(1,5)-linked galactofuranose sequence. Since the antigenicity disappears upon enzyme treatment, it can also be concluded that the antibodies used in this study bind only to sequences of ß(1,5)-linked galactofuranose residues, with an unsubstituted non-reducing galactofuranose terminal.

In conclusion, this epitope-degrading enzyme appeared to be very useful for the structural analysis of the epitopes of these antigenic polysaccharides. The unique epitope-degrading properties of the exo-ß-D-galactofuranosidase enables specific application of this enzyme. It can be very useful for the detection of false-positive reactions in immunological methods such as ELISA and latex-agglutination assay as it provides an alternative to the use of synthetic ß(1,5)-linked galactofuranose epitopes as specific blocking agents (Kamphuis *et al.*, 1989; Notermans *et al.*, 1988). Also, the purification and availability of the exo-ß-D-galactofuranosidase allows the specific removal of the non-reducing ß(1,5)-D-galactofuranose sequences, down to close to the ß-D-(1,6)-branchpoint, leaving the intact ß(1,6)-D-galactofuranose sequences freely exposed. This enables the possibility to raise antibodies against this enzymatically modified EPS and to study their specificity towards *Aspergillus, Penicillium* and other mould species.

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CHAPTER 6

H.J. Kamphuis, G.A. De Ruiter, S.H.W. Notermans and F.M. Rombouts.
Production of antigenic extracellular polysaccharides (EPS) by Penicillium aurantiogriseum *and* Penicillium digitatum, *(submitted).*

PRODUCTION OF ANTIGENIC EXTRACELLULAR POLYSACCHARIDES (EPS) BY *PENICILLIUM AURANTIOGRISEUM* AND *PENICILLIUM DIGITATUM*

ABSTRACT

Antigenic extracellular polysaccharides (EPS) were produced by *Penicillium aurantiogriseum* M 91 and *P.digitatum* M 58 under various conditions, including different carbon and nitrogen sources, glucose concentrations and incubation periods. With lactate as carbon source no antigenic EPS was produced. EPS produced consisted mainly of glucose, mannose and galactose. Differences in the monosaccharide composition occurred. The amount of glucose in the medium (1-30 g/l) influenced growth and the production of antigenic EPS moderately. The antigenicity of *P.aurantiogriseum* EPS decreased in time. In contrast, the antigenicity of *P.digitatum* EPS did not decrease in time. It was observed that *P.aurantiogriseum* grows well with nitrate as nitrogen source, this in contrast to *P.digitatum*.

INTRODUCTION

Fungi produce numerous extracellular macromolecules. These oligomers or polymers may be degradation products of complex substances and in all probability they originate from the mycelial cell wall. Haworth *et al.* (1935 and 1937) isolated and characterised from the culture filtrate of *Penicillium charlesii* galactocarolose, a 5-O-ß-D-galactofuranosyl containing decasaccharide and mannocarolose, a α-D-mannopyranosyl containing nonasaccharide. It was shown, that these oligosaccharides were derived from complex glycoproteins, later referred to as peptidophosphogalactomannan (Gander *et al.*, 1974). Peptido(phospho)galactomannans were also found to be produced by *Cladosporium werneckii* and *C.fulvum* (Lloyd, 1970; 1972; Dow and Callow, 1979), species of the dermatophytes *Trichophyton* and *Microsporum* (Barker *et al.*, 1963; Bishop *et al.*, 1962; 1965), species of the genera *Aspergillus* and *Penicillium*

(Bardalaye and Nordin, 1977; Miyazaki and Yadomae, 1968; Preston *et al.*, 1970). Interest in the production and characterisation of these peptidogalactomannans increased, due to the antigenic properties of these glycoproteins. Notermans and Soentoro (1986) showed that nearly all species belonging to the genera *Aspergillus* and *Penicillium* produce antigenic extracellular polysaccharides (EPS). It was demonstrated that EPS was water soluble, heat stable and consisted mainly of mannose, galactose and glucose. The EPS production was correlated with the mycelium dry weight (Notermans *et al.*, 1986). It was concluded that EPS may be a potential marker for the determination of the mycological quality of food and feed. With the enzyme-linked immunosorbent assay (ELISA), using antibodies raised against EPS of *P.digitatum*, only the genera *Aspergillus* and *Penicillium* could be detected (Notermans and Soentoro, 1986). It was shown that antibodies were directed to the ß(1,5)-linked galactofuranosyl residues of the EPS (Notermans *et al.*, 1988). The reliability of the immunoassay could be enhanced by using a synthetic tetramer of ß(1,5)-linked galactofuranosyl residues as specific blocking agent (Kamphuis *et al.*, 1989). A latex-agglutination assay based on this principle was used successfully in comparative studies for the detection of *Aspergillus* and *Penicillium* species in different types of food (Notermans and Kamphuis, 1990; Kamphuis *et al.*, 1992). However, it was observed that no EPS of *Penicillium* could be detected in mould ripened cheese, such as Roquefort and Camembert.

It is known that the production of extracellular polysaccharides can be influenced by growth conditions such as differences in aeration, carbon- and nitrogen source. In earlier studies it was observed that the polysaccharide production of *Aspergillus parasiticus* was approximately two times higher in submerged than in surface cultures (Ruperez and Leal, 1981). High carbon/nitrogen (C/N) ratios in the culture medium favoured the ß-glucan production by *Botrytis cinerea* (Leal *et al.*, 1979). The ß-glucan production increased with glucose concentration increasing to 20 g/l. However, the extracellular polysaccharide production by various mould species decreased when the glucose concentration in the medium exceeded 20-30 g/l (Leal-Serrano *et al.*, 1980; Leal and Rupe´rez, 1978). It has been shown that the preference for a carbon source

can be influenced by the C/N ratio (Graham *et al.*, 1976). The carbon, nitrogen, phosphorus and sulphur containing nutrients in *P.charlesii* cultures influenced primarily the growth characteristics and the final medium pH. Production of a peptidophosphogalactomannan by *P.charlesii* increased when the final medium pH decreased while the composition of the pPGM was only indirectly influenced throught pH effects (Salt and Gander, 1988).

This study describes the influence of culture conditions on the production of antigenic EPS. *P.aurantiogriseum* and *P.digitatum* were cultured with various carbon sources, glucose concentrations and nitrogen sources.

MATERIALS AND METHODS

Fungal isolates. Penicillium aurantiogriseum M 91 and *P.digitatum* M 58 from the collection of this laboratory were used. The strains were grown on Malt Extract Agar (Oxoid L 39) slants at 24°C and stored at 4°C. Spores were harvested by addition of 10 ml sterile physiological saline containing 0.05% (v/v) Tween 20 to each tube. Inoculum for *P.aurantiogriseum* and *P.digitatum* contained approximately 2×10^7 and 5×10^6 colony forming units per ml, respectively.

Mould count. Mould count was determined on malt extract agar plates (9 cm plates) containing 20 ml malt extract agar. Colonies were counted after 5 days of incubation at 24°C.

EPS production and isolation. EPS was produced in Yeast Nitrogen Base (YNB; Difco) supplemented with different carbon sources: galactose, maltose, mannose (all purchased from Sigma), lactose (BDH chemicals), glucose, lactate (Merck) and saccharose (Difco). For this, 900 ml portions of different carbon sources (30 g/l in distilled water) were heat sterilised (120°C for 15 min) in 3 l flasks. After addition of 100 ml 10-fold concentrated filter sterilised YNB (6.7 g/100 ml distilled water), spores were inoculated. EPS was also produced in Yeast Carbon Base (YCB; Difco)

supplemented with 20 g/l glucose and different nitrogen sources. For this, 100 ml filter sterilised 10-fold concentrated YCB (11.7 g/100 ml distilled water) was added to a sterilised solution of glucose (20 g/l in water), containing the nitrogen sources ammonium sulphate (5.2 g/l; Merck), casamino acids (5.0 g/l; Difco), potassium nitrate (7.8 g/l; Merck), urea (2.3 g/l; Merck) and ammonium sulphate/urea (2.5 and 1.25 g/l), respectively.

In some experiments at different time intervals, 1 ml culture fluid was taken and filtered. In general after incubation periods of 7 days at 24°C with orbital shaking at 100 rpm, mycelia were removed by filtration (Schleicher & Schüll 520 B) and the culture filtrate was freeze-dried. The freeze-dried material was dissolved in 40 ml distilled water and dialysed against running tap water (24 hrs) and distilled water (24 hrs). The retentate was freeze-dried and subsequently dissolved in 20 ml distilled water, water-insoluble materials were removed by centrifugation (30 min 18000 x g). EPS was collected from the supernatant after three 80% (v/v) ethanol precipitations, freeze-dried and weighed.

Mycelium dry weight (MDW) estimation. Mycelium was removed by filtration on a pre-weighed filter (Schleicher & Schüll 520 B). The mycelium was washed three times with distilled water and dried at 80°C for 24 hours and then weighed.

Chemical analysis. The neutral sugar composition of the EPS was determined before and after acid hydrolysis by conversion to alditol acetates as described by Englyst and Cummings (1984) and analysed by gas-liquid chromatography. Hydrolysis was performed by using 2 M trifluoro acetic acid (1 hr, 121°C) and inositol was used as internal standard (Albersheim *et al.*, 1967). Protein content was determined as described by Lowry (1951) with Bovine Serum Albumin (BSA) as standard.

Detection of antigenic EPS. Antigenic EPS was determined by the sandwich ELISA and was carried out as described by Notermans and Heuvelman (1985). To wells of polyvinyl trays (Cooke, Dynatech) 0.1 ml 10 μg/ml IgG 332 anti-*P.digitatum* EPS

diluted in 0.07 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) was added. The trays were incubated overnight (100 rpm) at room temperature and subsequently washed with running tap water containing 0.05% (v/v) Tween 20. Then 0.1 ml of EPS dilutions in PBS containing 0.05% Tween 20 (PBST) were added to each well. After 1 hour incubation with shaking at room temperature the trays were washed as described above. Then 0.1 ml IgG 658 anti-*P.digitatum* EPS conjugated to horse-radish peroxidase in PBS containing 0.05% (v/v) Tween 20 and 1% (w/w) BSA was added. The incubation (1 hour) and the washing procedure was carried out as described above. The enzyme activity was determined spectrophotometrically at 450 nm. For this, 1 ml of 42 mM of 3,5,3',5'-tetramethylbenzidine (Merck) in dimethyl-sulphoxide (Sigma) was added under vigorous shaking to 100 ml 0.1 M sodium acetate buffer pH 5.6. Just before adding 0.1 ml substrate to the wells, 7 μl 30% H_2O_2 was added to the solution. After incubation for 20 min at room temperature 0.05 ml 2M H_2SO_4 was added to each well to stop the enzyme reaction. The IgG binding capacity to EPS could completely be inhibited by addition of a tetramer of ß(1-5)-linked galactofuranosyl residues. The antigenicity of EPS is expressed as minimal detectable quantity (ng/ml) and the ELISA reaction of the culture filtrates is expressed as titre, i.e. reciprocal of the highest dilution giving a positive ELISA reaction.

RESULTS

Reproducibility of the experiments. *P.aurantiogriseum* and *P.digitatum* EPS was produced in four-fold in YNB supplemented with 30 g/l glucose (Table 1). Higher average mycelium dry weight values and EPS yields were observed for *P.aurantiogriseum*. EPS produced by *P.aurantiogriseum* contained more carbohydrate material and the protein content was lower than that of EPS produced by *P.digitatum*. Differences in the monosaccharide composition of the EPS produced by both isolates were observed. In general, the standard deviations observed were acceptable.

Table 1. Growth of *P.aurantiogriseum* and *P.digitatum* at 23°C for 7 days in 1000 ml YNB supplemented with 30 g/l glucose in four separate experiments.

Growth characteristics	*P.aurantiogriseum* (average ± s.d.)	*P.digitatum* (average ± s.d.)
Final pH	2.25 ± 0.02	2.09 ± 0.03
Mycelium dry weight (mg/ml)	6.5 ± 0.5	4.1 ± 0.1
EPS: yield (mg/ml)	176 ± 18	82 ± 9
protein (w/w %)	7 ± 1	37 ± 5
carbohydrate (w/w %)	91 ± 3	42 ± 2
mannose (mol %)	20 ± 1	36 ± 1
galactose (mol %)	35 ± 2	53 ± 9
glucose (mol %)	44 ± 2	10 ± 7
antigenicity[a]	40	120

[a] Minimal detectable quantity EPS in sandwich ELISA in ng/ml.

Incubation time. Time experiments were carried out with *P.aurantiogriseum* and *P.digitatum*, in YNB supplemented with 30 g/l glucose. The pH, mycelial dry weight, EPS yield and the ELISA titre of the culture filtrate of *P.aurantiogriseum* as function of the time are presented in Figure 1. In general for both isolates the same responses concerning the pH, mycelial dry weight, EPS yield were observed (Tables 2 and 3). However, there were differences in absolute values. After a quick drop of the medium pH within 2-4 days, the pH stabilised at approximately pH 2.0. Higher mycelium dry weights were observed for *P.aurantiogriseum* than for *P.digitatum*. *P.digitatum* produced less EPS than *P.aurantiogriseum*, 93 versus 223 mg/l, respectively.

The monosaccharide content, protein content and the antigenicity of EPS, after different incubation times isolated from *P.aurantiogriseum* and *P.digitatum* are shown in Tables 2 and 3, respectively. The protein contents of the *P.aurantiogriseum* EPS were considerably lower than the protein contents of the *P.digitatum* EPS, 6-15% and 13-34%, respectively.The carbohydrate content for both species varied from 12-76% and 48-57%, respectively. Galactose, glucose and mannose were the main sugars both

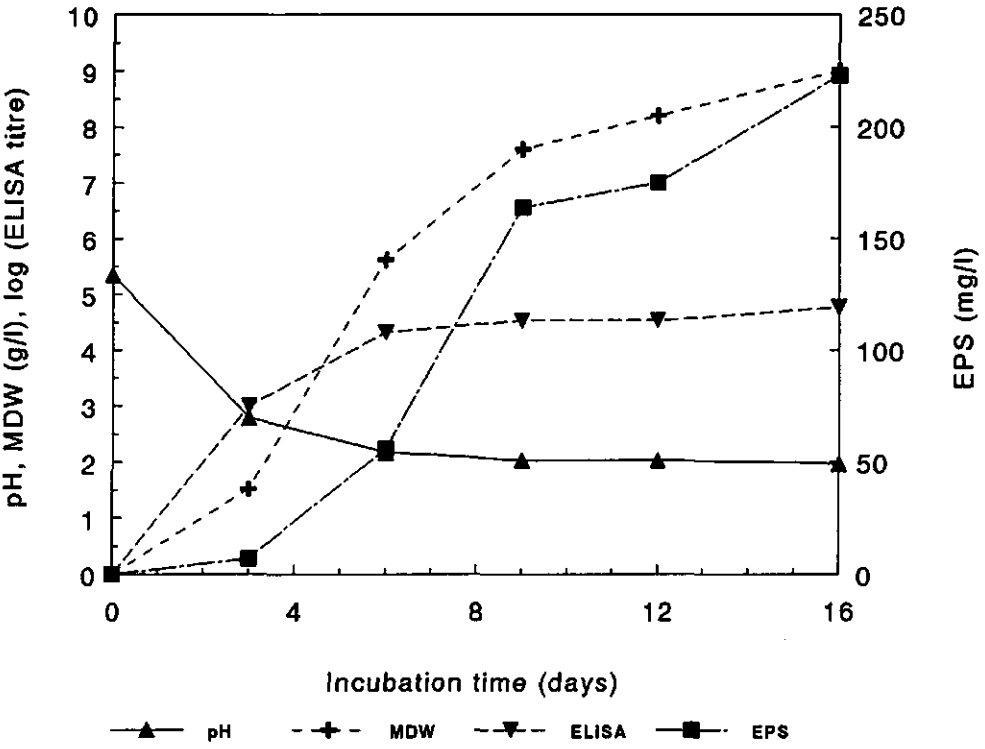

Figure 1. Growth characteristics of *P.aurantiogriseum* in YNB medium supplemented with 30 g/l glucose.

in the EPS of *P.aurantiogriseum* and *P.digitatum* EPS (Table 4). High glucose contents were determined in EPS produced by *P.aurantiogriseum* when the incubation period exceeded 3 days (approximately 50 mol%). High performance size-exclusion chromatography revealed that high molecular weight glucans were produced (results not shown).

In general, *P.digitatum* EPS isolated at different time intervals was more antigenic than *P.aurantiogriseum* EPS. The antigenicity of *P.aurantiogriseum* EPS decreased after 6 days incubation period.

Table 2. Medium pH values, mycelium dry weight, EPS yield and the carbohydrate content, protein content and antigenicity of *P.aurantiogriseum* EPS produced under different conditions.

Growth condition	pH		MDW	EPS			
				Yield	Carb	Protein	Antigenicity
	initial	final	(g/l)	(mg/l)	(w/w %)		
Effect incubation time (days)							
3	5.3	2.8	1.5	7	12	6	+++[a]
6	5.3	2.2	5.6	56	76	7	+++
9	5.3	2.0	7.6	164	75	10	++
12	5.3	2.0	8.2	175	68	15	++
16	5.3	2.0	9.0	223	71	15	+
Effect carbon source							
galactose	5.3	2.7	1.4	56	17	11	+++
lactose	5.3	2.8	1.2	24	39	16	+++
maltose	5.3	2.3	5.8	76	76	20	+++
mannose	5.3	2.1	6.6	86	71	16	++
saccharose	5.3	2.3	5.5	90	73	12	++
glucose	5.3	2.1	6.6	116	87	6	+++
Effect glucose concentration (g/l)							
1	5.3	3.5	0.3	9	28	10	++
5	5.3	2.6	1.9	16	62	10	+++
10	5.3	2.4	2.8	24	73	ND	+++
20	5.3	2.3	3.6	54	69	ND	+++
30	5.3	2.4	2.8	26	87	6	+++
Effect nitrogen source							
nitrate	5.4	4.4	2.1	28	27	5	+++
ammonium	5.3	2.5	2.7	28	46	6	+++
urea	6.4	4.0	2.9	30	39	9	+++
ammonium/urea	6.0	3.5	3.3	19	38	11	+++
casamino acids	5.7	3.6	5.1	26	33	11	+++

Note. Abbreviations used: MDW, mycelium dry weight; Carb, carbohydrate; ND, not detected.

[a] +++ ≤ 100 ng/ml EPS gives a positive ELISA result; ++ > 100 - ≤ 1,000 ng/ml EPS gives a positive ELISA result; + > 1,000 - ≤ 10,000 ng/ml EPS gives a positive ELISA result; - >10,000 ng/ml EPS gives a positive ELISA result.

Carbon source. EPS of *P.aurantiogriseum* and *P.digitatum* were produced in YNB supplemented with different carbon sources. *P.aurantiogriseum* was capable to grow on all tested carbon sources. The highest mycelial dry weights were observed on mannose, maltose, saccharose and glucose, respectively (Table 2). The EPS yields varied from 24-116 mg/l. The pH of the culture medium decreased to 2.1-2.8,depending on the carbon source. In case of lactate and lactate/glucose the pH increased (results not shown).

Differences were observed in the carbohydrate and protein content of the *P.aurantiogriseum* EPS. On lactate and lactose/glucose both the carbohydrate and protein content of the "EPS" was approximately 5 mol% (results not shown). The EPS consisted of mannose, galactose and glucose (Table 4). High molar percentages of glucose were determined in EPS produced on mannose and saccharose, 67 and 79%, respectively. In general, the minimal detectable quantity of all the isolated *P.aurantiogriseum* EPS was $\leq$ 100 ng/ml, with exception of the EPS produced on mannose and saccharose (Table 2).

Reduced growth of *P.digitatum* was observed with maltose and mannose as carbon source (Table 3). On maltose and mannose only a slight pH decrease of the culture medium was observed of approximately 1.1-1.7 pH units. On lactate and lactate/glucose the pH drop was 0.1-0.3 pH units (results not shown). The yield of EPS varied from 8 mg/l (galactose and mannose) to 81 mg/l (glucose). Differences in the molar percentage monosaccharides composition of the produced EPS by *P.digitatum* were observed (Table 4). Remarkable was the high molar percentages rhamnose (66%) in EPS produced on lactate (results not shown).

The minimal detectable quantity of EPS produced by *P.digitatum* on the different carbon sources was $\leq$ 1000 ng/ml for EPS produced on galactose, lactose, maltose and saccharose (Table 3). EPS produced on mannose was detectable in quantities of 1000-10,000 ng/ml. However, EPS produced on lactate and lactate/glucose were not antigenic (results not presented).

Table 3. Medium pH values, mycelium dry weight, EPS yield and the carbohydrate content, protein content and antigenicity of *P.digitatum* EPS produced under different conditions.

Growth conditions	pH initial	pH final	MDW (g/l)	EPS Yield (mg/l)	EPS Carb (w/w %)	EPS Protein (w/w %)	Antigenicity
Effect incubation time (days)							
3	5.3	2.5	2.4	9	48	13	+++[a]
6	5.3	2.2	4.3	35	56	21	+++
9	5.3	2.2	4.2	63	57	25	+++
12	5.3	2.2	4.4	70	52	34	+++
16	5.3	2.1	4.4	93	56	32	+++
Effect carbon source							
galactose	5.3	2.6	2.4	8	22	6	++
lactose	5.3	3.0	0.8	10	12	6	++
maltose	5.3	4.2	0.04	9	5	1	++
mannose	5.3	3.6	0.05	8	22	2	+
saccharose	5.3	2.2	5.6	28	51	34	++
glucose	5.3	2.2	4.2	81	42	41	++
Effect glucose concentration (g/l)							
1	5.3	3.5	0.1	13	67	26	+++
5	5.3	2.8	1.4	46	67	22	+++
10	5.3	2.3	3.3	54	68	24	+++
20	5.3	2.2	3.9	89	65	27	+++
30	5.3	2.2	3.9	69	40	40	++
Effect nitrogen source							
ammonium	5.4	2.5	3.1	18	34	20	+++
urea	6.4	4.1	2.7	28	35	26	++
ammonium/urea	6.0	3.6	4.2	44	58	21	+++
casamino acids	5.7	3.7	4.2	50	60	38	+++

For note and abbreviations see Table 2.

Glucose. *P.aurantiogriseum* and *P.digitatum* were grown in YNB supplemented with different glucose concentrations (1-30 g/l). In this experiment after seven days incubation EPS was isolated, MDW was determined and the pH of the culture medium was measured.

In general, when the glucose concentration increased the final pH of the culture medium decreased and the MDW and EPS yield increased (Tables 2 and 3). However, when *P.aurantiogriseum* was grown on 30 g/l glucose the MDW decreased in comparison to that of 20 g/l glucose. The same was observed for the yield of EPS. Protein was not detectable in *P.aurantiogriseum* EPS produced in 10 and 20 g/l glucose. The antigenicity of the *P.aurantiogriseum* EPS produced in different glucose concentrations was fairly constant, with the exception of EPS using 1 g/l glucose (Table 2).

The mycelial dry weight and the yield of EPS for *P.digitatum* increased with increasing glucose concentration (Table 3). The carbohydrate content and the protein content of *P.digitatum* EPS, produced in different concentrations glucose were comparable (Table 3). The antigenicity of the isolated *P.digitatum* EPS was comparable with exception of the EPS produced in 30 g/l glucose (Table 3). The monosaccharide composition of *P.digitatum* EPS produced with 30 g/l glucose was slightly different from that of the other EPS (Table 4). The mannose and glucose content were higher and the galactose content was somewhat lower than those of the EPS produced in cultures with less than 30 g/l glucose.

Nitrogen source. EPS of *P.aurantiogriseum* and *P.digitatum* was produced in YCB with 30 g/l glucose and different nitrogen sources (Tables 2 and 3, respectively). With exception of casamino acids, the nitrogen content was the same in all cases. Hardly any mycelial growth of *P.digitatum* with potassium nitrate (results not shown) while *P.aurantiogriseum* showed growth. For *P.aurantiogriseum*, the highest MDW was observed in YCB with casamino acids (Table 2). Highest mycelial dry weights for *P.digitatum* were observed with casamino acids and with a mixture of ammonium sulphate and urea.

Table 4. Monosaccharide composition in Mol % of *P.aurantiogriseum* and *P.digitatum* EPS produced under different conditions.

Growth condition	*P.aurantiogriseum*			*P.digitatum*			
	Man[a]	Gal	Glc	Rha	Man	Gal	Glc
Effect incubation time (days)							
3	15	70	12	-	12	47	41
6	15	42	43	-	28	66	5
9	19	17	64	-	31	62	6
12	29	18	52	-	45	46	9
16	28	17	54	-	41	45	13
Effect carbon source							
galactose	26	57	12	-	22	48	31
lactose	28	68	3	-	41	46	12
maltose	29	47	23	-	43	45	10
mannose	21	11	67	-	36	26	38
saccharose	11	10	79	-	43	41	15
glucose	21	35	44	-	38	40	21
Effect glucose concentration (g/l)							
1	58	33	8	-	34	63	2
5	30	68	1	-	26	71	2
10	25	63	10	-	25	72	2
20	24	58	17	-	32	65	4
30	28	61	10	-	37	55	8
Effect nitrogen source							
nitrate	17	34	43	14	15	20	51
ammonium	20	38	41	-	30	63	5
urea	24	54	22	-	38	25	36
ammonium/urea	31	64	3	-	28	67	4
casamino acids	35	31	33	-	29	66	4

[a] Man: mannose; Gal: galactose; Glc: glucose; Rha: rhamnose.

The EPS preparations produced on various nitrogen sources by the two mould isolates had antigenic properties. The quantities of EPS produced by *P.aurantiogriseum* on the various nitrogen sources varied between 19 and 30 mg/ml (Table 2). All *P.aurantiogriseum* EPS produced on those nitrogen sources showed a positive reaction in the ELISA and the minimal quantity EPS detectable was in all cases less than 100 ng/ml (Table 2). Differences in molar percentages of the monosaccharide composition of the *P.aurantiogriseum* EPS were observed (Table 4).

Yields of EPS of *P.digitatum* on various nitrogen sources varied between 10 and 50 mg/ml (Table 3). On potassium nitrate, 13 mg/l EPS was isolated although only traces mycelium were observed (results not shown). The antigenicity of the EPS of *P.digitatum* produced on various nitrogen sources is shown in Table 3. The minimal detectable quantities EPS were lower than 100 ng/ml with the exception of EPS produced on urea and potassium nitrate. When *P.digitatum* was grown on nitrate the EPS produced contained rhamnose (Table 4).

DISCUSSION

Antigenic extracellular polysaccharides (EPS) were produced by *P.aurantiogriseum* and *P.digitatum* in liquid cultures under various conditions. Under standard conditions, within an experiment, there was only a small variation in mycelial dry weight, amount of EPS and its monosaccharide composition, carbohydrate and protein content as observed for both *P.aurantiogriseum* and *P.digitatum* (Table 1). However, mycelial growth depended on the carbon source, its concentration and the nitrogen source. Under nearly all growth conditions antigenic EPS was produced. The antigenicity of the EPS depended amongst others on medium composition and incubation time and was apparently not dependent on protein content of the EPS (Tables 2, 3 and 4). During prolonged incubation of *P.aurantiogriseum* the antigenicity of the EPS decreased. Reasons may be enzymatic degradation of the antigenic part of EPS or the relatively increased production of non-antigenic polysaccharides. However, the latter

possibility is not supported by protein content or sugar composition, which does not change dramatically after 6 days incubation (Tables 2 and 4). No decrease of the antigenicity of *P.digitatum* EPS was observed upon prolonged incubation.

Despite growth, neither *P.aurantiogriseum* nor *P.digitatum* produced antigenic EPS when grown on lactate. In other experiments it was shown that also *P.camemberti* isolated from Camembert cheese also did not produce antigenic EPS on lactate, while this species did produce antigenic EPS on dialysed malt extract and YNB supplemented with lactose and glucose (unpublished results). These findings may explain the absence of antigenic EPS in Camembert and Roquefort cheese, in which lactate, peptides and amino-acids are the major carbon nutrients. The antibodies used in this study were reactive with the ß(1,5)-linked galactofuranosyl residues of the EPS (Notermans *et al.*, 1988). Therefore, the presence of ß-galactofuranosidases capable to hydrolyse galactofuranosyl residues of *Penicillium* EPS may be another possibility for the absence of antigenic extracellular polysaccharides in these cheeses. It is known that *Penicillium* species are capable to produce ß-galactofuranosidase (Cousin *et al.*, 1989) and high levels of this enzyme activity have been determined in Roquefort and Camembert cheeses (unpublished results). Consequently, the sandwich ELISA and the latex-agglutination assay for the detection of *Aspergillus* and *Penicillium* EPS may not be suitable for the detection of these genera in mould cheeses.

Glucose concentrations influenced mycelium dry weight and production of antigenic EPS moderately. Growth and EPS production at higher glucose concentrations may be limited by pH which fairly rapidly drops to a value of about 2 (Fig. 1). Growth of *P.aurantiogriseum* on nitrate was comparable with growth on ammonium sulphate but with *P.digitatum* nearly no growth was observed with nitrate as nitrogen source. These findings indicate differences in nitrogen metabolism by *P.aurantiogriseum* and *P.digitatum*.

In most EPS fractions mannose, galactose and glucose were the dominant monosaccharides present, as observed earlier by Notermans *et al.* (1987) and Kamphuis *et al.* (1992). Remarkable, however, was the high molar percentage of arabinose (66%) in EPS produced by *P.digitatum* on lactate (result not shown). Addition of a small

quantity of glucose to the lactate medium resulted in the absence of arabinose in EPS. EPS of *P.aurantiogriseum* produced on lactate did not contain arabinose. These EPS preparation did not show antigenic properties against antibodies used in this study. With nitrate as nitrogen source *P.digitatum* produced EPS containing a fairly high proportion of rhamnose (14 mol %). Presence of this monosaccharide has never been reported in extracellular polysaccharides produced by *Aspergillus* and *Penicillium* species. Up till now rhamnose has only been found in EPS of *Botrytis* as one of the constituent sugar residues (Cousin *et al.*, 1990). These findings indicate that the monosaccharide composition of EPS depends amongst others on the mould species and substrate composition. As a consequence comparison of results concerning the monosaccharide composition of EPS and quantitative growth characteristics of fungi found by several investigators is rather difficult.

In this study antigenic EPS were produced by *P.aurantiogriseum* and *P.digitatum* under various conditions. Under all circumstances tested antigenic EPS were produced, except when the moulds were grown on lactate. It should be of interest to test whether the EPS produced on lactate shows immunogenic properties in animals. If so, the antibodies obtained can be used for detecting these mould species in for example cheese.

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CHAPTER 7

H.J. Kamphuis, G.A. De Ruiter, G.H. Veeneman, J.H. van Boom, F.M. Rombouts and S.H.W. Notermans (1992). Detection of Aspergillus *and* Penicillium *extracellular polysaccharides (EPS) by ELISA: Using antibodies raised against acid hydrolyzed EPS. Antonie van Leeuwenhoek, 61, 323-332.*

DETECTION OF *ASPERGILLUS* AND *PENICILLIUM* EXTRACELLULAR POLYSACCHARIDES (EPS) BY ELISA: USING ANTIBODIES RAISED AGAINST ACID-HYDROLYSED EPS

ABSTRACT

Species of the fungal genera *Aspergillus* and *Penicillium* produce antigenic extracellular polysaccharides (EPS) in which galactofuranose residues are immunodominant. The antigenic determinant of the EPS of *A.fumigatus*, *A.niger* and *P.digitatum* could be removed by acid hydrolysis. Due to the hydrolysis of the EPS the immunological reaction between IgG anti-native EPS and hydrolysed EPS disappeared. Antibodies raised in rabbits against the acid-hydrolysed EPS revealed new antigenic determinants that were exposed as a result of the acid hydrolysis. Immunological inhibition experiments showed that the antibodies were no longer directed to galactofuranose residues.

Enzyme-linked immunosorbent assay, carried out with antibodies raised against the acid-hydrolysed EPS showed that the antibodies raised against the acid-hydrolysed EPS were more species specific than the antibodies raised against the native EPS.

INTRODUCTION

Fungi produce immunogenic and antigenic polysaccharides (Suzuki and Takeda, 1975; Notermans and Soentoro, 1986). Notermans and Heuvelman (1985) and Lin *et al.* (1986) have developed an enzyme-linked immunosorbent assay (ELISA) for the detection of these EPS in mould contaminated food.

Many investigators have tried to elucidate the structure of the EPS produced by *Aspergillus* and *Penicillium* species (Bennett *et al.*, 1985; Gander *et al.*, 1974; Gomez-Miranda and Leal, 1981; Preston *et al.*, 1969). EPS produced by species of *Aspergillus* and *Penicillium* consisted mainly of galactose, mannose, and glucose. In general, the mannopyranosyl residues are linked to one another primarily through $\alpha(1,2)$- and

α(1,6) O-glycosidic linkages. β(1,5)-Linked polygalactofuranosyl chains of variable length are linked to the mannan main core. The location of glucose in the EPS has not been elucidated.

The EPS produced by moulds are immunologically almost genus-specific and cross-reactivity occurred only between closely related genera, for example *Aspergillus* and *Penicillium*. (Notermans and Soentoro, 1986). Bennett *et al.* (1985) and Notermans *et al.* (1987) determined the immunodominant structure of the EPS produced by species of *Aspergillus* and *Penicillium*. It was demonstrated by Notermans *et al.* (1988) that the β(1,5)-linked D-galactofuranosides are immunodominant in *Aspergillus* and *Penicillium* EPS. It was shown that the paratope present on the IgG molecule, which was reactive with *Aspergillus* and *Penicillium* EPS interacted with four galactofuranosyl units.

With these findings, Kamphuis *et al.* (1989) developed a highly reliable latex-agglutination assay for the detection of EPS produced by *Aspergillus* and *Penicillium*. The reliability of the test could be enhanced by including a synthetic tetramer of β(1,5)-linked D-galactofuranose as a specific blocker in the assay.

It is known that EPS produced by genera other than *Aspergillus* and *Penicillium* possess similar structural elements. For example, mannose residues (1,2) and/or (1,6)-linked are present in EPS of *Cladosporium herbarum* (Miyazaki and Naoi, 1974), *Cladosporium tricoides* (Miyazaki and Naoi, 1975[a]), *Alternaria solani* and *Fusarium solani* (Miyazaki and Naoi, 1975[b]), and *Mucor hiemalis* (Miyazaki *et al.*, 1979). Therefore it might be expected that these structures are more generally present in mould EPS and probably they may have common antigenic activity. Using the acid lability of galactofuranosyl residues (Preston *et al.*, 1969), the immunodominant galactofuranosyl residues were removed and antibodies were raised against these hydrolysed EPS. The antibodies obtained were tested for their reaction with native and hydrolysed EPS.

The aim of this work was to obtain antibodies directed to the mannose part of the EPS, which would react with the EPS of *Aspergillus* and *Penicillium* and in all probability also with EPS produced by species of other genera.

MATERIALS AND METHODS

Fungal isolates. Penicillium digitatum M 58, *Aspergillus fumigatus* M 3, *A.niger* CBS 553.65, *A.fischeri* M 85, *P.aurantiogriseum* M 91, *P.dierckxii* M 90, *P.citrinum* CBS 117.64, *P.funiculosum* M 50, *Botrytis tulipae* M 18, *Cladosporium cladosporioides* M 20, *Fusarium oxysporum* M 28 and *Mucor racemosus* M 45 were used.

The strains were grown for 10 days on slopes of Malt Extract Agar (Oxoid L39) at 24°C and stored at 4°C until use. Spores were harvested for inoculation by adding 5 ml of sterile physiological saline containing 0.05% Tween 20 to each tube.

Production of native EPS. Native EPS of *A.niger*, *A.fumigatus* and *P.digitatum* (EPS I) was produced in Yeast Nitrogen Base (YNB; Difco) glucose medium. For this, 900 ml portions of glucose solution (30 g/l in water) were sterilised in 3 l flasks. Next 100 ml 10-fold concentrated (6.7 g/100 ml water) filter sterilised YNB was added, and spores were inoculated. After 6 days incubation at 24°C with orbital shaking at 120 rpm, mycelia were removed by filtration (Whatman no. 4) and the culture filtrate was freeze-dried. The freeze-dried material was dissolved in 40 ml distilled water and dialysed against running tap water (24 hours) and distilled water (24 hours). After freeze-drying of the retentate the material was dissolved in 20 ml distilled water and water insoluble materials were removed by centrifugation (30 min 18,000 x g). EPS was collected after three precipitations with 80% ethanol.

EPS of *A.fischeri*, *P.aurantiogriseum*, *P.dierckxii*, *P.citrinum*, *P.funiculosum*, *B.tulipae*, *C.cladosporioides*, *F.oxysporum*, *M.racemosus* and EPS II of *P.digitatum* were produced in dialysed malt extract medium according Notermans *et al.* (1987). EPS was isolated and purified using ammonium-sulphate precipitation and size-exclusion chromatography.

Chemical analysis. The neutral sugar composition of the EPS was determined by converting them to their alditol acetates as decribed by Englyst and Cummings (1984) and analysed by GLC. Hydrolysis was performed by using 2 N TFA (1 hr, 121°C) and

inositol was used as internal standard (Albersheim *et al.*, 1967). The protein content was determined as described by Lowry (1951) with Bovine Serum Albumin (BSA) as standard.

Epitope degradation by acid hydrolysis. Acid hydrolysis was carried out by treating EPS (5 mg/ml) with 0.01 N HCl at 100°C for 100 min. After neutralisation the hydrolysed EPS was dialysed against distilled water (24 hours). Then, the retentate and dialysate were freeze-dried. The freeze-dried retentate was used for immunisation experiments.

Antibody production. Antibodies against native EPS and hydrolysed EPS were raised in rabbits. They were injected subcutaneously on day 0 and 30 with 0.5 mg EPS. For this, EPS was mixed on day 0 and day 30 with Freund's complete adjuvant and Freund's incomplete adjuvant, respectively. Rabbits were bled for collecting sera on day 40. The IgG fraction was isolated by the method of Steinbuch and Audran (1969) and freeze-dried.

Sandwich ELISA for detection of EPS. The sandwich ELISA was carried out as described by Notermans *et al.* (1987). To wells of polyvinyl trays (Cooke, Dynatech) was applied 0.1 ml 10 μg/ml of rabbit 658 IgG anti-native EPS of *P.digitatum* diluted in 0.07 M phosphate buffer pH 7.2, containing 0.15 M NaCl (PBS). After overnight incubation at room temperature with shaking, the trays were rinsed in PBS with 0.05% (v/v) Tween 20 (PBST). Then 0.1 ml EPS, diluted in PBST was added and incubated for 60 min at room temperature with shaking. After rinsing, 0.1 ml of an optimal dilution of rabbit IgG anti-native EPS *P.digitatum* conjugated to horseradish peroxidase in PBST containing 1% (w/w) Bovine Serum Albumin (BSA) was added. After 60 min incubation, the trays were rinsed and the enzyme activity was determined spectrophotometrically at 450 nm.

For this 1 ml of 42 mM 3,5,3',5'-tetramethylbenzidine (Merck) in DMSO (Sigma) was added under vigorous shaking to 100 ml 0.1 M sodium acetate buffer pH 5.6. Just before adding 0.1 ml substrate solution to the wells, 7 μl of 30% H_2O_2 was added to

the solution. After incubation for 20 min at room temperature 0.05 ml of 2 M H_2SO_4 was added to the wells to stop the enzyme reaction.

Determination of the antibody titre in rabbits. To determine the titre of antibodies raised in rabbits against the acid-hydrolysed EPS (immunogen), the following ELISA was carried out. Polyvinyl trays (Cooke, Dynatech) were coated with 0.1 ml immunogen (2.5 μg/ml) diluted in PBS. After 1 night incubation at room temperature with orbital shaking, the trays were rinsed and 0.1 ml portions of rabbit serum diluted in PBST were added. After 60 min of incubation and another rinse, 0.1 ml of an optimal dilution of sheep anti-rabbit immunoglobulin conjugated to horseradish peroxidase in PBST containing 1% (w/w) BSA was added. After 60 min incubation the trays were rinsed and the enzyme activity was determined as described before.

Inhibition experiments (competitive ELISA). Inhibition experiments were carried out as follows: 0.1 ml of 2.5 μg/ml of an immunogen (acid-hydrolysed EPS of *A.niger*, *A.fumigatus* and *P.digitatum*, respectively) in PBS was added to each well of the trays. After overnight incubation with shaking at room temperature the trays were rinsed as described above. A concentration range of the inhibitor was made in PBST. Then 25 μl of the inhibitor together with 25 μl of the optimal dilution in PBST of the immunoglobulin (tested by checkerboard titration), raised against the immunogen in question, was added. After incubation for 60 min at room temperature the trays were rinsed and 0.1 ml of sheep anti-rabbit immunoglobulin conjugated to horseradish peroxidase in PBST containing 1% (w/w) BSA was added. The optimal concentration of the conjugate was tested by checkerboard titration. After incubation for 60 min, plates were rinsed and 0.1 ml of the substrate was added as described above. The enzyme activity was measured spectrophotometrically at 450 nm. The percent inhibition was calculated as 100-100 (absorbance with inhibitor/absorbance without inhibitor).

Chemical synthesis of several β(1,5)-linked galactofuranosyl saccharides. Methyl-β-D-galactofuranoside was obtained by chromatography of a mixture of methyl-D-galacto-

sides over Whatman cc 31 microgranular cellulose (Augestad and Berner, 1954). The β(1,5)-linked galactofuranosyl tetramer was synthesised by coupling of properly substituted monomer residues via the Helferich procedure (Veeneman *et al.*, 1987[a]). Additionally, dimers to heptamers of β(1,5)-linked galactofuranosides were synthesised by a solid phase approach (Veeneman *et al.*, 1987[b]). The homogeneity and identity of the synthesised oligosaccharides mentioned above were ascertained by NMR [^{1}H]- and [^{13}C]-spectroscopy and FAB mass spectrometry.

Conjugation procedure of IgG to horseradish peroxidase. The conjugation of IgG anti-acid hydrolysed EPS to horseradish peroxidase was carried out as described by Nakane and Kawaoi (1974).

RESULTS

Hydrolysis experiments. Initially, tests were carried out with EPS of *P.digitatum* partially hydrolysed, at 100°C for 15 min with 0.01 N HCl. It was shown that this EPS of *P.digitatum* could not be detected in a sandwich ELISA using IgG anti-native EPS. The galactose content of this partially hydrolysed EPS was decreased only slightly (from 42 to 37 mol %). Rabbits were immunised with this partially hydrolysed EPS. It was demonstrated that the immunological reaction between the antibody raised and the partially hydrolysed EPS was completely inhibited by a dimer of β(1,5)-linked galactofuranosyl residues (results not shown). Therefore the hydrolysis of the EPS of *A.fumigatus*, *A.niger*, and EPS I and II of *P.digitatum* were carried out at 100°C for 100 min. The monosaccharide compositions and the sugar and protein contents before and after this prolonged acid-hydrolysis are presented in Table 1. The most dominant monosaccharides present were mannose, galactose and glucose. In some EPS small quantities of xylose, arabinose and fucose were detected.

The molar % galactose of the native EPS of *A.fumigatus*, *A.niger*, and EPS I and II of *P.digitatum* were 25, 45, 55 and 8%, respectively. The acid hydrolysis resulted in a decrease of the molar percentage of galactose to 6, 9, 23 and 3%, respectively. The

Table 1. Monosaccharide, sugar and protein contents of EPS of *A.fumigatus*, *A.niger* and *P.digitatum* before and after acid-hydrolysis at 100°C for 100 min in 0.01 N HCl.

EPS	Acid hydrolysis	Mol % monosaccharide			Sugar	Protein
		Man[a]	Gal	Glc	(w/w %)	
A.fumigatus	no	12	25	62	73	13
A.fumigatus	yes	18	6	76	86	11
A.niger	no	50	45	4	46	18
A.niger	yes	83	9	6	71	38
I *P.digitatum*	no	22	55	21	50	22
I *P.digitatum*	yes	42	23	34	53	49
II *P.digitatum*[b]	no	10	8	82	71	6
II *P.digitatum*	yes	15	3	82	88	9

[a] Man: mannose; Gal: galactose; Glc: glucose.
[b] Production and purification of this EPS as described by Notermans *et al.*, 1987 (see materials and methods).

sugars in the dialysates of the acid-hydrolysed EPS were mainly galactose (up to 90 mol%).

As a result of the decrease of galactose after the hydrolysis, the molar % of mannose and glucose increased. The sugar content of the EPS of *A.fumigatus*, *A.niger*, and EPS I and II of *P.digitatum* increased from 73 to 86%, from 46 to 71%, from 50 to 53%, and from 71 to 88%, respectively. In general, due to the hydrolysis the protein content of the EPS increased. The protein content of EPS *A.niger*, EPS I and II of *P.digitatum* increased from 18 to 38%, from to 22 to 49% and from 6 to 9%, respectively. However, the protein content of EPS *A.fumigatus* decreased slightly from 13 to 11%. Molecular weight distribution patterns of the EPS before and after the hydrolysis showed, that the molecular weight of the main fraction decreased from about 40 to 10 kD (results not shown).

Antigenicity of the hydrolysed EPS. Acid-hydrolysed EPS did not react with IgG anti-native EPS. However, in case of EPS I of *P.digitatum* after dialysing and freeze-

drying, 200,000 ng/ml of the acid-hydrolysed EPS could be detected in the sandwich ELISA. Before hydrolysis 50 ng/ml of the EPS gave a positive result (Table 2). Before hydrolysis the minimal quantity EPS of *A.fumigatus* and *A.niger* detectable in the sandwich ELISA was < 50 ng/ml and after the acid-hydrolysis 200,000 ng/ml gave a negative result. The minimal detectable quantity of EPS I of *P.digitatum* before and after the acid-hydrolysis was 50 and 200,000 ng/ml, respectively. For EPS II of *P.digitatum* these values were 300 and >200,000 ng/ml, respectively. Whether the acid-hydrolysed polysaccharides possessed inhibitory activity in an ELISA was tested using *P.digitatum* EPS and IgG anti-native EPS. This inhibitory experiment is specific for the detection of $\beta(1,5)$-linked galactofuranosyl residues. It was shown that only EPS I of *P.digitatum* had inhibitory activity after the hydrolysis (Table 2). Before hydrolysis all EPS preparations possessed inhibitory activity.

Table 2. Minimal detectable quantity and inhibitory activity of extracellular polysaccharides (EPS) of *A.fumigatus*, *A.niger* and EPS I and II of *P.digitatum* before and after acid-hydrolysis at 100°C for 100 min in 0.01 N HCl.

Origin of EPS	Acid hydrolysis	Minimal detectable quantity (ng/ml)[a]	50% Inhibitory concentration (ng/ml)[b]
A.fumigatus	no	<50	300
A.fumigatus	yes	>200,000	>200,000
A.niger	no	<50	250
A.niger	yes	>200,000	>200,000
I *P.digitatum*	no	50	500
I *P.digitatum*	yes	200,000	25,000
II *P.digitatum*	no	30	2,000
II *P.digitatum*	yes	>200,000	>200,000

[a] Determined by the sandwich ELISA with IgG anti-native EPS of *P.digitatum*.
[b] Determined by the competitive ELISA with IgG anti-native EPS of *P.digitatum*.

Immunisation experiments. Rabbits were immunised with the acid-hydrolysed EPS of *A.fumigatus*, *A.niger* and EPS I and II of *P.digitatum*. High specific serum titres were

Table 3. Serum titres of rabbits that were immunised with acid-hydrolysed[a] EPS of *A.fumigatus*, *A.niger* and EPS I and II of *P.digitatum*.

Rabbit no.	Acid-hydrolysed EPS	Serum titre[b]	
		day 0	day 38
666	*A.fumigatus*	1,250	6,250
667	*A.fumigatus*	250	1,250
668	*A.niger*	250	>31,250
669	*A.niger*	250	>31,250
670	I *P.digitatum*	1,250	>156,250
671	I *P.digitatum*	1,250	>156,250
672	II *P.digitatum*	1,250	>31,250
673	II *P.digitatum*	1,250	6,250

[a] 100°C for 100 min in 0.01 N HCl.
[b] reciprocal of highest dilution of serum analysed in sandwich ELISA giving a positive result.

obtained when rabbits were immunised with the hydrolysed EPS of *A.niger* and EPS I and II of *P.digitatum* (Table 3). Acid hydrolysed *A.fumigatus* EPS did not result in a high specific serum titre after immunisation.

Antibody characterisation. After isolation and purification of the IgG antibodies inhibitory experiments were carried out to characterise the IgG's obtained. Inhibitory experiments were done by coating the acid-hydrolysed EPS of *A.fumigatus*, *A.niger*, and EPS I and II of *P.digitatum*. The reaction between the acid-hydrolysed EPS and the antibodies raised against the same EPS could be inhibited by the native EPS as well as by the hydrolysed EPS (Table 4). However, the acid-hydrolysed EPS inhibited the reaction between the acid-hydrolysed EPS and the subsequent IgG better than the native EPS. Furthermore, the antibodies raised against the acid-hydrolysed EPS of *A.niger* (IgG 668 and 669), EPS I of *P.digitatum* (IgG 670 and 671) and EPS II of *P.digitatum* (IgG 672) were characterised by carrying out inhibitory experiments using specific inhibitors (Table 5). None of the antibodies raised against the acid-hydrolysed

EPS could be inhibited by a $\beta(1,5)$-linked galactofuranose tetramer when reacting with the acid-hydrolysed EPS to which they were raised. In case of IgG anti-acid-hydrolysed EPS of *A.niger* (IgG 668 and 669) the reaction could only be inhibited by methyl-α-D-mannopyranoside. The reaction of IgG 670 and 671 and the acid-hydrolysed EPS I of *P.digitatum* could be inhibited by methyl-α-D-glucopyranoside.

The reaction between IgG 672 and the hydrolysed EPS II of *P.digitatum* could be inhibited by different methyl-monosaccharides. Of the methyl-monosaccharides methyl-α-D-glucopyranoside showed the highest inhibitory activity (50% inhibitory concentration of < 5mg/ml).

Table 4. Inhibition of antibody binding to acid-hydrolysed[a] extracellular polysaccharides (EPS) by the native EPS from different moulds.

Origin of EPS used as inhibitor	Acid hydrolysis	50% Inhibitory concentration (μg/ml)[b]				
		IgG 668	IgG 669	IgG 670	IgG 671	IgG 672
A.niger	no	7.8	2.9	-[c]	-	-
A.niger	yes	2.0	0.8	-	-	-
P.digitatum I	no	-	-	2.7	2.4	-
P.digitatum I	yes	-	-	1.1	1.0	-
P.digitatum II	no	-	-	-	-	1.9
P.digitatum II	yes	-	-	-	-	<0.8

[a] 100°C for 100 min in 0.01 N HCl.
[b] Determined by the competitive ELISA with the IgG's anti-acid-hydrolysed EPS from *A.niger* (IgG 668 and 669), acid-hydrolysed EPS I of *P.digitatum* (IgG 670 and 671) and acid-hydrolysed EPS II of *P.digitatum* (IgG 672), respectively.
[c] not tested.

Antigenic specificity of the antibodies raised against the acid-hydrolysed polysaccharides. The sandwich ELISA was done with IgG anti-native *P.digitatum* EPS (IgG 658), IgG anti-acid-hydrolysed *A.niger* EPS (IgG 668), IgG anti-acid-hydrolysed EPS I of *P.digitatum* (IgG 670) and IgG anti-acid-hydrolysed EPS II of *P.digitatum* (IgG 672). For this the IgG's were conjugated to horseradish peroxidase. EPS of different fungal species, before and after an acid-hydrolysis at 100°C for 100 min in 0.01 N HCl, were

tested (Table 6).

IgG 658 reacts only with the native EPS produced by different *Aspergillus* and *Penicillium* species. As observed earlier, no reaction occurred with the native *P.funiculosum* EPS (Notermans and Soentoro, 1986). No positive results, with the exception of *P.aurantiogriseum* EPS, were observed with the hydrolysed *Aspergillus* and *Penicillium* EPS and with native and hydrolysed EPS produced by species of genera other than *Aspergillus* and *Penicillium*.

Table 5. Inhibition studies with antibodies raised against native EPS of *P.digitatum* (IgG 658) and the acid-hydrolysed EPS of *A.niger* (IgG 668 and 669) and acid-hydrolysed EPS I and II of *P.digitatum* (IgG 670 to 672).

Inhibitor[a]	50% Inhibitory concentration (mg/ml)					
	IgG 658	IgG 668	IgG 669	IgG 670	IgG 671	IgG 672
Me-α-galactopyr.	>150	>150	>150	>150	>150	90
Me-ß-galactopyr.	>150	>150	>150	>150	>150	>150
Me-α-glucopyr.	>150	>150	>150	35	30	<5
Me-ß-glucopyr.	>150	>150	>150	>150	>150	>150
Me-α-mannopyr.	>150	<5	<5	>150	>150	32
ß(1,5)-linked[b] tetramer	0.025	>0.1	NT	>0.1	NT	>0.1

[a] Me: methyl; pyr: pyranoside.
[b] galactofuranoside.

When the sandwich ELISA was carried out with the IgG's raised against the acid-hydrolysed EPS of *A.niger* (IgG 668), EPS I of *P.digitatum* (IgG 670) and EPS II of *P.digitatum* (IgG 672) no reactions were observed with native or hydrolysed EPS produced by species of other genera than *Aspergillus* and *Penicillium* (Table 6).

The reactions observed with the native and hydrolysed EPS produced by species of *Aspergillus* and *Penicillium* were more species specific in comparison with the reactions obtained with the sandwich ELISA carried out with IgG anti-native *P.digitatum* EPS (IgG 658). IgG 668 did not react with both the native and the hydrolysed EPS of *P.citrinum*, *P.funiculosum* and EPS II of *P.digitatum*. *A.fumigatus*

Table 6. Minimal detectable quantity of extracellular polysaccharides (EPS) as determined by the sandwich ELISA with IgG anti-native EPS of *P.digitatum* (IgG 658) and with IgG anti-acid-hydrolysed EPS of *A.niger* (IgG 668), IgG anti-acid-hydrolysed EPS I of *P.digitatum* (IgG 670) and IgG anti-acid-hydrolysed EPS II of *P.digitatum* (IgG 672).

Origin of EPS	Acid hydrolysis	Detectable quantity of EPS[a]			
		IgG 658	IgG 668	IgG 670	IgG 672
A.fumigatus	no	+++	-	+++	-
	yes	-	+	-	-
A.niger	no	+++	+	++	-
	yes	-	++	++	-
I *P.digitatum*	no	+++	+	+++	+
	yes	-	++	+++	++
II *P.digitatum*	no	++	-	++	+
	yes	-	-	++	+
P.dierckxii	no	++	-	-	-
	yes	-	+	-	-
A.fischeri	no	+++	+	-	-
	yes	-	+	-	-
P.aurantiogriseum	no	+++	+	+	+
	yes	+	+	-	+
P.citrinum	no	+++	-	-	-
	yes	-	-	-	-
P.funiculosum	no	-	-	-	-
	yes	-	-	-	-
F.oxysporum	no	-	-	-	-
	yes	-	-	-	-
M.racemosus	no	-	-	-	-
	yes	-	-	-	-
C.cladosporioides	no	-	-	-	-
	yes	-	-	-	-
B.tulipae	no	-	-	-	-
	yes	-	-	-	-

[a] Detectable quantity; - ≥ 10 μg/ml; + ≥ 1 - < 10 μg/ml; ++ ≥ 0.1 - < 1 μg/ml; +++; < 0.1 μg/ml.

and *P.dierckxii* EPS could only be detected after the acid-hydrolysis. EPS I of *P.digitatum* and EPS of *A.niger*, *A.fischeri* and *P.aurantiogriseum* gave a positive result in the sandwich ELISA carried out with IgG 668 before and after the acid-hydrolysis. When IgG 670 (anti-acid-hydrolysed EPS I of *P.digitatum*) was used in the sandwich ELISA, no reactions could be observed with the native and hydrolysed EPS of *P.dierckxii*, *A.fischeri*, *P.citrinum* and *P.funiculosum*. Native EPS of *A.fumigatus* and *P.aurantiogriseum* could be detected in this ELISA. However, the acid-hydrolysed EPS of these two species gave a negative result. Also the native and acid-hydrolysed EPS of *A.niger* and EPS I and II of *P.digitatum* gave positive results. Using IgG 672 (anti-acid-hydrolysed EPS II of *P.digitatum*) in the sandwich ELISA, no reactions were observed with both the native and acid-hydrolysed EPS from *A.fumigatus*, *A.niger*, *P.dierckxii*, *A.fischeri*, *P.citrinum*, and *P.funiculosum*. EPS I and II of *P.digitatum* and *P.aurantiogriseum* EPS could be detected both before and after the acid-hydrolysis.

DISCUSSION

In an attempt to develop a sandwich ELISA for the detection of EPS produced by several species of different genera, antibodies were raised to common parts (for example, mannose) of these immunogens. Antibodies were raised in rabbits against acid-hydrolysed EPS of *A.niger*, *A.fumigatus* and *P.digitatum*. The immunodominant galactofuranosyl residues were hydrolysed after 15 minutes at 100°C in 0.01 N HCl.

The analysis of the sugar compositions of EPS after 15 min hydrolysis showed that the galactose content decreased approximately 5 mol%, indicating that only a small amount of the galactose content is responsible for the antigenicity in the sandwich ELISA. Although the antigenicity in the sandwich ELISA of the acid-hydrolysed EPS disappeared, the inhibitory activity in the competitive ELISA using IgG anti-native *P.digitatum* EPS did not. This means that there must be still β(1-5)-linked galactofuranosyl residues in the acid-hydrolysed EPS present. Therefore it was decided to hydrolyse the EPS of *A.niger*, *A.fumigatus*, and *P.digitatum* for 100 minutes. The molar % galactose decreased further upon this treatment. However, differences in the

decrease between the four hydrolysed EPS were observed. According to Gander *et al.* (1974) conditions of 0.01 N HCl, 100°C, 100 min may account for up to 90-95% hydrolysis of the galactofuranosyl residues. Therefore galactose residues still present in the hydrolysed EPS will be in the pyranose form, mainly. The hydrolysis reaction was checked by analysis of the dialysis products of hydrolysed EPS. These products had a carbohydrate content of approximately 80% of which 80 to 90% was galactose.

Two different EPS of *P.digitatum* were produced by the same strain, however, the culture medium was different. EPS I was produced in YNB/glucose medium and EPS II was produced in dialysed malt extract medium of Notermans *et al.* (1987). Ammonium-sulphate precipitation of EPS II of *P.digitatum* was followed by Sepharose CL-6B chromatography. As indicated by sugar analysis (Table 1) this EPS II contains substantially more glucose residues. In separate studies we have performed that *P.digitatum* if maltose is used as carbon source produces an extracellular glucan in addition to the galactomannan. This glucan is apparently not separated from the galactomannan by the purification procedure as applied by Notermans *et al.* (1987). However, the antigenicity in the sandwich ELISA, using IgG anti-native *P.digitatum* EPS, was nearly the same for both the EPS I and II, despite the difference in composition. The antigenicity of the EPS of *A.fumigatus*, *A.niger* and EPS II of *P.digitatum* in the sandwich ELISA, using IgG-anti native *P.digitatum* EPS and the competitive ELISA, disappeared completely after the acid-hydrolysis. However, EPS I of *P.digitatum* retained some antigenicity in both ELISA's after the acid-hydrolysis, indicating that the $\beta(1,5)$-linked galactofuranosyl residues in EPS I of *P.digitatum* are more resistant to the acid-hydrolysis than in other EPS. It was also observed that hydrolysis of *P.digitatum* EPS for 15 min in 0.01 N HCl at 100°C results in an EPS with different antigenicity in the sandwich ELISA, using IgG-anti native *P.digitatum* EPS and the competitive ELISA, respectively. This may be due to a different three dimensional structure of the acid-hydrolysed EPS in the sandwich and competitive ELISA, respectively.

All the acid-hydrolysed EPS gave strong increases in the specific serum titre with the exception of *A.fumigatus* EPS and (one) EPS II of *P.digitatum*. The antibodies of

these three rabbits were not isolated because earlier results showed that such an increase (factor 5) of the serum titre is normal. Generally, the rabbit feed is contaminated with *Aspergillus* and *Penicillium* species, leading to a low specific serum titre. This means that when these rabbits are immunised, this acts as a kind of booster resulting in higher specific serum titres. It has been shown that when feeding rabbits fresh vegetables instead of the pellet feed, the level of *Aspergillus* and *Penicillium* circulating antigens in the serum decreased, implicating that before the immunisation with the hydrolysed EPS *Aspergillus* and *Penicillium* antigens are present (personal observations). The difference in immunogenicity of the acid-hydrolysed EPS may be explained by a decrease of the main molecular weight fraction (from about 40 to 10 kD), or a possibly lower epitope density on the acid-hydrolysed EPS (Kabat and Bezer, 1958; Richter, 1981) or to different protein contents in comparison with the native EPS. Presently, the immune response of EPS is under investigation.

It can be concluded, that the reaction between the hydrolysed EPS and the subsequent IgG can be inhibited by both the native- and acid-hydrolysed EPS. After hydrolysis of the EPS, the 50% inhibitory concentrations of these EPS were lower. In comparison with the native EPS, the epitopes on the hydrolysed EPS are better exposed, probably due to less steric hindrance. In general, the reaction of the acid-hydrolysed EPS with subsequent IgG could only be inhibited by methyl-mannose or methyl-glucose residues, especially the α-isomers. No inhibitions could be achieved with a tetramer of $\beta(1,5)$-linked galactofuranosyl residues. Thus, the immunodominant sugar residues in the acid-hydrolysed EPS are probably mannose and/or glucose. This result indicates, that when the immunopotent galactofuranose residues are hydrolysed other (immunosilent) parts of the EPS become immunodominant for example the mannose and glucose containing parts of the acid-hydrolysed EPS. However, when galactofuranose residues are still present in the hydrolysed EPS, these residues are so immunodominant, that antibodies are raised against the remaining galactofuranose residues. This was observed when EPS of *P.digitatum* was hydrolysed at 100°C for 15 min in 0.01 N HCl. The reaction of this partially hydrolysed EPS with the subsequent IgG could be inhibited completely by a dimer of $\beta(1,5)$-linked galactofuranosyl

residues. Probably, this antibody combining site size is the smallest which is possible toward sugar residues (Kabat, 1966).

As shown in Table 6, the genus specificity did not disappear when the sandwich ELISA was carried out with the IgG's 668, 670 and 672. However, the reaction pattern with the different EPS within the *Aspergillus* and *Penicillium* genera changed. This alteration in the reaction pattern within *Aspergillus* and *Penicillium* could be the starting point for the development of a sandwich ELISA for the detection of only a few important mycotoxin producing species belonging to the genus *Aspergillus* for example *A.flavus* or *A.parasiticus*.

The change in the reaction pattern with the EPS produced by species belonging to the genera *Aspergillus* and *Penicillium* reflects differences in structure of galactomannans from different species of *Aspergillus* and *Penicillium*. Antibodies against the acid-hydrolysed EPS were more species specific and it was not possible to develop a universal sandwich ELISA with the IgG's anti-acid hydrolysed EPS for the detection of EPS produced by species of different genera.

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CHAPTER 8

H.J. Kamphuis, S. Notermans, G.H. Veeneman, J.H. van Boom and F.M. Rombouts (1989) A rapid and reliable method for the detection of molds in foods: using the latex-agglutination assay. J. Food Prot., 52(4), 244-247.

A RAPID AND RELIABLE METHOD FOR THE DETECTION OF MOULDS IN FOODS: USING THE LATEX-AGGLUTINATION ASSAY

ABSTRACT

An agglutination test by using latex beads (0.8 μm diameter) coated with IgG antibodies raised against extracellular polysaccharides of *Penicillium digitatum* has been developed. As low as 5 to 10 ng/ml of the purified extracellular polysaccharide of the same species can be detected by this preparation. Analysis of culture filtrates from 25 different moulds showed that the positive reactivity was obtained only with the species of the genera *Aspergillus* and *Penicillium*. The application of this test was confirmed in testing samples of spices and nuts. Further, the reliability could be enhanced by including a specific blocker in the assay, the synthetic epitopes consisting of four ß(1,5)-linked D-galactofuranosyl residues.

INTRODUCTION

Contamination of foods with moulds lead to spoilage, economic losses and represent a health hazard due to the production of mycotoxins (Elling *et al.*, 1975; Northolt, 1979; Umeda, 1977). Most methods for the detection of moulds are limited due to a number of drawbacks as summarised by Jarvis *et al.* (1983). Recently, Notermans and Heuvelman (1985) and Lin *et al.* (1986) reported a immunological method using the ELISA for the detection of heat stable extracellular polysaccharides (EPS) produced by various moulds. This technique is useful for testing both heated or unheated food samples. EPS consists mainly of mannose, galactose and glucose. It was demonstrated that the ß(1,5)-linked D-galactofuranose part of the EPS produced by the species of *Aspergillus* and *Penicillium* is immunodominant (Notermans *et al.*, 1988). It was estimated that an IgG molecule reacting with EPS combines with four ß(1,5)-linked galactofuranosyl residues. Such synthetic galactofuranosyl hapten when combined with the ELISA for EPS, specific blocking due to the competition by

haptens can be observed. The ELISA test takes 5 to 10 hours, while similar results can be achieved in 10 to 20 min by the latex-agglutination method reported here.

A simple, rapid, and sensitive technique (latex-agglutination) is described, including the use of a synthetic epitope for specific blocking, enhancing the reliability of this method.

MATERIALS AND METHODS

Fungal isolates. Various fungal strains as listed in Table 2 were cultured on the slopes of Malt Extract Agar (OXOID L 39) and stored at 4°C. Spores were harvested from these cultures with 5 ml of sterile physiological saline containing 0.05% Tween 20, then used as inoculum in various experiments.

Antigen production. The polysaccharide antigen from moulds was prepared according to Notermans *et al.* (1987). Briefly, mould spores were inoculated in dialysed malt extract broth, and incubated at 24°C for 6 days with orbital shaking at 120 rpm. Mycelia were removed by filtration and the culture filtrate was freeze-dried. After vigorous shaking of the freeze-dried material in 80% ammonium sulphate (3 hrs), insoluble components were removed by centrifugation followed by filtration. The filtrate was dialysed against distilled water and applied to a distilled water equilibrated Sepharose CL-6B column (2.5 x 100 cm).

Antibody production. Rabbits were immunised with 0.25 mg of purified EPS along with Freund complete adjuvant on days 1 and 4. On day 30, 0.5 mg EPS along with Freund incomplete adjuvant was used. The rabbits were bled on day 40 for collecting sera. The IgG fraction was isolated by the method of Steinbuch and Audran (1969) and lyophilized until further use.

Latex-agglutination

A) *Coating*: Beads were coated with IgG according to the method of Horiguchi *et al.* (1984). Briefly, after two washes in 0.07 M phosphate buffered saline (PBS, pH 7.2), the concentration of latex beads (0.8 μm, Sigma) was adjusted such that a 1:100 dilution would give 0.3 to 0.33 absorbance at 650 nm. This was equivalent to a 35 to 40 fold dilution from the original suspension. Optimal concentration of IgG 658 anti-*P.digitatum* EPS in 1 ml was mixed with equal volume of the bead suspension, incubated at 37°C in an orbital shaker at 120 rpm for 1.5 hours. After two washes with PBS containing 0.2% (w/w) Bovine Serum Albumin (BSA), the beads were resuspended in 2 ml PBS containing 0.2% BSA and 0.1% sodium azide, then stored at 4°C.

B) *Agglutination test*: IgG coated beads and the sample in the volumes of 25 μl were mixed in the wells of a Boerner slide (MERCO), and agglutination was determined after 10 to 20 minutes of shaking. Dilutions of *P.digitatum* EPS were used as a standard.

C) *Specific Blocking*: Prior to the addition of the test sample to IgG coated beads, dilutions of a tetramer of ß(1,5)-linked D-galactofuranosides was added. The tetramer was prepared as described by Veeneman *et al.* (1987).

Analysis of culture filtrates from different moulds. Spores of different mould species were added to 30 ml dialysed malt extract broth. After incubation at 24°C for 6 days with orbital shaking at 100 rpm, the cultures were filtered. The filtrate was diluted in PBS for use in the latex-agglutination test.

Analysis of nuts and spices. Nuts and spices were collected from various commercial sources. The samples were homogenised for 1 min in 10 fold amount of 0.1% peptone water in a Waring blender. The supernatant was diluted in PBS for use in the latex-

agglutination test.

Mould count. Colony forming units from sample homogenates and their 1:10 dilutions were determined on agar plates (9 cm plates) containing 20 ml malt extract agar. (20 g malt extract, 15 g agar and 1 l distilled water). Filter sterilised chlortetracycline was added to the sterilised medium just before pouring at a final concentration of 10 μg/ml. 0.1 ml of the test samples were surface plated in duplicate. Plates were incubated at 24°C for 4 to 10 days. Colonies grown were identified to genus level according the standard taxonomic schemes.

RESULTS

Latex-agglutination. For coating of beads 50 μg/ml IgG was found to be optimal. The diluent for beads contained 0.2% BSA, without auto-agglutination among the beads would occur. The coating procedure described in this report is reproducible. As shown in Table 1, as low as 5 to 10 ng/ml of purified EPS from *P.digitatum* was readily detected.

Table 1. Sensitivity of the latex agglutination: Blocking of agglutination by the synthetic tetramer of ß(1,5)-linked D-galactofuranosides in a dose response manner.

Tetramer (μg/ml)	Purified extracellular polysaccharides (EPS) of *Penicillium digitatum* (ng/ml)[b]						
	250,000	5,000	500	125	25	5	0
0	+[c]	+	+	+	+	+	-
20	+	+	+	+	-	-	-
100	+	+	±	-	-	-	-
500	NT[d]	NT	-	-	-	-	-
1000	-	-	-	-	-	-	-

[a] concentration in latex bead suspension.
[b] concentration in reaction mixture.
[c] + agglutination, - no agglutination.
[d] NT, not tested.

Table 2. Latex-agglutination of culture filtrates of different mould species. Latex beads were coated with antibodies raised against the extracellular polysaccharide produced by *Penicillium digitatum*. Specific blocking was carried out using a tetramer of ß(1,5)-linked D-galactofuranosides at a final concentration of 100 µg/ml.

Mould species	Latex-agglutination reaction	Specific blocking[a]
Alternaria alternata M 13	-[b]	NT[c]
Aspergillus candidus M 1	+++	yes
A.fischeri M 85	+++	yes
A.flavus M 2	+++	yes
A.fumigatus M 3	+++	yes
A.ostianus M 8	+++	yes
A.parasiticus M 9	+++	yes
A.repens M 5	+++	yes
A.versicolor M 11	+++	yes
Botrytis cinerea M 17	-	NT
Cladosporium cladosporioides M 20	-	NT
Fusarium dimerum M 25	-	NT
Geotrichum candidum M 32	-	NT
Mucor racemosus M 45	-	NT
Penicillium diversum var. *aureum* M 48	+++	yes
P.expansum M 51	+++	yes
P.implicatum M 52	+++	yes
P.islandicum M 53	-	NT
P.palitans M 54	+++	yes
P.roqueforti M 56	+++	yes
P.rubrum M 57	-	NT
P.velutinum M 92/CBS 318.59	+++	yes
P.verrucosum var. *cyclopium* M 60	+++	yes
P.viridicatum M 62	+++	yes

[a] Specific blocking was tested using 1/20 diluted culture filtrate.
[b] -, No reaction observed in 1/20 dilution, + reaction observed in 1/20 dilution; reaction observed in 1/2,000.
[c] NT, not tested.

Blocking capacity of ß(1,5)-linked D-galactofuranosides. The blocking capacity of tetramer ß(1,5)-linked D-galactofuranosides was determined by combining it with the

IgG coated latex beads prior to the addition of the test sample. Data in Table 1 show that 100 μg/ml of tetramer was sufficient for completely inhibiting the agglutination when as much as 125 ng/ml of purified EPS was present. For other inhibition tests, a concentration of 100 μg/ml of the tetramer was used.

Analysis of culture filtrates of different mould species. The culture filtrates of 24 different species from 8 different genera were tested in the latex-agglutination test with or without specific blocking by tetramer. As presented in Table 2, agglutination was observed only with the species from the genera *Aspergillus* and *Penicillium*. The blocking of agglutination due to the tetramer of ß(1,5)-linked D-galactofuranosides was observed in all those cases. Within the genus *Penicillium*, *P.rubrum* and *P.islandicum* were the only exceptions which failed to agglutinate the IgG coated latex beads.

Analysis of nuts and spices. A number of nut and spice samples as shown in Table 3, were analysed with the latex-agglutination test for the presence of EPS produced by the species of *Aspergillus* and *Penicillium*. Sample 1 (peanuts), and sample 8 (pistachio) did not show any agglutination (Table 3), while all other nut samples showed positive agglutination. Samples 2, 3 and 9 at dilution 1/20 and samples 4, 6 and 10 at dilution 1/2,000 of the extracts caused agglutination of the IgG coated latex beads. When the synthetic tetramer was included in the reaction mixtures, complete inhibition of agglutination was observed in all but one case. The failure of inhibition of agglutination with sample 3 (Table 3) suggests that the reaction was probably not due to EPS produced by *Aspergillus* and *Penicillium*. All extracts of spices caused agglutination at a dilution of 1/2,000, which was also inhibited by the addition of the synthetic tetramer.

The mould count of the samples varied from <500 up to 40,000 cfu/g of products. Most of the moulds present in nuts (sample 1-10) and in spices (samples 15, 16, 18 and 19) belongs to the genus *Aspergillus* and *Penicillium*. Moulds of the genus *Neurospora* were present in chillipowder (sample 13) and curry (sample 18). No correlation between latex-agglutination reaction and mould counts were observed.

Table 3. Latex-agglutination reactions and mould counts of extracts of nuts and spices. Latex beads were coated with antibodies raised against the extracellular polysaccharides (EPS) produced by *Penicillium digitatum*. Specific blocking was carried out using a tetramer of ß(1,5)-linked D-galactofuranosides at a final concentration of 100 µg/ml.

No.	Type of food	Latex-aggl. reaction	Specific blocking[a]	Mould count	Genus distribution[d] (%)			
				(log cfu/g)	*Pen/Asp*	*Muc/Rhiz*	*Clad*	Others
1	peanuts	-[b]	NT[c]	<2.0	-	-	-	-
2	peanuts	+	yes	<2.0	-	-	-	-
3	walnuts	+	no	4.3	100	-	-	-
4	walnuts	+++	yes	3.6	100	-	-	-
5	walnuts	++	yes	4.5	95	5	-	-
6	hazelnuts	+++	yes	3.0	80	-	20	-
7	pistachio	++	yes	<2.0	-	-	-	-
8	pistachio	-	NT	2.1	NT	NT	NT	NT
9	mixture of nuts	+	yes	2.3	100	-	-	-
10	almonds	+++	yes	2.9	90	-	-	10
11	nutmeg	+++	yes	3.0	-	-	10	90
12	cayenne powder	+++	yes	<2.5	-	-	-	-
13	chilli powder	+++	yes	2.5	-	50	-	50
14	paprika powder	+++	yes	NC	40	50	10	-
15	white pepper	+++	yes	4.5	100	-	-	-
16	black pepper	+++	yes	4.1	90	-	-	10
17	cloves	+++	yes	<2.5	-	-	-	-
18	curry	+++	yes	4.6	90	5	-	5
19	cinnamon	+++	yes	4.0	60	10	30	-

[a,b,c] See Table 2.

[d] *Pen*: *Penicillium*; *Asp*: *Aspergillus*; *Muc*: *Mucor*; *Rhiz*: *Rhizopus*; *Clad*: *Cladosporium*.

DISCUSSION

A rapid, simple, reliable, and sensitive test for the detection of mould in foods is needed. Over 100 different mycotoxins have been described (Rodricks, 1976), which represent a major hazard if present in foods. Testing for various mycotoxins may be difficult, while the absence of viable and non-viable moulds may provide sufficient assurance to consider food materials to be free from potential mycotoxins. The ELISA as described by Notermans and Heuvelman (1985) and Lin *et al.* (1986) are suitable for such purpose because the heat stable polysaccharides of moulds are detected. Nearly all moulds are known to produce such polysaccharides (Notermans and Soentoro, 1986). Although the ELISA is a relatively simple technique, it takes 5 to 10 hours for providing results. This report describes a latex-agglutination test which is simpler, equally reliable, and rapid, requiring only 10 to 20 min. When compared to ELISA, an agglutination test is less sensitive by 5 to 10 fold. However, the sensitivity may be increased by recording results at 1 to 2 hour period, and also by increasing the concentration of antibody reagent (data not shown).

The observation that the samples of all species of *Aspergillus* and *Penicillium* except *P.rubrum* and *P.islandicum* gave positive reaction in the latex-agglutination test (Table 2), is similar to that obtained by ELISA as reported by Notermans and Soentoro (1986).

Results presented in Table 3 showed no correlation between the latex-agglutination reaction and the mould count. With the latex-agglutination test heat stable EPS produced by moulds are determined. Therefore the presence of non-viable moulds are determined as well. Especially dry products such as nuts and spices can be contaminated by moulds which are killed during drying of these products. The results demonstrate that the latex-agglutination assay, as described here, is suited for testing dried or (heat) processed products.

Further, a random selection of nut and spice samples were also used in the latex-agglutination test for determining the scope of application for such test. The agglutination reaction between EPS and the antibody on the beads can be blocked by the

presence of haptens consisting of a tetramer of ß(1,5)-linked D-galactofuranosides. Since EPS is produced by all species of *Aspergillus* and *Penicillium*, the inhibition of agglutination by the synthetic tetramer should be achieved. In case of agglutination which can not be blocked by the tetramer may be considered as a non-specific reaction, as seen with sample 3, in Table 3.

Preliminary results show that when the latex beads were coated with IgG anti-EPS of *Mucor racemosus* or of *Cladosporium cladosporioides* the latex-agglutination test is also sensitive for the detection of EPS produced by *Mucor racemosus* and *Cladosporium cladosporioides*.

The described latex-agglutination test can detect heat stable polysaccharide antigens which may be related to the mycelial weight (Notermans *et al.*, 1986). Thus, the contamination can be detected in the absence of viable moulds. The reliability of the latex-agglutination test is further enhanced by combining the use of a specific blocking agent, a tetramer of ß(1,5)-linked D-galactofuranosides in this case. Both, the latex-agglutination test and the control test based on the specific blocking are commercially available for more applications.

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CHAPTER 9

GENERAL DISCUSSION

The presence and outgrowth of moulds in food may result in spoilage causing economic losses. Additionally, mycotoxins may be produced which is a threat to human health. To control the presence of moulds in food it is of importance to dispose of convenient and reliable detection methods. Existing detection methods of moulds have their specific advantages and disadvantages. A promising technique is the immunoassay based on the detection of antigenic extracellular polysaccharides (EPS) produced by moulds. Further characterisation of the immunoassay for the detection of *Aspergillus* and *Penicillium* and evaluation of the assay were the main goals of this study. Antigenically identical EPS is produced by most *Aspergillus* and *Penicillium* species. It has been revealed that ß(1,5)-linked galactofuranosyl residues are only responsible for the antigenic properties of these EPS.

Experiments were carried out to prove whether ß(1,5)-linked galactofuranosyl residues are indeed the immunodominant part of EPS produced by *Aspergillus* and *Penicillium* species. For this, amongst others, artificial immunogens consisting of ß(1,5)-linked galactofuranosyl tetramers and heptamers conjugated to tetanus toxoid were produced (Chapter 4). Also, an exo-ß-D-galactofuranosidase produced by *Trichoderma harzianum* was purified, characterised and used as a structure probe. To evaluate the immunoassay for practical application the production of antigenic EPS by *P.digitatum* and *P.aurantiogriseum* were studied under various conditions. It was also tried to raise antibodies against so-called immunosilent structural features of EPS produced by *Aspergillus* and *Penicillium*. It was anticipated that these antibodies would be reactive with EPS produced by other mould genera as well. This would result in an immunoassay enabling simultaneous detection of more genera than only *Aspergillus* and *Penicillium*. Finally a rapid and reliable latex-agglutination assay was developed and food samples were investigated for the presence of antigenic extracellular polysaccharides produced by *Aspergillus* and *Penicillium*.

From the studies carried out it became clear that EPS produced by *Aspergillus* and *Penicillium* contain the common sugars, galactose, glucose and mannose, in combina-

tion with a minor part of protein. The ß(1,5)-linked galactofuranosyl residues are immunodominant as proved by immunisation experiments using artificial immunogens and specific enzymatic hydrolysis (Chapter 5). It is remarkable that antibodies raised against EPS of *Aspergillus* and *Penicillium* are always directed to the carbohydrate part, notably the ß(1,5)-linked galactofuranosyl residues and not to the protein or mannan part. Antibodies raised against immunosilent structures of EPS produced by *Aspergillus* and *Penicillium*, which are probably also present in EPS of other genera did not result in antibodies reacting with EPS of genera other than *Aspergillus* and *Penicillium* (Chapter 7). In contrast, these antibodies were more species specific, indicating that the ß(1,5)-linked galactofuranosyl residues are responsible for the genus specificity. Further reseach is recommended concerning the immunogenicity of EPS, with special attention for the protein part and the carbohydrate/protein ratio of the EPS produced by *Aspergillus* and *Penicillium*. For this it is important to determine whether EPS produced by various *Aspergillus* and *Penicillium* species are T-cell dependent or T-cell independent immunogens. For a T-cell dependent immune response the protein part is important.

Galactofuranosidases are generally produced by *Aspergillus* and *Penicillium* species (Cousin *et al.*, 1989). Nevertheless, in food EPS produced by these genera can easily be detected. The production of this enzyme by *Aspergillus* and *Penicillium* in certain types of food should be studied in more detail. Especially in *Penicillium* ripened cheese relatively high galactofuranosidase activities were found (personal observation). In these samples no EPS was detected. It became clear that ß(1,6)-linked galactofuranosyl residues are present in the EPS of *Aspergillus* and *Penicillium* as side chains of the ß(1,5)-linked galactofuranosyl residues. The use of a specific enzyme as structure probe and the reductive cleavage method for glycosidic bonds allowed the discovery of these structural features. However, no antibodies were produced against the ß(1,6)-linked galactofuranosyl residues upon immunisation of rabbits with *Aspergillus* and *Penicillium* EPS. In contrast, the ß(1,6)-linked galactofuranosyl dimers possess the lowest inhibitory capacity in the reaction between EPS produced by *P.digitatum* and antibodies raised against this EPS (Notermans *et al.*, 1988). These findings

invite for new interesting experiments e.g. immunisation with EPS, treated with exo-ß-D-galactofuranosidase. This enzyme is specific for ß(1,5)-linked galactofuranosyl residues and requires an unsubstituted non-reducing terminal for attack. It would be interesting to see if enzyme-treated EPS, which still has sequences of ß(1,5)-linked and ß(1,6)-linked galactofuranosyl residues (Chapter 5) raises antibodies in rabbits and, if it does, what the antigenic specificity of these antibodies would be.

Antigenic polysaccharides have been produced by *P.digitatum* and *P.aurantiogriseum* under various conditions. However, with lactate as carbon source no antigenic EPS was produced. This may be a reason why the immunoassay is not suitable for the detection of antigenic EPS produced by *Penicillium* in mould ripened cheeses. However, it is more likely that EPS is produced, but it may not react with the antibodies specific for ß(1,5)-linked galactofuranosyl residues. Tsai *et al.* (1990) demonstrated that production of EPS occurs in artificially infected cheeses and yogurt. Further research concerning the production of antigenic EPS on these types of food products is recommended, with special attention for the availability and metabolism of amino acids and carbon sources other than sugars. The EPS produced under cheese-ripening conditions can be used for immunisation experiments to produce antibodies which are directed to structural features present in these EPS. Recently, we have determined that the carbon/nitrogen ratio is also important for the production of antigenic extracellular polysaccharides by *Fusarium* (Kamphuis *et al.*, in prep[4].). Experiments described in Chapter 6 showed that comparison of the antigenicity of EPS as obtained by an immunoassay, with chemical parameters, such as carbohydrate content or composition is difficult and irrelevant. Firstly, the ELISA "detects" three dimensional structures which represent a minor portion of the polysaccharides and secondly, acceptable variations of ELISA results are in order of 20 to 25%. This in contrast to acceptable variation in chemical parameters such as sugar content or composition, which are much smaller. Comparison of the galactose content of *Aspergillus* and *Penicillium* EPS with an ELISA result, carried out with antibodies directed to ß(1,5)-linked galactofuranosyl residues is also irrelevant. Results in Chapter 5 show that the galactose residues are all in the furanose form,

but they may be ß(1,5)- or ß(1,6)-linked. Only the ß(1,5)-linked residues are antigenically reactive. Also, the lenght of the side chains may vary (Bardalaye *et al.*, 1977; Gander *et al.*, 1986), and it has been shown in Chapter 5 that a non-substituted non-reducing terminal ß(1,5)-linked residue is required for antigenicity. The results presented in Chapter 7 strenghten these arguments.

In the authors opinion ELISA results should be quantified by expressing them as titre (reciprocal of the highest dilution giving a positive result) or minimal detectable quantity of EPS. In both cases this is the smallest quantity that can reliably be detected by the method used.

The latex-agglutination assay developed for the detection of moulds in food and feed as described in Chapter 8 was tested in collaborative and comparative studies in

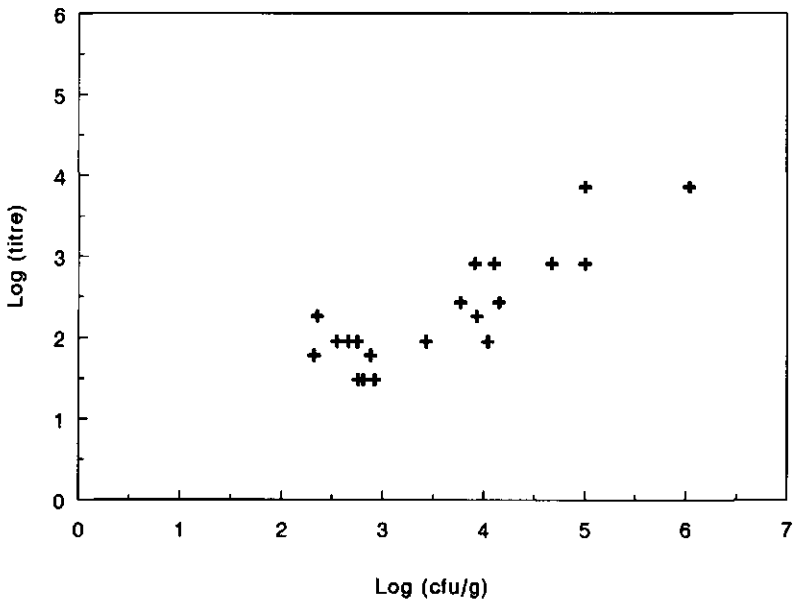

Figure 1. Relation between the mould count and the latex-agglutination test of maize samples as determined in a collaborative study (Kamphuis *et al.*, 1992)

order to assess its reliability and applicability (Notermans and Kamphuis, 1990; Kamphuis *et al.*, 1992). In these studies the mould counts of the investigated food and feed samples (cereals, spices, herbs, walnuts and maize) were also determined. The comparative study showed that the mould count was carried by various participants according to different procedures, thus for quantitative comparison the mould count needs to be standardised or reference materials should be incorporated (King *et al.*, 1986; Samson *et al.*, 1992; Kamphuis *et al.*, in prep[b].). In general, the mould count correlated positively with the latex-agglutination titre, but there was a fairly large variation in results (Fig. 1). With the latex-agglutination almost no false-positive or false-negative results were observed. Exceptions, giving false-positive results, probably due to the presence of polyphenols, were walnut samples. These substances crosslink the antibodies used in the assay. These findings confirm the necessity of including reliable detection of both false-positive and false-negative samples in test procedures. In contrast to the mould count, the latex-agglutination titre was not influenced by gamma-irradiation and heat treatment, indicating that the mould count is not a good reflection of the previous state of moulding, if any processing treatment was carried out.

This thesis has shown that immunological detection of *Aspergillus* and *Penicillium* in food and feed is rapid, reliable and simple and can be used for routine analysis of food and feed products. Nevertheless, antigenic EPS could not be detected in mould ripened cheeses such as Camembert and Roquefort. Immunoassays for the detection of other genera e.g. *Mucor*, *Rhizopus*, *Cladosporium*, *Fusarium*, *Botrytis* and *Monascus* are also available (Notermans and Soentoro, 1986; Cousin et al, 1990; De Ruiter *et al.*, 1991; Kamphuis *et al.*, in prep[a]). Since these assays are all genus-specific they may be suitable for rapid taxonomic classification purposes.

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SUMMARY

This thesis is devoted to the immunological detection of *Aspergillus* and *Penicillium* in food products. More specifically, the immunogenicity, antigenicity, production and structure of the water-soluble extracellular polysaccharides (EPS) of these moulds have been studied, and a latex-agglutination assay, based on the detection of EPS has been developed.

For the detection of moulds many methods are available, each of them with specific advantages and disadvantages, mostly related to reliability and applicability **(Chapter 2)**.

An overview of the immunogenicity and antigenicity of EPS produced by moulds is presented in **Chapter 3**. The role of ß(1,5)-galactofuranoside sequences as epitopes of galactomannans from *Aspergillus* and *Penicillium* is documented. Antigenically specific polyclonal antibodies raised against *P.digitatum* EPS are directed towards ß(1,5)-linked galactofuranosyl residues. These antibodies react specifically with EPS from *Aspergillus* and *Penicillium*.

Synthetic tetramers and heptamers of ß(1,5)-linked galactofuranosides are conjugated to tetanus toxoid and polyclonal antibodies are raised in rabbits against these synthetic immunogens **(Chapter 4)**. Antibodies obtained after immunisation with the heptamer conjugate possess the same genus specific antigenicity as the antibodies raised against *P.digitatum* EPS. No reactions are observed with the *Penicillium* subgenus *biverticillium* species and species belonging to genera other than *Aspergillus* and *Penicillium*. In contrast, antibodies raised against the tetramer conjugate reacted only with six out of 24 tested *Aspergillus* and *Penicillium* strains.

From Glucanex, a *Trichoderma harzianum* enzyme preparation, an exo-ß-D-galactofuranosidase is purified. This enzyme is used to hydrolyse specifically the immunodominant ß(1,5)-linked galactofuranosyl residues from *Aspergillus* and *Penicillium* EPS. This enzyme alleviates the antigenicity of the EPS completely **(Chapter 5)**.
Additionally, the reductive cleavage method for determination of the glycosidic bonds revealed that the ß(1,5)-linked galactofuranosyl side chains in *P.digitatum* EPS carry

side-chains of ß(1,6)-linked galactofuranosyl residues. These results allowed to propose a new structural model for the antigenically active galactofuranoside side chains of *Penicillium* galactomannans.

In **Chapter 6**, the production of antigenic EPS by *P.aurantiogriseum* and *P.digitatum* has been described under various growth conditions. Antigenic EPS was produced under almost all conditions investigated. However, both *P.aurantiogriseum* and *P.digitatum* do not produce antigenic EPS on lactate as the carbon source. Also, *P.camemberti* isolated from a mould fermented cheese (Camembert) does not produce antigenic EPS on lactate, althought, *P.camemberti* produces antigenic EPS on other substrates. The monosaccharide composition of the EPS produced by *P.aurantiogriseum* and *P.digitatum* under various conditions varies considerably.

Immunopotent acid-labile ß(1,5)-linked galactofuranosyl residues of *Aspergillus fumigatus, Aspergillus niger* and *Penicillium digitatum* EPS were acid-hydrolysed (**Chapter 7**). Antibodies are raised against these acid-treated extracellular polysaccharides. It was supposed that these acid-treated EPS preparations would elicit antibodies with a broader specificity, making them useful in the detection of nearly all or all moulds occurring in food products. However, the antibodies obtained are more species specific and are generally directed to glucosyl and/or mannosyl residues of the EPS.

Antibodies raised against *P.digitatum* EPS are used for the development of a rapid and reliable latex-agglutination assay for the detection of *Aspergillus* and *Penicillium* in food and feed (**Chapter 8**). The reliability of the assay is enhanced by using a synthetic epitope, a tetramer of ß(1,5)-linked galactofuranosides. With this tetramer false-positive results can easily be recognised.

Finally, in **Chapter 9** the applicability of the developed latex-agglutination assay is tested in both comparative and collaborative studies. The significance of extracellular polysaccharides produced by moulds for the detection of moulds in food and feed is discussed.

HET GEBRUIK VAN EXTRACELLULAIRE POLYSACHARIDEN VOOR HET AANTONEN VAN *ASPERGILLUS* EN *PENICILLIUM* IN LEVENSMIDDELEN

SAMENVATTING

Voor de bepaling van schimmels zijn veel detectiemethoden bekend. De meeste methoden hebben specifieke voordelen maar ook nadelen, zeker wat betreft de betrouwbaarheid en de toepasbaarheid.

Dit proefschrift behandelt het immunologisch onderzoek, betreffende de detectie van schimmels in levensmiddelen. De immunogene en antigene eigenschappen, produktie en structuur van water-oplosbare extracellulaire polysachariden (EPS) van *Aspergillus* en *Penicillium* werden bestudeerd en een latexagglutinatie test, gebaseerd op het aantonen van EPS, werd ontwikkeld en geëvalueerd.

Een overzicht van de antigene eigenschappen van schimmel EPS staat in **hoofdstuk** 3. Ingegaan wordt op de functie van de ß(1,5)-gebonden galactofuranose structuur als epitoop van de galactomannanen geproduceerd door *Aspergillus* en *Penicillium*. Om te bewijzen dat ß(1,5)-gebonden galactofuranose immunodominant is, werden synthetische tetrameren en heptameren van ß(1,5)-gebonden galactofuranose eenheden geconjugeerd aan tetanus toxoide. Tegen deze synthetische immunogenen werden polyklonale antilichamen opgewekt (**hoofdstuk** 4). De verkregen antilichamen met het heptameer-conjugaat bleken dezelfde eigenschappen (genus specificifiek) te hebben als de antilichamen opgewekt tegen *P.digitatum* EPS. Deze antilichamen reageerden met EPS geproduceerd door alle *Aspergillus* en *Penicillium* species (met uitzondering van de *Penicillium* subgenus *biverticillium* species). De antilichamen opgewekt tegen het tetrameer-conjugaat reageerden echter alleen met 6 van de 24 geteste *Aspergillus* en *Penicillium* species.

Verder werd uit Glucanex een enzympreparaat geproduceerd door *Trichoderma harzianum* een exo-ß-D-galactofuranosidase geïsoleerd. Dit enzym werd gebruikt voor de hydrolyse van de immunodominante ß(1,5)-gebonden galactofuranose eenheden

van *Aspergillus* en *Penicillium* EPS. Als gevolg hiervan verdwenen de antigene eigenschappen van het EPS (**hoofdstuk 5**). Met behulp van de 'reductive-cleavage' methode voor glycosidische bindingen kon worden aangetoond dat de ß(1,5)-gebonden galactofuranose zijtakken in het *P.digitatum* EPS zijketens hebben van ß(1,6)-gebonden galactofuranose. Dit alles resulteerde in een nieuw structuurmodel voor de galactofuranose zijketens met antigene eigenschappen van het *P.digitatum* galactomannaan.

In **hoofdstuk 6** wordt de produktie van antigene extracellulaire polysacchariden door *P.aurantiogriseum* en *P.digitatum* onder verschillende groei-omstandigheden beschreven. In alle gevallen werd antigeen EPS geproduceerd, zij het dat zowel *P.aurantiogriseum* als *P.digitatum* geen immunologisch aantoonbaar EPS produceerde bij groei op de koolstofbron lactaat. Ook *P.camemberti*, geïsoleerd van een Camembert kaas, produceerde geen immunologisch detecteerbaar EPS op lactaat. Dit was wel het geval op andere substraten. Ondanks de immunologisch sterke verwantschap bleken de monosachariden-samenstellingen van de EPS'en geproduceerd door *P.aurantiogriseum* en *P.digitatum* onder uiteenlopende groei-omstandigheden aanzienlijk te verschillen.

Ook werden de immunopotente zuur-labiele ß(1,5)-gebonden galactofuranose eenheden van *Aspergillus fumigatus*, *Aspergillus niger* en *Penicillium digitatum* EPS met behulp van zuur-gehydrolyseerd (**hoofdstuk 7**). Antilichamen opgewekt tegen deze zuur-gehydrolyseerde EPS'en bleken niet meer genus specifiek te zijn. Hierdoor waren ze niet geschikt voor het aantonen van meer schimmelgenera in levensmiddelen. De verkregen antilichamen waren duidelijk (species) specifieker en in het algemeen gericht tegen de glucose en/of mannose residuen van het EPS.

Antilichamen opgewekt tegen *P.digitatum* EPS en gericht tegen ß(1,5)-gebonden galactofuranose werden gebruikt voor de ontwikkeling van een snelle en betrouwbare latexagglutinatie test voor het aantonen van *Aspergillus* en *Penicillium* in levensmiddelen en veevoeders (**hoofdstuk 8**). De betrouwbaarheid van de test kon worden vergroot door gebruik te maken van een gesynthetiseerd tetrameer van ß(1,5)-gebonden galactofuranose eenheden als specifiek blokkeringsagens. Hierdoor konden

de zogenaamde vals-positieve resultaten eenvoudig worden opgespoord.

Tenslotte komt in **hoofdstuk 9** de toepasbaarheid van de ontwikkelde latexagglutinatie in zowel vergelijkende onderzoeken als ringonderzoeken aan de orde. Het belang van de antigene extracellulaire polysachariden voor het aantonen van schimmels in levensmiddelen en veevoeders wordt besproken.

NAWOORD

Dit proefschrift is tot stand gekomen dankzij de medewerking van velen. Ik wil hen allen bedanken voor de inzet en steun. Een aantal personen wil ik echter met naam noemen.

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 23 januari 1963 geboren te Vaassen. In 1981 werd het VWO-diploma behaald aan het Myrtus College te Apeldoorn. In datzelfde jaar begon hij met de studie Levensmiddelentechnologie (oude stijl) aan de (toentertijd) Landbouwhogeschool te Wageningen. Het doctoraalexamen, met de hoofdvakken levensmiddelenchemie en organische chemie en bijvak levensmiddelenmicrobiologie werd in november 1987 behaald. Hij bracht zijn praktijktijd door bij de Keuringsdienst van Waren te Zutphen en bij Gist-brocades te Séclin (Frankrijk). Vanaf 1 januari 1988 tot 1 januari 1992 was hij werkzaam als Assistent in Opleiding aan de Landbouwuniversiteit bij de vakgroep Levensmiddelentechnologie, sectie Levensmiddelenchemie en -microbiologie (Prof.dr.ir. F.M. Rombouts). Het onderzoek beschreven in dit proefschrift is grotendeels uitgevoerd op het Laboratorium voor Water- en Levensmiddelenmicrobiologie (Dr.ir. S.H.W. Notermans) van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne te Bilthoven. Sinds 1 juni 1992 is hij werkzaam bij Gerkens Cacao Industrie BV te Wormer.

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