Arabinase induction and carbon catabolite repression in *Aspergillus niger* and *Aspergillus nidulans*

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Arabinase induction and carbon catabolite repression in *Aspergillus niger* and *Aspergillus nidulans*

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1 In de conclusies die Dowzer and Kelly en Arst *et al.* trekken uit hun experimenten met *cre*A transformanten wordt geheel voorbij gegaan aan het feit dat twee keer zoveel *cre*A mRNA gedetecteerd wordt by groei van *Aspergillus nidulans* op L-arabinose dan op D-glucose. (Dowzer en Kelly (1989) Curr Genet 15:457-459; Arst *et al.* (1990) Molec Microbiol 4:851-854)

2 Tagawa and Terui tonen niet aan dat de glucose die zij vinden in mycelium van *Aspergillus niger* gekweekt op L-arabinose afkomstig is van een omzetting van L-arabinose in D-glucose zoals zij beweren. (Tagawa and Terui (1968) J Ferm Technol 46:693-700)

3 Het feit dat de variabiliteit van enkele physicochemische eigenschappen van hemicellulolytische enzymen gecorreleerd is met de bron waaruit ze gezuiverd zijn rechtvaardigt de conclusie dat voor nauwkeurige karakterisatie van extracellulaire enzymen beter uitgegaan kan worden van vers cultuur filtraat dan van een commercieel enzym preparaat (Rombouts et al. (1988) Carbohydr Polym 9:25-47; dit proefschrift)

4 De omstandigheden in het laboratorium waaronder de fysiologie van micro-organismen wordt bestudeerd zijn vaak niet gerelateerd aan de eco-fysiologische omstandigheden waarin het organisme normaal verkeert.

5 Het voorkomen in Canada van een Cree indianenstam impliceert niet direct dat naast de 4 gevonden *cre*A - *cre*D genen er ook een *cre*E gen bestaat.

6 Een microbioloog kan op zijn werk beter zijn handen wassen voor dat hij naar het toilet gaat dan erna.

7 Het aantal postdocs dat in het buitenland werkt is omgekeerd evenredig met de economische situatie van het thuisland van de desbetreffende postdoc.

8 Het huidige tempo van informatisering binnen de wetenschap heeft tot gevolg dat binnen een laboratorium altijd iemand aanwezig dient te zijn met voldoende kennis van die informatisering.

Wageningen, 24 januari 1994 Peter van der Veen

aan mijn ouders

voor Margryt

Contents

Introduction

CHAPTER 1

INTRODUCTION

Conversion of agricultural biomass, which consists mainly of plant cell walls, is of technological relevance for *e.g.*:

- bioconversion to useful fuels or chemicals
- upgrading waste pulps and virgin pulps in the manufacture of paper
- extracting of oils from seeds
- process and product innovation in the processing of fruit and vegetables
- starch/gluten separation in wheat processing.

For these conversions a whole range of commercial enzyme preparations, often produced by fungi like *Aspergillus niger*, are available. These enzyme preparations contain a whole spectrum of cell wall degrading enzymes which roughly can be divided into three major groups: cellulolytic, pectinolytic and hemicellulolytic enzymes. Some compounds are only present in small amounts in these commercial enzyme preparations and therefore called 'minor enzymes'. Nevertheless, minor enzymes can be very important in industrial processes.

Plant cell walls are composed of approximately 90% of polysaccharides and 10% of proteins. The main polysaccharides that have been identified are cellulose, hemicelluloses like xyloglucans, arabinoglucuronoxylans, glucomannans, arabinans, galactans, arabinogalactans and pectic polysaccharides (McNeil et al. 1984).

The enzymes involved in the breakdown of these polysaccharides in general are hydrolases and, in the case of pectin degradation also lyases. Furthermore esterases are of importance which are able to remove ester linked substituents like methoxyl, acetyl, feruloyl and coumaryl groups. The hydrolases can be classified as depolymerases and can be subdivided

Table 1. Industrial application of cell-wall-degrading enzymes after Voragen (1990)

Enzymes	Industry	Applications
Xylanase	Brewing Baking	Beer filtration Baking volume, water binding
Pectin esterase	Fruit and vegetables	Juice clarification
Polygalacturonase	Fruit and vegetables	Maceration, citrus juice clarification/viscosity reduction
Pectinesterase + polygalacturonases/pectin lyase	Fruit and vegetables	Juice clarification, juice oil extraction, citrus peel oil, citrus pulp wash
Pectin lyase, pectate lyase	Fruit and vegetables	Maceration
Arabinases (galactanases)	Fruit and vegetables	Juice clarification (ultrafiltration)
Pectinesterase, polygalacturonase, pectin lyase, cellulases, hemicellulases	Fruit and vegetables	Liquefaction, clear cloudy juices Enhancement of natural product extraction
Pectinases, (hemi)cellulases	Byproducts	Improvement of feed digestibility
Glycosidases, esterases	Biomass	Saccharification of biomass
Pectinases, hemicellulases	Fibrous byproducts	Improvement of functionalities
Cellulases, hemicellulases	Paper	Enhancement of fibre-fibre bonding

in enzymes which degrade the polysaccharide backbone at random (endo-enzymes) and enzymes which cleave off monomeric sugars from polymeric or oligomeric substrates from the non-reducing end (exo-enzymes).

Table 1 shows a summary of cell-wall-degrading enzymes and their use in industrial applications.

Hemicellulose and hemicellulases

Hemicelluloses can be defined as those non-cellulosic cell wall polysaccharides which are soluble in alkali. They are usually classified according to their constitutive sugar residues: xylans, mannans, arabinans and galactans. Most hemicelluloses occur as heteropolysaccharides containing different carbohydrate residues (D-xylose, L-arabinose, D-mannose, D-

glucose, D-galactose, D-glucuronic acid, D-galacturonic acid) which are sometimes esterified (acetyl, feruloyl, coumaryl), in the backbone chain and/or in the side chains thus generating structural variation in these polysaccharides (Poutanen et al. 1991). In turn, a large number of enzymes, termed hemicellulases, are required to hydrolyse these multiple and complex structures. The major groups of hemicellulases are xylanases, mannanases, arabinases and galactanases. If one considers the fact that already several cellulase families have been identified to play a role in the biodegradation of a chemically simple polysaccharide as cellulose (Henrissat et al. 1989; Béguin 1990; Gilkes et al. 1991), the actual number of hemicellulase families required is expected to exceed this considerably.

Arabinan and arabinases

Arabinans are present in large amounts in seeds, fruits, vegetables, bark and wood. In general they are associated with galactans and pectins. Structurally, arabinans consist of 1,5 linked chains of *α*-L-arabinofuranosyl residues, which are substituted with *α*-L-arabinofuranosyl residues mainly at the O-3 and/or also at the O-2 position (Stephen 1983). There are two types of arabinan degrading enzymes, *α*-L-arabinofuranosidases and endo-arabinases. Some *α*-L-arabinofuranosidases, which are exo-enzymes, have activity on polymeric branched arabinans and a strongly reduced activity on linear 1,5-*α*-L-arabinan, but also on synthetic substrates like *p*-nitrophenyl-*α*-L-arabinofuranoside (PNPA). Other *α*-L-arabinofuranosidases prefer low molecular weight substrates like arabino-oligomers. The endo-arabinases only have activity on linear arabinans resulting in the formation of oligomers. Fig. 1 shows the structure of a hypothetical plant arabinan and the sites of attack by arabinolytic enzymes.

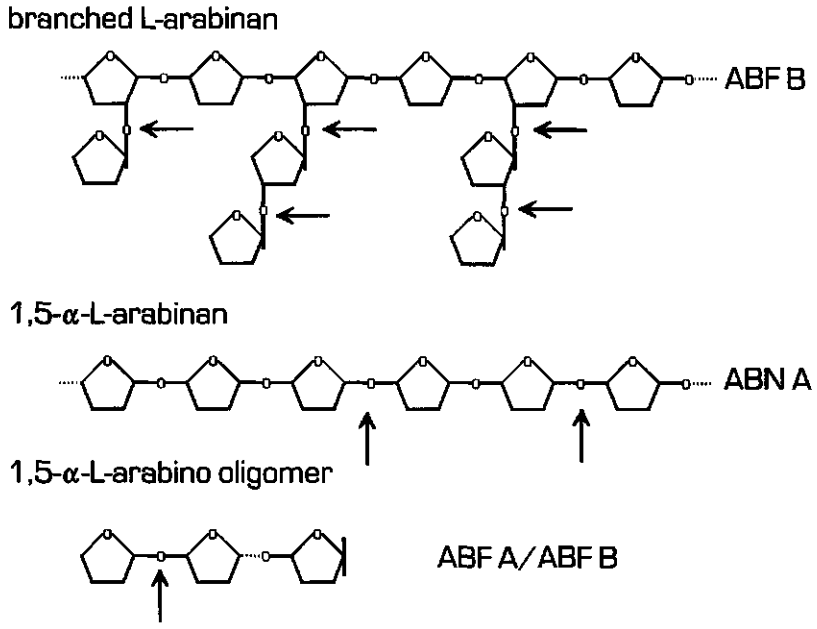

Fig. 1 Structure of a hypothetical arabinan and the sites of attack by arabinolytic enzymes. ABF A: α-L-arabinofuranoside A; ABF B: α-L-arabinofuranoside B; ABN A: endo-arabinase.

Arabinan degrading enzymes have been isolated from a large number of sources including bacteria and fungi like *Streptomyces purpurascens* (Komae et al. 1982), *Bacillus subtilis* and other *Bacillus* strains (Weinstein and Albersheim 1979; Yoshikara and Kaji 1983; Kaji and Saheki 1975), *A. niger* (Kaji and Tagawa 1970; Waibel et al. 1980) and certain *Clostridium* sp. (Kaji 1984).

Thus far three types of arabinan degrading enzymes have been identified from a commercial *A. niger* enzyme preparation by Rombouts et al. (1988). These are α-L-arabinofuranosidases A and B, both active on the synthetic substrate and arabino oligomers, but only the latter one also

active on polymeric branched or linear arabinans, and endo-arabinase which is only active on linear arabinan.

Arabinose catabolic pathway

The final degradation products of (hemi)cellulolytic activity are usually small oligomers or monomeric sugars which are used as carbon source. These breakdown products often play an important role in the induction of the (hemi)cellulolytic enzyme systems.

Mandels et al. (1962) found that in *Trichoderma* the disaccharide sophorose (ß-1,2-glucobiose) induces the whole cellulolytic system, *viz* cellobiohydrolase I and -II and endoglucanase. Furthermore for pectinases it is known that for the induction of pectate lyases in *Erwinia chrysanthemi* an intermediate of the degradation pathway of pectin (2-keto, 3-deoxygluconate) (KDG) is responsible (Collmer and Bateman 1981; Condemine et al. 1986). From studies done by Rombouts et al. (1988) it is known that the end product of arabinan degradation by the arabinase complex is monomeric L-arabinose. This pentose sugar can be taken up by *A. niger* and converted in several steps to D-xylulose-5-P which then enters the pentose phosphate pathway. Fig. 2

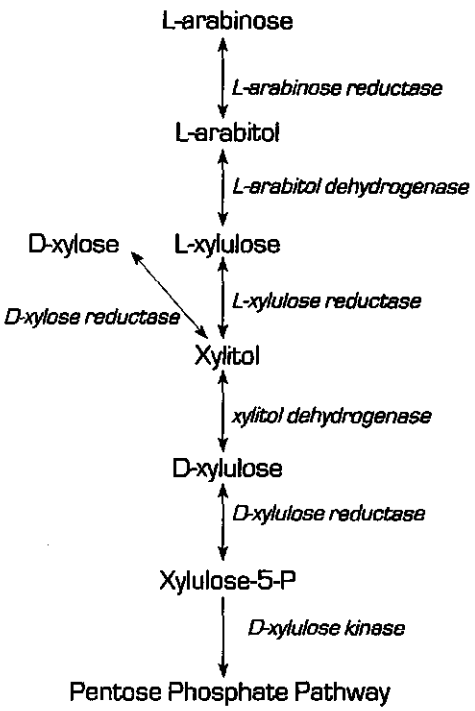

Fig. 2 Degradation pathway of L-arabinose and D-xylose in *Aspergillus niger* (*cf* Witteveen et al. 1989)

shows the degradation pathway of L-arabinose and D-xylose in *A. niger*. It is not known however whether these sugars play a role in the induction of the arabinolytic system in *A. niger*.

Carbon catabolite repression

Degradation of biomass by a complex of (hemi)cellulolytic enzymes releases a large number of different monomeric sugars. For some of these sugars, D-glucose for instance, it is known that they cause strong carbon catabolite repression. Fungi like *A. niger* and *A. nidulans* prefer the utilisation of easy degradable carbon sources and therefore repress the synthesis of enzymes necessary for the degradation of less preferred substrates.

It is known that the regulation of biosynthesis of (hemi)cellulolytic and pectinolytic enzymes normally is controlled by two mechanisms, pathway specific induction and carbon catabolite repression as was found, for example, for polygalacturonase and pectin esterase in *A. niger* (Maldonado et al. 1989) and cellulase in *A. nidulans* (Bagga et al. 1989).

Wide domain gene control including carbon catabolite repression is most extensively studied in the fungus *A. nidulans*. Arst and Cove (1973) and Bailey and Arst (1975) already isolated mutations in *A. nidulans* leading to carbon catabolite derepression. The *cre*A gene thus identified was thought to be a negatively acting wide domain regulator (Arst and MacDonald 1975; Arst and Bailey 1977). Cloning of the *cre*A gene of *A. niger* and *A. nidulans* confirmed this and demonstrated this gene to encode a 'zinc finger' DNA binding protein of the Cys_2His_2 type (Dowzer and Kelly 1989,1991; Drysdale et al. 1993).

Using similar strategies as Arst and Cove (1973), Hynes and Kelly (1977) obtained also mutations in two other genes (*cre*B and *cre*C) which

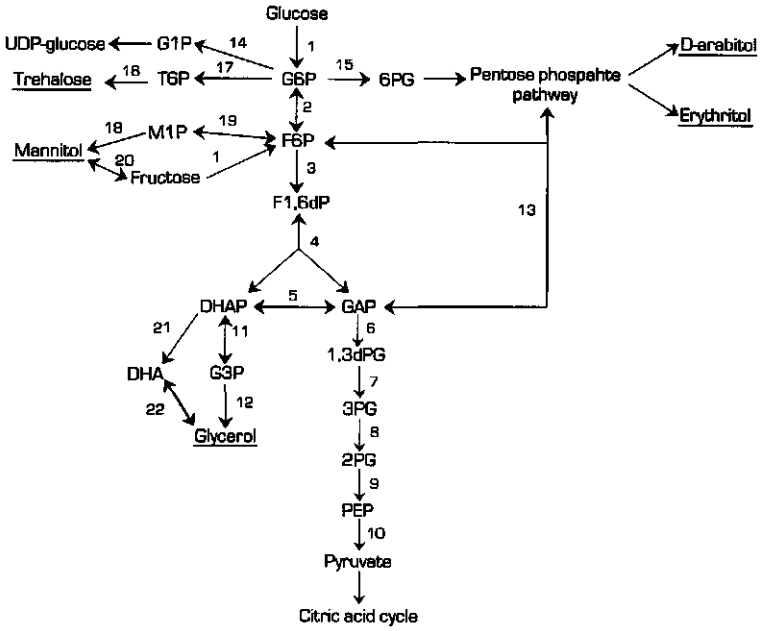

Fig. 3 Schematic representation of a part of the hexose monophosphate pathway and metabolic routes involved in polyol synthesis. 1: hexokinase; 2: phosphoglucose isomerase; 3: phosphofructokinase; 4: aldolase; 5: triose phosphate isomerase; 6: glyceraldehyde-3-phosphate dehydrogenase; 7: phosphoglycerate kinase; 8: phosphoglyceromutase; 9: enolase; 10: pyruvate kinase; 11: glycerol-3-phosphate dehydrogenase; 12: glycerol-3-phosphate phosphatase; 13: transketolase; 14: phosphoglucomutase; 15: glucose-6-phosphate dehydrogenase; 16: trehalose-6-phosphate synthase; 17: trehalose-6-phosphate phosphatase; 18: mannitol-1-phosphate phosphatase; 19: fructose-6-phosphate dehydrogenase; 20: mannitol dehydrogenase; 21 dihydroxyacetonephosphate phosphatase; 22 glycerol dehydrogenase; 23 UDP-glucose pyrophosphorylase

do not have a direct role in carbon catabolite repression but are thought to affect toxicities and/or utilisation of a wide range of compounds and thus play a more indirect metabolic role (Arst 1981). The existence of such carbon catabolite derepressed mutations opens a way to study induction and carbon catabolite repression of extracellular enzymes at more detail.

Intracellular metabolite and polyol concentrations

Together with enzyme activities, intracellular metabolite concentrations and polyol levels can give information about the flux of metabolites through the different pathways and the regulation of these pathways. The concentration of polyols formed gives information about the flux of the different metabolites towards the formation of polyols especially if an overflow in the metabolism occurs. For the formation of polyols in *Aspergilli* the following functions have been postulated:

- balancing the osmotic pressure
- carbon source reserve compounds
- regulation of cofactor balances
- transport of carbon substrates through the hyphae

The formation and breakdown of metabolites and polyols are strongly influenced by growth conditions. Also genetic changes, especially in regulatory genes, influence the composition of the polyol spectrum. Using the levels of the metabolite and polyol concentration, information about changes which have occurred in the physiology of the mutated cell can be obtained by making a comparison with the levels found in the wild type strain under controlled growth conditions. For carbon catabolite repression the degradation pathway of D-glucose will play an important role. Fig 3. gives an overview of the metabolites and enzymes involved in the breakdown of D-glucose. Also the possible routes for the formation of different polyols is given.

AIM AND OUTLINE OF THE THESIS

The organisms of study in this thesis are *Aspergillus niger* and *Aspergillus nidulans*. The first organism is widely used to make technical enzyme preparations which can be used to degrade agricultural biomass whereas the second organism is often used as a model system for studying biological problems on a more molecular level. This thesis therefore can be divided into two main topics: (1) a study on the arabinan degrading enzymes and enzymes present in the degradation pathway of L-arabinose in both organisms and (2) a study on the phenomena of carbon catabolite repression in *A. nidulans* with respect to induction/repression of enzymes and pathways involved. The aim of this thesis is to try to reach some understanding of the arabinan degrading enzymes and in particular of the induction mechanism of arabinan degrading enzymes and enzymes present in the intracellular metabolic pathway. Also a study was initiated on the physiological effects of carbon catabolite repression on glycolysis and po;yol metabolism.

Before studying the induction mechanism of the arabinases these enzymes were purified and characterised. Although there are some reports about the purification and characterisation of arabinan degrading enzymes from *Aspergilli* (Kaji et al. 1970) these usually handle only the α-L-arabinofuranosidase B, which is the major arabinase. Only one report from Rombouts et al. (1988) described the purification and characterisation of three arabinan degrading enzymes from a commercial *A. niger* enzyme preparation. Chapter 2 describes growth condition for the induction of the three known arabinases in a genetically well defined *A. niger* N400 strain as well as the purification and characterisation of the enzymes found.

Because the arabinase complex releases the monomeric sugar L-

arabinose from crude arabinan (Rombouts et al. 1988) the catabolic pathway of L-arabinose in *A. niger* could play an important role in the induction of the extracellular arabinases. L-arabinose and metabolites derived from L-arabinose were examined for their inducing abilities in Chapter 3.

Although *A. niger* N400 is a genetically well defined strain, *A. nidulans* has been considered to be a more advanced model system for studying biological problems, mainly due to the possibility of sophisticated genetic analysis in combination with the metabolic versatility of this organism. Thus *A. nidulans* represents an attractive microorganism to study biosynthesis of arabinan degrading enzymes at the molecular level. Arabinase induction in *A. nidulans* together with the purification and characterisation of the arabinases produced by this fungus are described in Chapter 4.

Carbon catabolite repression plays an important role in the breakdown of polymeric substrates. The release of repressing sugars affects the synthesis of other enzymes necessary for the complete degradation of the polymeric substrate. From *A. nidulans* a number of carbon catabolite derepressed mutants are available and characterised. Chapter 5 describes the comparison of these different carbon catabolite derepressed mutant strains and wild type *A. nidulans* at the level of induction/repression of the arabinan degrading enzymes.

The measurements of intracellular metabolite concentrations and polyol levels together with enzyme activities give us information about physiological processes occurring within the cell. The metabolite concentrations and polyol levels are strongly influenced by growth conditions, but also changes made by mutations in regulatory genes can completely change the internal metabolite concentrations, polyol pools and enzyme levels.

Measuring these values and comparing them with values of a wild type strain information can be obtained about changes which have occurred in the mutant strain with respect to the different pathways involved.

In Chapter 6 a comparison between *A. nidulans* wild type and the *creA*^{d}-30 mutant strains was made upon growth on D-glucose by measuring enzyme activities, metabolite concentrations and polyol levels present in the degradation pathway of D-glucose. D-glucose plays an important role in the occurrence of carbon catabolite repression of for instance arabinases, and the availability of different carbon catabolite derepressed mutants opens a way to investigate which physiological changes occur in the different *cre* strains available compared to the wild type under specific growth conditions.

In Chapter 7 a summary of the thesis together with some general conclusions are given.

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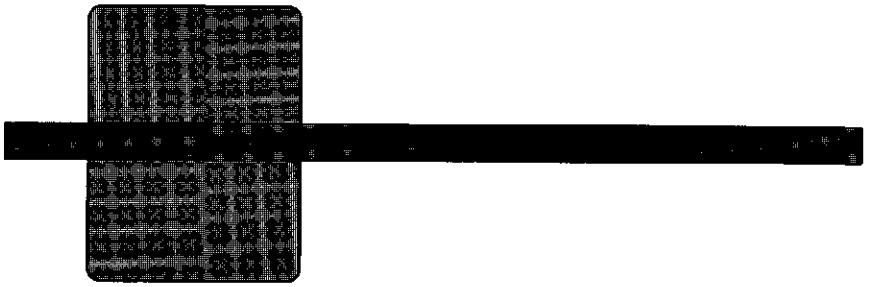
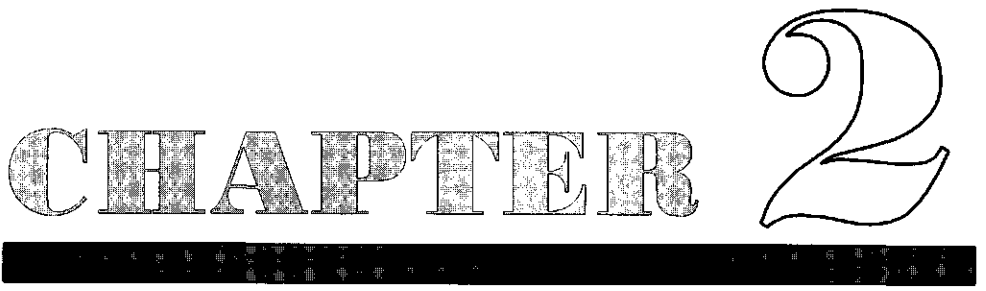


Induction, purification and characterisation of arabinases produced by *Aspergillus niger*



INDUCTION, PURIFICATION AND CHARACTERISATION OF ARABINASES PRODUCED BY *ASPERGILLUS NIGER*[1]

Peter van der Veen, Michel J.A. Flipphi, Alphons G.J. Voragen and Jaap Visser

SUMMARY

The induction of arabinases in *Aspergillus niger* N400 was studied on different simple and complex carbon sources. Sugar beet pulp was found to be an inducer of three arabinan degrading enzymes (*α*-L-arabinofuranosidase A, *α*-L-arabinofuranosidase B and endo-arabinase). These enzymes were purified from *A. niger* culture fluid after growth of the fungus in medium employing sugar beet pulp as the carbon source and were characterised both physio-chemically (Mw 83000, 67000, 43000 Da and, Pi 3.3, 3.5 and 3.0 for *α*-L-arabinofuranosidases A and B and endo-arabinase, respectively) and kinetically (K_m on *p*-nitrophenyl-*α*-L-arabinofuranoside 0.68 and 0.52 mM for *α*-L-arabinofuranosidases A and B, resp.; K_m on sugar beet arabinan 0.24 and 3.7 g/l for *α*-L-arabinofuranosidase B and endo-arabinase, resp.). The amino acid compositions of the three enzymes were determined also. The enzymic properties were compared with those from arabinases purified from a commercial *A. niger* enzyme preparation. Differences were found though the kinetic data suggest considerable similarity between the enzymes from the different sources.

[1]published in: Arch Microbiol (1991) 157:23-28

Antibodies raised in mice against the three enzymes were found to be highly specific and no crossreactivity with other proteins present in culture filtrates was observed. A mixture of these antibodies has been used to analyze specific induction of these individual enzymes on simple and complex substrates by Western blotting.

INTRODUCTION

The main component of plant biomass is cellulose which occurs as microfibrils embedded in pectic and hemicellulolytic material. Hemicelluloses (xylans, arabinans, galactans, glucans and mannans) rank next to cellulose as the second most abundant group of renewable polysaccharides in agricultural biomass. In the processing of agricultural products like fruits, vegetables and cereals (Voragen et al. 1982; Siliha 1985), hemicellulosic substances play an important role and their enzymatic modification is of technological relevance.

Commercial enzyme preparations used for this purpose are usually made by fungi such as *Aspergillus niger* and contain a large number of different enzyme activities. Some are only present in small amounts in these commercial preparations although thought to be important in particular industrial processes. We focus here on one such class - the arabinases. L-arabinose residues are widely distributed in structural polysaccharides (arabinans, arabinogalactans and arabinoxylans) and in glycoconjugates (Stephen 1983; Kaji 1984) and linear arabinans can cause haze formation in apple juice preparation. (Voragen et al. 1982).

Arabinan-degrading enzymes have been isolated from a number of sources including fungi and bacteria. Some species that produce arabinan-

degrading activities are *A. niger*; (Kaji and Tagawa 1970; Waibel et al. 1980); *Streptomyces purpurascens* (Komae et al. 1982) (α-L-arabinofuranosidase (EC 3.2.1.55)); *Bacillus subtilis* and other *Bacillus* strains (Weinstein and Albersheim 1979; Yoshikara and Kaji 1983; Kaji and Saheki 1975) and certain *Clostridium* spp. (Kaji 1984) (endo-1,5-α-L-arabinase (EC 3.2.1.99)). The only arabinan-degrading enzyme purified from the filtrate of a defined *A. niger* culture was an α-L-arabinofuranosidase (Kaji and Tagawa 1970).

Recently three enzymes with arabinan-degrading activity were purified from a commercial *A. niger* enzyme preparation (Rombouts et al. 1988).

In this paper, growth conditions are presented which induce these three arabinases in a genetically well defined *A. niger* strain and the purification and characterisation of the enzymes is given.

MATERIALS AND METHODS

Materials

Sugar beet pulp was a gift from CSM (Breda, The Netherlands). All chromatography media used were supplied by Pharmacia (Uppsala, Sweden). All chemicals used were of p.a. quality and obtained from Merck (Darmstadt, FRG) except for the Gum arabic, Gum ghatti, Gum tragacanth, Gum storax, Gum guaiac, Locust bean gum and PNPA which was obtained from Sigma (St. Louis, Mo., USA). Alkaline phosphatase labelled goat anti-mouse IgG was obtained from Bio-Rad (Richmond, Calif., USA).

Strain and growth condition

The *Aspergillus* strain used was *Aspergillus niger* N400 (CBS 120-49). The strain was grown on media which contained per litre: 5 g Neopepton; 2 g NH_4NO_3; 1 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$ and trace metals as described before (Witteveen et al. 1989). The pH was set to 5.5 with HCl. Carbon sources were added to a concentration of 1% (w/v). For the induction experiments growth was carried out in 100 ml Erlenmeyer

flasks containing 30 ml medium in a waterbath shaker for a maximum of 72 h at 30°C. For enzyme purification *A. niger* N400 was grown for 48 h at 30°C in a 10 litre flask using 8 litre medium; sugar beet pulp (1% w/v) was added as the carbon source. Aeration took place as previously described using a sparger to distribute the air (Dijkema et al. 1985). All media were inoculated with 1 x 10^6 spores/ml.

Determination of enzyme activities

α-L-arabinofuranosidase and endo-arabinase activities were determined as described by Rombouts et al. (1988) with the following modifications. For α-L-arabinofuranosidase activity a McIlvaine buffer pH 3.5 was used and the reaction was stopped with 0.5 ml 0.25 *M* sodium carbonate. One unit is defined as the amount of enzyme which releases 1 μmole *p*-nitrophenol min^{-1} from PNPA. The molar extinction coefficient (16208 M^{-1} cm^{-1}) was calculated using a standard curve for *p*-nitrophenol under the same conditions. Endo-arabinase activity was determined using sugar beet arabinan as a substrate. After incubation at 30°C the enzyme activity was determined by measuring the increase in reducing end groups using the Neocuproïne method as described by Stephens et al. (1974). One enzyme unit is defined as the amount of enzyme which produced 1 μmole of reducing end groups per minute. Sugar beet arabinan was prepared according to the procedure described by Hirst and Jones (1948).

Column chromatography

Column chromatography was carried out on DEAE-Sephadex A50, DEAE-Sepharose Fast Flow and Superose 12 and MONO Q HR 5/5 column. All steps in the purification were carried out at 4°C except for those using the FPLC system (Superose 12/MONO Q) which were carried out at room temperature. All buffers contained 0.02% sodium azide. Details of the purification are given under 'Results'.

Protein determination

Protein concentrations were measured using the commercial BCA protein assay reagent (Sigma) according to the supplier's instructions using bovine serum albumin as a standard.

Electrophoresis and isoelectric focusing

The purification procedure was monitored by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS according to Laemmli (1970) on a midget gel electrophoresis system (LKB). Protein molecular masses were determined by SDS-PAGE (10%) with the

following protein standards: carbonic anhydrase (29000 Da), ovalbumin (45000 Da), bovine serum albumin (68000 Da) and phosphorylase B (92500 Da). The pI of each purified native enzyme was determined by isoelectric focusing in the pH range 2.5-7 using a FBE-3000 apparatus (Pharmacia) and by subsequent staining with Coomassie Brilliant Blue. The pI calibration kit from Pharmacia (broad range) was used as a standard.

pH and temperature optima and kinetic data

α-L-arabinofuranosidase activity and endo-arabinase activity were measured as described above. A McIlvaine buffer was used in the pH range 2.5-8.0. Enzyme activity was measured at 30°C. Temperature optima were determined at the pH optima of each of the different enzymes. The temperature ranged from 25 to 75°C. Kinetic experiments were carried out at 30°C at the pH optima of each of the different enzymes.

Antibodies and Western blotting

Antibodies against the pure enzymes were raised in Balb/c mice as follows: equal volumes of filter sterilised phosphate buffered saline (PBS) and Freund's complete adjuvant (Difco, Detroit, USA) were vortexed for 4 h at room temperature. Desalted, lyophilised purified protein (50 μg) was dispersed in approx. 300 μl of adjuvant emulsion. This mixture was vortexed and immediately injected intraperitoneally into a mouse. Seven weeks after immunisation the mice were boostered by intraperitoneal injection of an emulsion of PBS and incomplete adjuvant (Difco) containing 25 μg of purified protein per mouse. After seven days, blood samples were taken and serum recovered. Six weeks after sampling, the mice were again boostered and seven days later they were bled. Antisera were stored at -70°C.

Crossreactivity between the three antisera and the three enzymes was tested by Western blotting. Incubation of nitrocellulose blots with the specific antisera followed by staining with alkaline phosphatase labelled goat anti-mouse IgG was done as described by the manufacturer (Bio-Rad).

Amino acid composition

Purified enzymes were reduced according to the procedure described by Amons (1987) followed by gas-phase hydrolysis with 6 N hydrochloric acid and 1% (v/v) redistilled phenol for 24 h at 110°C. Derivatization of amino acids was done according to Bidlingmeyer et al. (1984). PTC amino acids were analyzed on a SP 8000 HPLC (Spectra

Physics, San José, Calif., USA) using the separation conditions described by Janssen et al. (1986).

RESULTS

Induction of arabinases

In order to find a substrate which induces all three known arabinan-degrading enzymes (Rombouts et al. 1988) experiments were carried out using different carbon sources. These are listed in Table 1. As the arabinase complex releases monomeric sugars from polymeric substrates it is possible that the monomeric sugars so produced may induce arabinase activity. The complex carbon sources listed all contain arabinose residues in different amounts. After growth for 24 and 72 h samples of the culture broth were taken, filtered and the PNPA hydrolysing activities in the filtrate were measured (Table 1). On D-arabinose, arabinogalactan, Gum guaiac, Gum storax and Locust bean gum *Aspergillus niger* showed very poor growth resulting in complete absence or in very low amounts of arabinase activity.

Activities are all expressed in U/ml because of the difficulty in determining the total biomass or total fungal protein content of those cultures in which solids were used as complex carbon sources.

As we cannot distinguish between the activities of α-L-arabinofuranosidase A and α-L-arabinofuranosidase B on PNPA, media showing high activities on this substrate have been partially fractionated. The media (sugar beet pulp, apple pulp, L-arabinose) were dialysed against 0.02 M piperazine buffer pH 5.0 and loaded onto a MONO Q column. Elution was

Table 1. Arabinase activity on PNPA of *Aspergillus niger* culture filtrate after growth on different carbon sources. Activities are expressed in units per ml culture filtrate.

	Activity	
Carbon source	24 h	72 h
D-Xylose	0.003	0.038
L-Arabinose	0.024	0.316
D-Arabinose	0.002	0.011
D-Fructose	0.002	0.011
Glucose	0.001	0.039
Sorbitol	0.001	0.012
Pectin	0.020	0.251
Polygalacturonate	0.006	0.053
Arabinogalactan	0.022	0.208
Wheat bran	0.029	0.141
Millet	0.004	0.042
Sugar beet pulp	0.407	0.662
Apple pulp	0.577	0.632
Gum arabic	0.008	0.161
Gum ghatti	0.094	0.528
Gum tragacanth	0.127	0.329
Locust bean gum	0.064	0.113
Gum storax	0.001	0.009
Gum guaiac	0.002	0.013

performed with a 30 ml linear sodium chloride gradient (0-0.5 M) in the same buffer. Fractions of 1 ml were collected and examined for activity on sugar beet arabinan and PNPA. From this analysis sugar beet pulp was found to be a good inducer of all three known arabinan-degrading enzymes. As can be seen in Fig. 1 there are three peaks of enzyme activity. One peak (A) is only active on PNPA (*α*-L-arabinofuranosidase A), one peak (C) is only active on sugar beet arabinan (endo-arabinase) and one (B) is active on both substrates (*α*-L-arabinofuranosidase B). As the total content of arabinose residues in arabinan extracted from sugar beet pulp

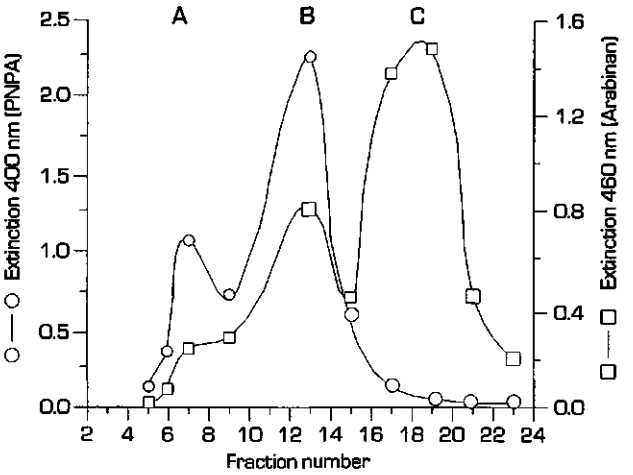


Fig. 1. Fractionation of the three arabinase activities from the culture filtrate of *Aspergillus niger* grown on sugar beet pulp as the carbon source by chromatography on MONO Q. A α-L-arabinofuranosidase A; B α-L-arabinofuranosidase B; C endo-arabinase; ○ activity on PNPA; □ activity on sugar beet arabinan.

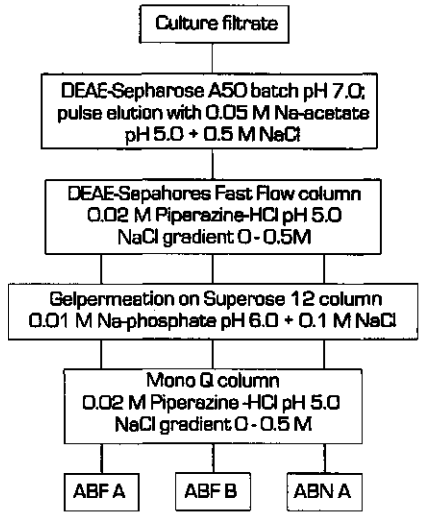
is very high (88.5 % (w/w) of the total sugar content according to Rombouts et al. (1988)), sugar beet pulp was chosen as the carbon source to cultivate *A. niger*.

Purification of arabinan degrading enzymes from culture filtrate

A. niger was grown for 48 h on sugar beet pulp. The culture broth (8 l) was filtered and the resulting supernatant brought to pH 7.0 with 0.1 N NaOH. The purification procedure is shown in Scheme 1.

DEAE-Sephadex A-50 (8 g dry weight) was equilibrated with 20 mM sodium phosphate buffer pH 7.0 and poured into a glass filter holder (12 cm diameter). Culture filtrate was passed through this DEAE-Sephadex A-50 column, which retained all the arabinase activities.The column was

then washed with sodium acetate buffer pH 5.0 and arabinase activity was pulse eluted with 200 ml sodium acetate buffer pH 5.0 + 0.5 M NaCl. Fractions containing enzyme activity were pooled and dialysed against 0.02 M Piperazine-HCl buffer pH 5.0 and loaded onto a DEAE-Sepharose Fast Flow column (14 x 2,7 cm) equilibrated in 0.02 M Piperazine-HCl buffer pH 5.0. Elution was performed with a 500 ml linear sodium chloride gradient (0-0.5 M) resulting in three distinct partially overlapping activity peaks. By measuring enzyme activities on PNPA and sugar beet arabinan it was found that pool I contained α-L-arabinofuranosidase A, pool II contained α-L-arabinofuranosidase B whereas pool III contained endo-arabinase. The three pools were subsequently dialysed against 0.02M Piperazine-HCl buffer pH 5.0, loaded onto a small DEAE-Sepharose Fast Flow column (7 x 1.6 cm) and eluted with a 100 ml linear sodium chloride gradient (0-0.5 M) in the same buffer. All three enzymes were then dialysed against 'Millipore' water and concentrated to a final volume of 1-2 ml in a Speed Vac. The three enzyme preparations were subsequently individually loaded onto a Superose 12 GPC column (56 x 2.7 cm) which was equilibrated with 0.01 M sodium phosphate pH 6.0. Final purification was achieved by repeating chromatography on a MONO Q column. On SDS-PAGE the three



Scheme 1. Flow sheet of the purification of three arabinases from *Aspergillus niger* culture filtrate after growth on sugar beet pulp as the carbon source.

Table 2. Physio-chemical and kinetic properties of the three arabinases from *Aspergillus niger* N400 culture filtrate.

	α-L-arabinofuranosidase		endo-arabinase
	A	B	
Mr (SDS-PAGE) (Da)	83000	67000	43000
Isoelectric point	3.3	3.5	3.0
pH optimum	3.4	3.8	4.6
Temperature optimum (°C)	46	56	51
Kinetic parameters:			
on PNPA			
K_m (x 10^{-4} M)	6.8	5.2	
V_{max} (I.U.)	33	23.5	
on sugar beet arabinan			
K_m (g/l)		0.24	3.7
V_{max} (I.U.)		3.5	0.16

enzymes resolve as single bands and hence appear to be pure.

Physio-chemical and kinetic properties

The physio-chemical properties of all three purified enzymes are listed in Table 2. On an isoelectric focusing gel the three enzymes each appear as a single band. The isoelectric points are all very low (viz. 3.3, 3.5 and 3.0 for α-L-arabinofuranosidase A, α-L-arabinofuranosidase B and endo-arabinase resp.). That single bands are observed on SDS-PAGE and I.E.F gels we may conclude that the three enzymes are pure. Isoelectric focusing gel suggested an elution order from the MONO Q of α-L-arabino-furanosidase B (I.E.P. 3.5), α-L-arabinofuranosidase A (I.E.P. 3.3) and endo-arabinase (I.E.P. 3.0). However, the first two enzymes eluted in reverse order which maybe due to an uneven distribution of the surface

Table 3. Amino acid composition of three *Aspergillus niger* arabinases.

	Number of residues[a]		
	α-L-arabinofuranosidase		endo-arabinase
Amino acid	A	B	
Aspartic acid[b]	102	64	38
Glutamic acid[b]	105	61	32
Serine	102	83	63
Glycine	89	94	54
Histidine	9	8	5
Arginine	19	18	7
Threonine	65	70	33
Alanine	67	70	33
Proline	37	34	20
Tyrosine	33	34	30
Valine	36	32	21
Methionine	9	8	7
Cysteine[c]	3	6	3
Isoleucine	28	21	17
Leucine	54	34	21
Phenylalanine	21	20	11
Tryptophan	nd	nd	nd
Lysine	16	11	6
Total number	795	668	403

Notes:

[a] Based on Mr values of 83000, 67000 and 43000.

[b] Sum of acid and amide forms

[c] The number of cysteine residues was determined for the reduced and S-carboxymethylated protein

nd Not determined

charge of these enzymes.

The K_m and V_{max} values of the three enzymes were measured on their preferred substrates (Table 2). *α*-L-arabinofuranosidase A has higher K_m and V_{max} values on the synthetic substrate (PNPA) than *α*-L-arabinofuranosidase B. The K_m value of *α*-L-arabinofuranosidase B on sugar beet

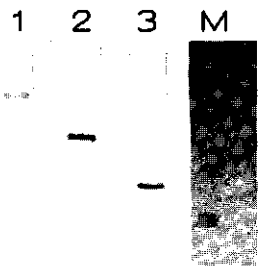


Fig. 3. Western blot of the three purified arabinases. Each lane contains a mixture of the three enzymes. The lanes were separately incubated with the individual antibodies prepared against each of the three arabinases.
Lane 1 anti-*α*-L-arabinofuranosidase A; lane 2 anti-*α*-L-arabinofuranosidase B; lane 3 anti-endo-arabinases; M molecular weight markers (92500, 68000, 45000 and 29000 Da)

arabinan is also very low if compared with that of endo-arabinase but the V_{max} value of *α*-L-arabinofuranosidase B is much higher on this substrate.

The pH optimum of all three enzymes is quite broad but the use of narrow pH intervals results in the pH optima reported in Table 2.

The amino acid compositions of the three purified arabinases are summarized in Table 3. These were determined after a standard hydrolysis time of 24 h. Besides having a high content of acidic residues, all three enzymes have a rather low cysteine content.

Immunological relationships between arabinan-degrading enzymes

The immunological properties of all three purified arabinases have been examined on Western blots. All the antisera proved to be highly specific for the enzyme they were raised against when tested at a 1:3,000 dilution. No cross-reactivity was found between the three enzymes indicating that they are quite distinct proteins (Fig. 3).

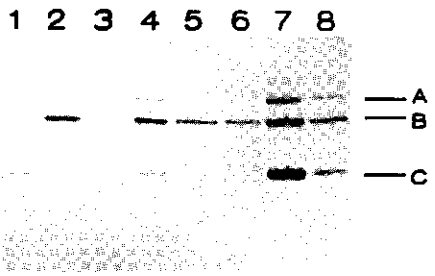


Fig. 4. Western blot of *Aspergillus niger* culture filtrates obtained after growth on different carbon sources. The blot was incubated with a mixture of antibodies against the three arabinases.
1. Arabinogalactan; 2. L-Arabinose; 3. Pectin; 4. Gum ghatti; 5. Wheat bran; 6. Gum tragacanth, 7. Sugar beet pulp; 8. Apple pulp.
A. α-L-arabinofuranosidase A; B. α-L-arabinofuranosidase B; C. endo-arabinase.

Analysis of induction patterns by Western blotting

Western blotting with the specific antibodies resulted in a fast analysis of the induction patterns of the arabinases on different substrates. From the culture filtrates showing high enzymic activity on PNPA (Table 1) an immunoblot was made (Fig. 4). This immunoblot was incubated with a mixture of the three antibodies.

All culture filtrates on the blot showed α-L-arabinofuranosidase B protein present in different amounts. The monomeric sugar arabinose, pectin, wheat bran and Gum tragacanth only showed induction of α-L-arabinofuranosidase B protein. Arabinogalactan, on which *A. niger* grows very poorly, led to induction of α-L-arabinofuranosidase B and endo-arabinase in low amounts. The other culture filtrates showed the presence of all three arabinases. Sugar beet pulp had the highest amount of the three arabinases. Thus, Western blotting confirmed the conclusions derived from the screening of the culture filtrates on FPLC MONO Q in combination with enzyme measurements.

DISCUSSION

It is apparent from Table 1 that there is a large number of substrates which can induce arabinase activities to different degrees. These range from very complex substrates containing arabinose such as sugar beet pulp to simple substrates such as L-arabinose itself.

After partial purification of culture filtrates of *Aspergillus niger* it was demonstrated that sugar beet pulp induces all three known arabinases to relatively high levels (see also Figs. 1 and 4). Apple pulp induces the three arabinases in lower amounts whereas L-arabinose only induces α-L-arabinofuranosidase B.

Comparison of the properties of the enzymes purified from the commercial enzyme preparation 'Pectinase 29' (Rombouts et al. 1988) with those of the enzymes purified from the culture filtrate of *A. niger* indicates differences in molecular weight and pI. In contrast to the molecular weights determined by Rombouts et al. (1988) (128000 Da for α-L-arabinofuranosidase A, 60000 Da for α-L-arabinofuranosidase B and 35000 Da for endo-arabinase) we find these to be 83000 Da, 67000 Da and 43000 Da respectively. However, direct comparison by SDS-PAGE of the enzymes isolated from the commercial enzyme preparation with those purified from culture filtrate revealed no differences in migration between them. The values reported previously appear to arise from the use of another gel system (Pharmacia Fast System) which leads to wrong estimates.

Furthermore, the isoelectric points found in this study are different from those of the enzymes found in the commercial enzyme preparation (viz. 3.3 vs. 6-6.5 for α-L-arabinofuranosidase A, 3.5 vs. 5.5-6.0 for α-L-arabinofuranosidase B and 3.0 vs. 4.5-5.5 for endo-arabinase). The

migration problems described by Rombouts et al. (1988) were not encountered in this study for the enzymes directly purified from culture filtrate. These enzymes reached their final positions within 1.5 h.

Comparison of the K_m values on PNPA of α-L-arabinofuranosidases A and B from the commercial enzyme preparation (resp. 6 and 4.8×10^{-4} M) with the K_m values found for the enzymes purified from the culture filtrate (resp. 6.8 and 5.2×10^{-4} M) reveal no actual differences.

The K_m of α-L-arabinofuranosidase B found on sugar beet arabinan is in the same range as found by Kaji and Tagawa (1970) (0.24 against 0.26 g/l). Also the isoelectric point of α-L-arabinofuranosidase B (pI 3.5) is in the same range as the isoelectric point found by Kaji and Tagawa (1970) for their α-L-arabinofuranosidase (pI 3.6). These data gives an indication that the enzymes are either the same or very similar.

Comparison between the endo-arabinase cannot be made because of the use of different substrates. Even between various batches of substrate differences are found. Nevertheless, the optimum pH (5.0) and optimum temperature (50°C) of the commercial enzyme lies within the same range.

The low pI values of the enzymes can also be understood in view of the amino acid compositions which show large numbers of negatively charged residues (Asp, Glu) and low numbers of positively charged residues (Lys, Arg, His).

Finally, the specificity of the antibodies prepared against the three enzymes provided a better tool to screen different media for their inducing properties, and to analyze their individual induction features as well as to screen other *Aspergillus* strains for the presence of arabinases.

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Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*

CHAPTER 3

INDUCTION OF EXTRACELLULAR ARABINASES ON MONOMERIC SUBSTRATES IN *ASPERGILLUS NIGER*[2]

Peter van der Veen, Michel J.A. Flipphi, Alphons G.J. Voragen and Jaap Visser

SUMMARY

The induction of extracellular arabinases by pentose sugars and polyols generated by the metabolic pathway of L-arabinose and D-xylose catabolism in *Aspergillus niger* was investigated. Induction occurred with L-arabinose and L-arabitol but not with D-xylose or xylitol. L-arabitol in particular was found to be a good inducer for α-L-arabinofuranosidase and endo-arabinase activities. Western blotting analysis showed both α-L-arabinofuranosidase A and B to be present. No induction was observed using D-arabitol. Unlike the wild type *A. niger* N402 strain, the *A. niger* xylulose kinase negative mutant N572 also showed induction of α-L-arabinofuranosidases A and B and endo-arabinase activity on D-xylose and xylitol. This is due to metabolic conversion of these compounds leading to the accumulation of both xylitol and L-arabitol in this mutant, the latter of which then acts as inducer. The induction of the two α-L-arabinofuranosidases and endo-arabinase is under the control of two regulatory systems namely pathway specific induction and carbon catabolite repression. Under derepressing conditions in the wild type only α-L-arabinofuranosi-

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dase B could be detected by Western blotting analysis. This indicates that *α*-L-arabinofuranosidase B is of importance in the initiation of specific induction of the various arabinase activities in *A. niger* grown on arabinose containing structural polysaccharides.

INTRODUCTION

The induction mechanism of extracellular polysaccharide degrading enzymes and the nature of the inducers involved has been widely studied in bacteria, for instance in bacilli (Martin et al. 1989) and clostridia (Schink and Zeikus 1983) and to a lesser extent in the fungi. Large polysaccharide structures, often complex in their chemical and/or physical composition, normally lead to a variety of different enzyme activities necessary for the degradation of these substances to oligomers and finally to monomers. Often, one or several of the degradation products of these polymer substrates is involved in the induction mechanism of these enzymes.

In 1962 Mandels et al. found that the disaccharide sophorose (ß-1,2-glucobiose) was able to induce the whole cellulase system in *Trichoderma* (cellobiohydrolase I and II, endo-glucanase). Although sophorose is converted very rapidly by a membrane bound ß-glucosidase, a very small amount enters the cell by an active uptake system (Kubicek et al. 1990). This small amount is thought to induce the cellulase complex. It was also found that oligosaccharides of cellulose can induce the cellulase complex but to a lesser extent than sophorose (Wang et al. 1988). For *T. reesei* it is clear that one of the enzymes of the cellulase system (ß-glucosidase) is present constitutively (Umile and Kubicek 1988) and is located in the cell

membrane.

For the induction of the pectate lyases in *Erwinia chrysanthemi* it is known that an inducer of these enzymes is an intermediate of the degradation pathway of pectin (Collmer and Bateman 1981; Condemine et al. 1986), namely 2-keto,3-deoxygluconate (KDG).

Not much is known about the induction of arabinases in *Aspergillus niger*. It has been reported that sugar beet pulp is a good inducer (Kaji and Tagawa 1970; v. d. Veen et al. 1991) but other complex substrates with a high content of arabinose residues also induce one or more arabinases (v. d. Veen et al. 1991).

The arabinase complex releases monomeric L-arabinose from polymer substrates containing L-arabinose (Rombouts et al. 1988). The monomeric sugar is taken up by *A. niger* and converted in several steps to D-xylulose-5-phosphate which enters the pentose phosphate shunt (L-arabinose → L-arabitol → L-xylulose → xylitol → D-xylulose → D-xylulose-5-phosphate → pentose phosphate pathway; D-xylose → xylitol → D-xylulose → D-xylulose-5-phosphate → pentose phosphate pathway)(Witteveen et al. 1989).

It has been shown previously that growth of *A. niger* on L-arabinose induces arabinase activity (Tagawa and Terui 1968, v.d. Veen et al. 1991). This activity was due to the presence of α-L-arabinofuranosidase B (v.d. Veen et al. 1991).

The relevance of L-arabinose itself and its intermediates in the degradation route of pentoses for the induction process have been further investigated.

MATERIALS AND METHODS

Materials

All chemicals used were of p.a. quality and obtained from Merck (Darmstadt, FRG) except for PNPA which was obtained from Sigma (St. Louis, Mo., USA). Novozyme 71 was obtained from Novo Nordisk (Bagsvaerd, Denmark). Alkaline phosphatase labelled goat anti-mouse IgG was obtained from Bio-Rad (Richmond, Calif, USA).

Strains and growth conditions

The *Aspergillus* strains used were *Aspergillus niger* N402 and *A. niger* N572 which were derived from the parental strain N400 (CBS 120-49). The strain N402 is a morphological mutant with short conidiophores (*csp*A1) (Bos 1987). The xylulose kinase negative mutant strain N572 (*csp*A1, *nic*A1, *xki*A1) is an auxotrophic mutant with a nicotinamide deficiency (Witteveen et al. 1989). The strains were grown on minimal medium which contained per litre: 6 g $NaNO_3$; 1.5 g KH_2PO_4; 0.5 g KCl; 0.5 g $MgSO_4.7H_2O$ and trace metals as described before (Witteveen et al. 1989). The pH was set to 5.5 with HCl. The carbon sources were added in a concentration of 1 % (w/v). For the N572 strain nicotinamide was added at a final concentration of 1 mg/ml. For the induction experiments, growth was carried out in 100 ml Erlenmeyer flasks containing 30 ml medium under conditions described previously (v.d. Veen et al. 1991). In transfer experiments, mycelium was grown in 1 l Erlenmeyer flasks for 24 h containing 250 ml medium using 1% (w/v) D-glucose as carbon source. After harvesting the mycelium was washed with saline and aliquots of 0.75 g mycelium (wet weight) were transferred to 100 ml Erlenmeyer flasks.

Preparation of cell-free extracts and protein determination

The preparation of cell-free extracts was carried out as described by Witteveen et al. (1989). Protein concentrations were measured as described before (v.d. Veen et al. 1991) using the commercial BCA protein assay reagent (Sigma) according to the supplier's instructions using bovine serum albumin as a standard.

Preparation of protoplasts

Protoplasts of *A. niger* N402 mycelium induced on L-arabitol were made as described by Kusters-van Someren et al. (1991) using STC buffer (1.33 M sorbitol, 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$) as an osmotic stabilizer.

Determination of enzyme activities and glucose concentration

α-L-Arabinofuranosidase and endo-arabinase activities were determined as described before (v.d. Veen et al. 1991). The activities of L-arabinose reductase, D-xylose reductase, L-arabitol dehydrogenase and xylitol dehydrogenase were measured as described by Witteveen et al. (1989). The glucose concentration of culture filtrate was determined using a diagnostic kit of Sigma.

Electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis and Western blotting were carried out as described previously (v.d. Veen et al. 1991).

RESULTS

Induction of PNPA activity by L-arabinose and L-arabitol

Mycelia of *Aspergillus niger* N402 and N572 were grown on 1% (w/v) D-glucose in 100 ml Erlenmeyer flasks for 24 h at 30 °C. Different carbon sources were added to a final concentration of 1% (w/v), including D-glucose as a control, to test their ability to induce PNPA activity. After additional growth periods of 24 h and 48 h, samples were taken and extracellular α-L-arabinofuranosidase activity on PNPA was measured (Table 1).

From the results obtained it is obvious that an induction of PNPA hydrolysing activities on L-arabinose and L-arabitol occurs in the wild type *A. niger* N402. Whereas D-xylose and xylitol show little or no induction of arabinase activity, L-arabitol shows higher levels of induction.

In comparison to the wild type, the D-xylulose kinase negative mutant N572 showed higher levels of induction with L-arabitol and particularly with L-arabinose. Moreover, substantial induction of arabinase activity was found in the presence of D-xylose and xylitol. Witteveen et al. (1989)

Table 1. Arabinase activities on PNPA of *Aspergillus niger* N402 and N572 after induction on different monomeric carbon sources. Activities are expressed in mU/ml culture filtrate.

Carbon source	PNPA Activity			
	Strain N402		Strain N572	
	24 h	48 h	24 h	48 h
L-arabinose	56	213	278	486
L-arabitol	201	482	321	534
D-xylose	35	52	287	407
Xylitol	55	65	138	181
D-glucose	19	46	19	36

have noted that this mutant, when transferred to D-xylose and L-arabinose, intracellularly accumulates the two pathway related pentitols xylitol and L-arabitol. It thus seems that as a result of this higher internal level of arabitol and xylitol, induction of arabinases exceed the level reached in the wild type strain. Also D-xylose and xylitol give rise to an intracellular accumulation of L-arabitol which then leads to an induction of arabinase activity in this strain.

When glucose was added in the control experiment, very little arabinase activity was found in the culture filtrate (19 mU/ml after 24 h; 46 mU/ml after 48 h). Western blotting showed that this activity was due to the presence of a small amount of α-L-arabinofuranosidase B, as mentioned before (v.d. Veen et al. 1991).

To exclude the possibility of repression due to low levels of glucose present in the media, mycelium was first grown on D-glucose and then harvested, washed with saline and subsequently transferred to fresh media containing different carbon sources at a concentration of 1 % (w/v). The 100 ml Erlenmeyer flasks used for this purpose were inoculated with 0.75 g of mycelium (wet weight). Cultivation of *A. niger* N402

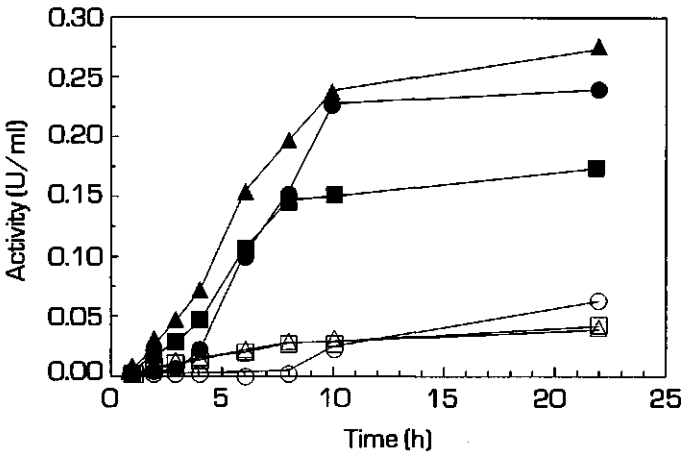

Fig. 1. Induction of arabinase activity in *Aspergillus niger* grown on different monomeric substrates. ■ 1% L-arabinose; ▲ 1% L-arabitol; ▵ 1% D-xylose; □ 1% xylitol; ○ 1% D-glucose; ● 1% L-arabitol + 0.2% D-glucose.

on glucose and transfer of the mycelium to media containing the pentoses and pentitols described above resulted in a maximal induction of arabinase activity within 10 h (Fig. 1). The first activity appeared within 1 h of induction and after 8-10 h the activity levelled off and hardly increased whilst growth was continued for another 12 h. In this case a two-fold higher activity was found on L-arabitol as compared to L-arabinose. On D-xylose and xylitol less activity was found, even after 22 h of induction.

Induction on L-arabitol in the presence of 0.2% glucose resulted in a delay of 4 h in the appearance of PNPA hydrolysing activity. As soon as the glucose level in the medium becomes very low (< 0.05 mg/ml) (Fig. 2) arabinase activity is found and after 10 h of growth the activity is almost as high as in the L-arabitol culture itself. In the control experiment the carbon source (1% D-glucose) is completely utilised after 10 h of growth, at which point a slight increase in arabinase activity is observed

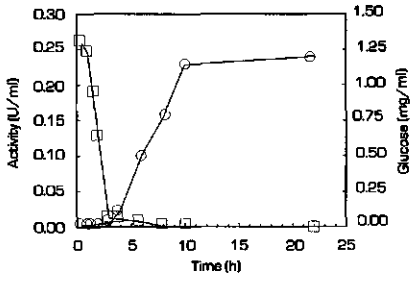

Fig. 2. The effect of glucose on arabinase induction under inducing conditions in *Aspergillus niger* grown on 1% L-arabitol + 0.2% D-glucose. □ amount of glucose in the medium; ○ PNPA activity.

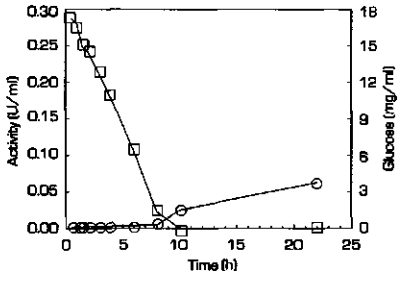

Fig. 3. Relationship between glucose concentration and arabinase activities in the culture filtrate of *Aspergillus niger* grown on 1% D-glucose. □ amount of glucose in the medium; ○ PNPA activity.

(Fig. 3). This phenomenon is most likely due to derepression leading to a basal level of α-L-arabinofuranosidase activity.

When D-arabitol instead of L-arabitol was used in these experiments, no induction was found.

Induction patterns analyzed by Western blotting

From the culture filtrates obtained as described above, Western blots were made and incubated with a mixture of antibodies which were separately raised against purified α-L-arabinofuranosidases A and B and endo-arabinase. The results are given in Fig. 4. When L-arabinose is used as the inducing substrate (Fig. 4A) only α-L-arabinofuranosidase B protein is present whereas the three arabinases are present upon induction by L-arabitol (Fig. 4B). In the latter case α-L-arabinofuranosidase B protein is already present after 1 h of induction. α-L-arabinofuranosidase A and endo-arabinase protein are present shortly after α-L-arabinofuranosidase B (1.5-2 h) though the amount of α-L-arabinofuranosidase A is low com-

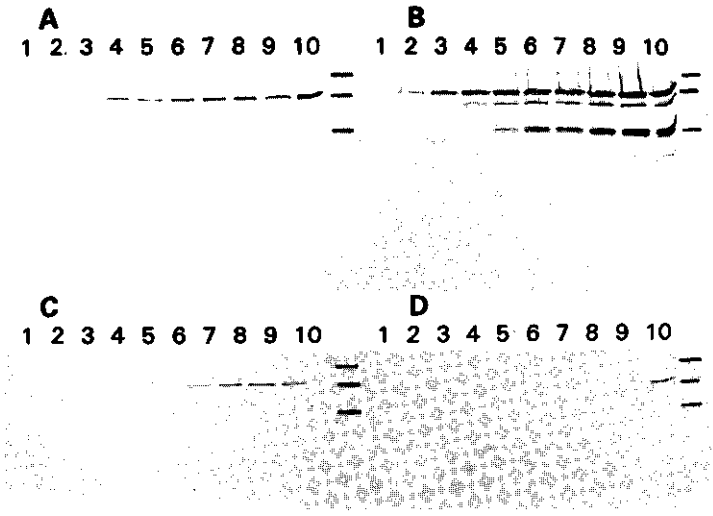

Fig. 4. Western blots of culture media from transferred mycelia, pregrown on D-glucose. The blots were incubated with a mixture of antibodies raised against the three arabinases. Marker lines indicate *α*-L-arabinofuranosidase A, *α*-L-arabinofuranosidase B and endo-arabinase proteins (from top to bottom). Blot A: transferred to L-arabinose (1%); Blot B: transferred to L-arabitol (1%); Blot C: transferred to D-glucose (1%); Blot D: transferred to L-arabitol (1%) + D-glucose (0.2%). Lane 1: 0.5 h; lane 2: 1 h; lane 3: 1.5 h; lane 4: 2 h; lane 5: 3 h; lane 6: 4 h; lane 7: 6 h; lane 8: 8 h; lane 9: 10 h; lane 10: 22 h.

pared to the other two arabinases.

In the control experiment using D-glucose as carbon source it is apparent that when the substrate is exhausted *α*-L-arabinofuranosidase B appears (Fig. 4C; see also Fig. 3). However, this state of derepression does not lead to detectable levels of the other two enzymes.

When analyzing the medium of a culture grown on a mixture of L-arabitol and D-glucose the PNPA activity which appears as the glucose concentration becomes reduced, can also be ascribed to the *α*-L-arabinofuranosidase B protein as detected by Western blotting (Fig 4D).

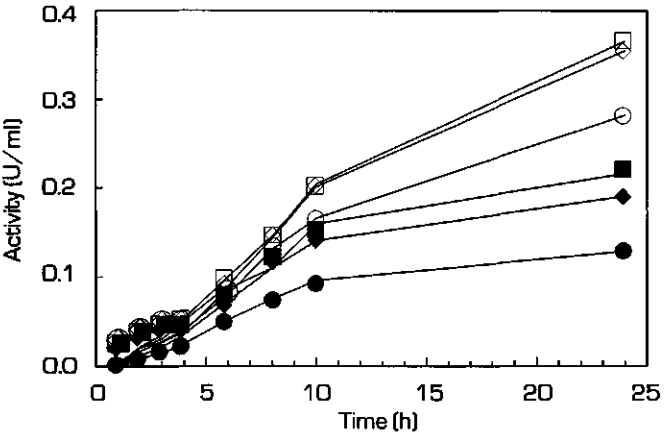

Fig. 5. Induction of arabinases on different amounts of L-arabitol. Concentrations are as follows: □ 65 mM; ◇ 30 mM; ○ 15 mM; ■ 6.5 mM; ● 3 mM; ◆ 1.5 mM

Concentration of inducer

In order to test the concentration range over which L-arabitol is effective as an inducer of PNPA activity, *A. niger* N402 was grown on D-glucose and then transferred to media containing different concentrations of L-arabitol. The concentrations used ranged from 1.5 to 65 mM. PNPA hydrolysing activity was measured at regular intervals over 10 h and the final enzyme levels were determined 24 h after transfer. The results are shown in Fig. 5. Although the enzyme induction levels reached clearly depend on the inducer concentration used, 1.5 mM L-arabitol is still quite effective. There is approximately a 3-fold increase in enzyme level compared to that reached in culture filtrates of derepressed cultures. Increasing the inducer concentration 25 times resulted in an only 2-3 fold difference in enzyme level over a 10 h time span. Since cultures become

Table 2. Intracellular activities of enzymes involved in L-arabinose and D-xylose catabolism. *Aspergillus niger* strains N402 and N572 were pregrown for 24 h on D-glucose and transferred to different carbon sources. Activities are expressed in mU/mg protein.

	Activity							
	L-arabinose reductase		D-xylose reductase		L-arabitol dehydrogenase		Xylitol dehydrogenase	
Carbon source (1% w/v)	N402	N572	N402	N572	N402	N572	N402	N572
D-glucose	22	27	2	9	5	9	2	11
L-arabinose	302	374	189	374	99	192	543	522
L-arabitol	164	183	74	102	160	274	405	451
D-xylose	368	368	577	404	82	231	446	475
Xylitol	55	222	60	255	38	103	93	225

carbon-limited over longer periods after transfer, particularly at low inducer concentrations, a slight increase of this ratio is observed after 24 h.

Induction of the catabolic pathway of L-arabinose

As L-arabinose and particularly L-arabitol effectively induce extracellular arabinase biosynthesis in *A. niger*, the induction of the catabolite pathway of L-arabinose itself was also investigated. Mycelia of *A. niger* N402 and N572 were grown on D-glucose and then transferred for 6 h to different media containing D-glucose, L-arabinose, L-arabitol, D-xylose and xylitol. Mycelia were harvested, washed and cell-free extracts prepared. The activities of different enzymes from the L-arabinose catabolic pathway were measured (Table 2).

In both the wild type strain N402 and the xylulose kinase mutant N572 a pathway-specific induction of all the enzymes involved occurred. On D-glucose only low basal levels for all enzymes tested were measured. The induction level of the first enzyme needed to degrade a particular inducing

carbon source is always higher on this substrate than on the other carbon sources except for xylitol dehydrogenase. This latter enzyme has the lowest value in xylitol-grown mycelium compared to values found for three other inducing carbon sources. As was noted previously by Witteveen and coworkers (1989) the two reductases involved have a broad substrate spectrum. Moreover, D-xylose reductase is also induced by L-arabinose and similarly L-arabinose reductase is induced on D-xylose.
When inducing the wild type on D-xylose and xylitol, L-arabitol dehydrogenase activity is low in comparison with induction on L-arabinose and L-arabitol as carbon sources.

In the xylulose kinase negative mutant N572 induction of the route-specific enzymes is in all cases higher than in the wild type. With xylitol as carbon source all activities are nearly 4 times higher. Also, the activity of the L-arabitol dehydrogenase is 3 to 4 times higher on D-xylose and xylitol whereas in the case of D-xylose the other enzyme levels are the same or only slightly higher in comparison to the wild type.

Witteveen et al. (1989) have shown by ^{13}C NMR spectroscopy that upon transfer of the xylulose kinase negative mutant strain N572 to D-xylose xylitol and L-arabitol accumulate intracellularly unlike the wild type. This accumulation could be responsible for the high induction level of L-arabitol dehydrogenase found in this mutant. The formation of high intracellular amounts of L-arabitol on D-xylose and xylitol leads in this mutant also to an induction of arabinase hydrolysing activities (Table 1), in contrast to the wild type N402.

Localization of arabinase activity

In the cell-free extracts obtained from L-arabitol induced mycelia, PNPA hydrolysing activity could also be detected. When analyzing these sam-

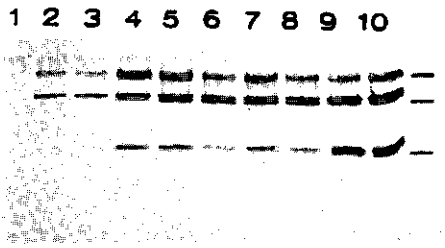

Fig. 6. Western blot of cell-free extract from L-arabitol induced mycelia. The blot was incubated with a mixture of antibodies raised against the three arabinases. Marker lines indicates α-L-arabinofuranosidase A, α-L-arabinofuranosidase B and endo-arabinase proteins (from top to bottom). Lane 1: 0.5 h; lane 2: 1 h; lane 3: 1.5 h; lane 4: 2 h; lane 5: 3 h; lane 6: 4 h; lane 7: 6 h; lane 8: 8 h; lane 9: 10 h; lane 10: 22 h.

ples by Western blotting (Fig. 6) α-L-arabinofuranosidase A and B and endo-arabinase proteins could be detected. After an induction period of half an hour, α-L-arabinofuranosidase A and B proteins were present, whereas after 1.5 h of induction all three arabinases could be detected.

A consequence of making extracts is that it is not possible to distinguish between those enzymes present intracellularly or those bound to the cell wall. Therefore protoplasts were made from L-arabitol induced *A. niger* N402 mycelia. After washing, the protoplasts were disrupted and PNPA hydrolysing activities were measured in the original culture filtrate, in the mycelium extract and in the disrupted protoplasts. No activity was found in the disrupted protoplasts, 27 % of the total activity was associated with the mycelium and therefore probably located in the fungal cell wall and 73 % of the total activity was found in the culture filtrate.

DISCUSSION

There are several arguments in favour of L-arabitol being responsible for the induction of both α-L-arabinofuranosidases A and B and the endo-arabinase in *Aspergillus niger*.

In transfer experiments L-arabitol is more effective than L-arabinose in inducing PNPA activity (Table 1) whereas in wild type *A. niger* both D-xylose and xylitol are ineffective as inducers. With the xylulose kinase negative *A. niger* mutant N572 induction of PNPA activity is also found using D-xylose and xylitol. Witteveen et al. (1989) have shown by ^{13}C NMR spectroscopy that upon transfer of this mutant to D-xylose both xylitol and L-arabitol accumulate intracellularly, unlike the wild type in which only xylitol is found.

The accumulation of L-arabitol in the xylulose kinase negative mutant is also in agreement with an approx. 2.5 to 3 fold increase in L-arabitol dehydrogenase activity (Table 2) resulting in equilibration between the xylitol and L-arabitol metabolite pools.

Western blot analysis indicates differences in the levels of expression of the three different enzymes. Only α-L-arabinofuranosidase B can be detected in derepressed cultures. This is also the most responsive system to induction. When L-arabinose is used with the wild type only α-L-arabinofuranosidase B is detected whereas using L-arabitol all three enzymes are found though in different amounts. The inducer has to fulfil certain stereospecific requirements since D-arabitol fails to induce.
Induction by L-arabitol was found to be influenced by carbon catabolite repression. No PNPA activity was found in the presence of D-glucose.

All these observations suggest that expression of α-L-arabinofuranosidase B may trigger the biosynthesis of the other arabinase activities

since α-L-arabinofuranosidase B is able to split 1-2, 1-3 and 1-5 bonds in polymers containing arabinose residues (Rombouts et al. 1988). The release of L-arabinose into the medium and uptake by *A. niger* will convert the monomeric substrate into L-arabitol and thus induce all three enzymes.

Finally no repression of the three arabinases was observed when using high amounts of L-arabitol in contrast to what was found for L-arabinose by Tagawa and Terui (1968).

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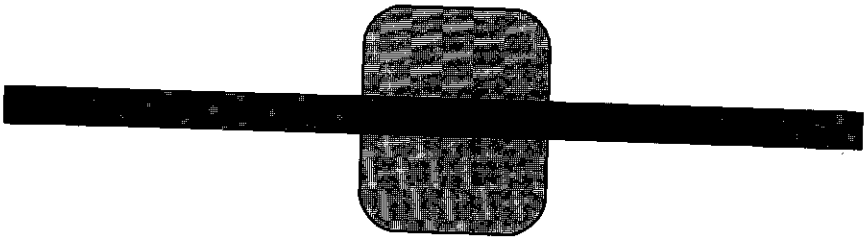

Arabinan degrading enzymes from *Aspergillus nidulans*: Induction and purification

CHAPTER 4

ARABINAN DEGRADING ENZYMES FROM *ASPERGILLUS NIDULANS*: INDUCTION AND PURIFICATION[3]

Daniel Ramón, Peter van der Veen and Jaap Visser

SUMMARY

The presence in *Aspergillus nidulans* of two enzymes related to the *Aspergillus niger* endo-arabinase and α-L-arabinofuranosidase B has been established using antibodies against the purified *A. niger* enzymes. Moreover, the absence of an equivalent in *A. nidulans* to the α-L-arabinofuranosidase A of *A. niger* has been confirmed both at the protein and at the DNA level. Both *A. nidulans* arabinases have been purified and physico-chemically and kinetically characterized. They have a much higher temperature optimum than the corresponding *A. niger* enzymes. The pattern of induction has been studied on media containing different carbon sources showing an important role of L-arabitol in the induction of these enzymes.

INTRODUCTION

Hemicellulose ranks next to cellulose as the second most abundant group of renewable polysaccharides in nature. Hemicellulose is composed

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out of xylans, arabinans, galactans, glucans and mannans, which contribute to the texture of plant tissues as structural components of the cell wall. In the processing of agricultural products like fruits, vegetables and cereals, hemicellulosic substances play an important role and their enzymatic modification is of technological relevance (Siliha 1985; Voragen et al. 1982). Also for the economics of biomass bioconversion to fuels and chemicals, the complete utilization of hemicelluloses is important. Enzymes capable of hydrolysing hemicellulose are usually produced by filamentous fungi such as *Aspergillus niger* or *Trichoderma reesei*. These mixtures contain a large number of different enzyme activities (*i.e.* xylanases, arabinases, galactanases, glucanases or mannanases) but some of these are only present in small amounts.

We are particularly interested in the arabinan-degrading enzymes. This class of hemicellulases has been isolated from a number of bacteria and fungi as *Bacillus subtilis* (Kaji and Saheki 1975; Weinstein and Albersheim 1979), *Erwinia carotovora* (Kaji and Shimokawa 1984), *Streptomyces purpurascens* (Komae et al. 1982), *A. niger* (Kaji and Tagawa 1979; Waibel et al. 1980), *Sclerotinia fructigena* (Fielding and Byrde 1969) or *Sclerotinia sclerotium* (Baker et al. 1979). Recently, we have studied the induction pattern of the arabinan-degrading enzymes of *A. niger* (Veen at al. 1991; Veen et al. 1993). From induced cultures, two α-L-arabinofuranosidases and one endo-arabinase were purified.

When analyzing other arabinan-degrading fungi, we focused our attention particularly on *Aspergillus nidulans*. Since the pioneer work of Pontecorvo et al. (1953), *A. nidulans* has become a model system for studying biological problems in fungi, mainly due to the possibility of sophisticated genetic analysis in combination with the metabolic versatility of this organism. A further advantage of *A. nidulans* is that efficient genetic

manipulation techniques are available. Thus, *A. nidulans* represents an attractive microorganism to study biosynthesis of arabinan-degrading enzymes at the molecular level. In the present work we have taken advantage of the sufficiently close relationship between *A. nidulans* and *A. niger* to first analyze the induction of the *A. nidulans* arabinan-degrading enzymes using antibodies previously raised against the *A. niger* enzymes. Also the purification and characterization of these activities from *A. nidulans* culture filtrates are shown.

MATERIALS AND METHODS

Materials

Sugar beet pulp was a gift from CSM (Breda, The Netherlands). Apple pectin was obtained from Obi-pektin (Bischofszell, Switzerland). All chromatography media used were supplied by Pharmacia (Uppsala, Sweden). All chemicals used were of p.a. quality and obtained from Merck (Darmstadt, Germany) except for Guar gum and *p*-nitrophenyl-*α*-L-arabinofuranoside (PNA) which were supplied by Sigma (St. Louis, MO., USA) and CM-linear 1,5-*α*-L-arabinan (linear 1,5-*α*-L-arabinan carboxymethyl form) which was obtained from Megazyme (Sydney, Australia). Alkaline phosphatase labelled goat anti-mouse IgG was obtained from Bio-Rad (Richmond, CA., USA). Restriction endonucleases were purchased from Promega (Madison, WI., USA). Arabinan was prepared from sugar beet pulp according to the procedure described by Hirst and Jones (14). All proteins for calibrations were obtained from Sigma.

Strains and growth conditions

Aspergillus nidulans WG096 (*paba*A_1, *y*A_2) was used throughout this work. *Aspergillus niger* CECT2088, *Aspergillus oryzae* CECT2094 and *Aspergillus terreus* CECT2663 were obtained from the Spanish Type Culture Collection (CECT) and used as a source of DNA. The *A. nidulans* strain was grown on minimal medium (Pontecorvo et al. 1953). Carbon sources were added to a final concentration of 1 % (w/v). Cultures

were grown in 100 ml Erlenmeyer flasks containing 25 ml medium, and incubated in a waterbath shaker for a maximum of 42 h at 37 °C. All media were inoculated with 10^6 spores/ml.

Determination of enzyme activities

Culture samples were ten times concentrated by evaporation in a Speed Vac centrifuge. Activities were assayed at 37 °C. α-L-arabinofuranosidase (PNAse) activity was determined as described previously (Veen et al. 1991) using PNA as substrate. Endo-arabinase activity was determined using the commercial arabinazyme tablets (Megazyme) following the instructions of the supplier and CM-linear arabinan for kinetic studies. One unit of enzyme was defined as the amount of enzyme required to release 1 μmol min^{-1} of arabinose reducing sugar equivalents from CM-linear 1,5-α-L-arabinan. Protein concentrations were measured using the commercial Bicinchoninic acid protein assay kit (Sigma) and using bovine serum albumin as a standard.

Column chromatography

Column chromatography was carried out on DEAE-Sephadex A-50, Sephacryl S200 and by using MONO Q HR 5/5 or a MONO P HR 5/20 columns. DEAE-Sephadex A-50 (4 g dry weight) was equilibrated with 20 mM sodium phosphate buffer pH 7.0 and poured into a glass filter holder (12 cm diameter). The Sephacryl S200 column was equilibrated with 0.01 M sodium phosphate pH 6.0 + 0.1 M NaCl. To couple crude arabinan to Sepharose CL6B, 10 g of Sepharose were washed with water on a glass filter. Then, 10 ml of 1,4-butanediol diglycidyl ether, 10 ml of 0.6 N NaOH and 20 mg of $NaBH_4$ were added, and the mixture was rotated for 16 h at 25 °C. After this, the Sepharose was washed with water and 100 ml of 0.1 N NaOH, and then, 30 ml of 0.01 N NaOH and 600 mg of crude arabinan were added. After 24 h of rotation at 40 °C, 2 ml of propanolamine were added and rotation was continued for 2 h. The mixture was washed with water, 0.02 M Piperazine pH 5.5, and 0.02 M sodium phosphate buffer pH 7.5 / NaCl 0.5 M. All steps in the purification were carried out at 4 °C except for those using the Pharmacia FPLC system (MONO Q) that were carried out at room temperature. All buffers contained 0.02 % sodium azide.

Electrophoresis and isoelectric focusing

The proteins were monitored by electrophoresis on 10 % polyacrylamide gels containing 0.1 % SDS (Laemmli 1970) using a midget gel electrophoresis system (Pharmacia).

Carbonic anhydrase (29000 Da), ovalbumin (45000 Da), bovine serum albumin (68000 Da) and phosphorylase B (92500 Da) were used as molecular mass standards. The pI of each purified native enzyme was determined by isoelectric focusing in the range from pH 2.5-7.0 using a FBE-3000 apparatus (Pharmacia) and the pI calibration kit (broad range) from the same supplier as a standard.

Optimal pH and temperature; kinetics

Optimal temperature was determined at the optimal pH of each of the different enzymes by measuring over a range from 30 to 75 °C for 10 minutes. α-L-arabinofuranosidase and endo-arabinase activities were measured as described above. McIlvaine buffers were used in the pH range between pH 2.5 and 7.0. They were made by mixing 0.1 M citric acid with 0.2 M Na_2HPO_4 . Sodium acetate buffers (0.05 M) were used in the range from pH 4.0 to 7.0.

Kinetic experiments were carried out in McIlvaine buffer at 37 °C at the optimal pH of each of the different enzymes.

Antibodies and Western blotting

Antibodies against the purified *A. niger* enzymes were obtained previously (Veen et al. 1991). Cross-reactivity between the antisera and the samples were tested by Western blotting. Incubation of nitrocellulose blots with the specific antisera followed by staining with alkaline phosphatase labelled goat anti-mouse IgG was done as described by the manufacturer (BioRad).

DNA manipulations

DNA isolation, restriction analysis, Southern blotting and heterologous hybridization were done as described previously (Ramón et al. 1987). A 1.5 kbp *Pst*I DNA fragment containing the main part of the *A. niger abf*A gene encoding the α-L-arabinofuranosidase A protein was used as a probe (Flipphi et al. 1993). This fragment was labelled with ^{32}P-dCTP using the random priming technique (Feinberg and Vogelstein 1983).

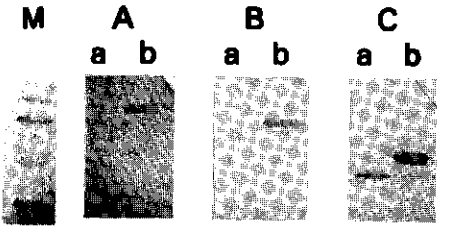

Fig. 1. Western blot of *A. nidulans* culture filtrates obtained after growth on sugar beet pulp medium. The different panels show the reaction with the *A. niger* α-L-arabinofuranosidase A (panel A), α-L-arabinofuranosidase B (panel B) or endo-arabinase (panel C) polyclonal antibodies. Lanes "a" contain in all panels culture filtrates of *A. nidulans*. Lanes "b" contain the purified *A. niger* enzymes α-L-arabinofuranosidase A, 83 kDa (panel A); α-L-arabinofuranosidase B, 67 kDa (panel B) or endo-arabinase, 43 kDa (panel C); M molecular weight markers (92500, 68000, 45000 and 29000 Da).

RESULTS AND DISCUSSION

Production of arabinan degrading enzymes by *A. nidulans*

In order to detect in culture filtrates of *A. nidulans* the presence of proteins related to the previously isolated arabinases from *A. niger*, *A. nidulans* was grown in minimal medium with sugar beet pulp as carbon source. Concentrated samples of the culture media were run on SDS polyacrylamide gels, transferred to nitrocellulose membranes and incubated with specific antisera against the enzymes α-L-arabinofuranosidase A, α-L-arabinofuranosidase B and endo-arabinase from *A. niger* (Veen et al. 1991). The result of this experiment is shown in Fig. 1 and clearly indicates the presence in *A.nidulans* of proteins related to α-L-arabinofuranosidase B and endo-arabinase of *A. niger*. In the case of the α-L-arabinofuranosidase B Western blot analysis (Fig. 1, panel B), two cross-reacting proteins of similar molecular mass (approximately 65 kDa) were detected. These bands showed, after purification, the same isoelectric

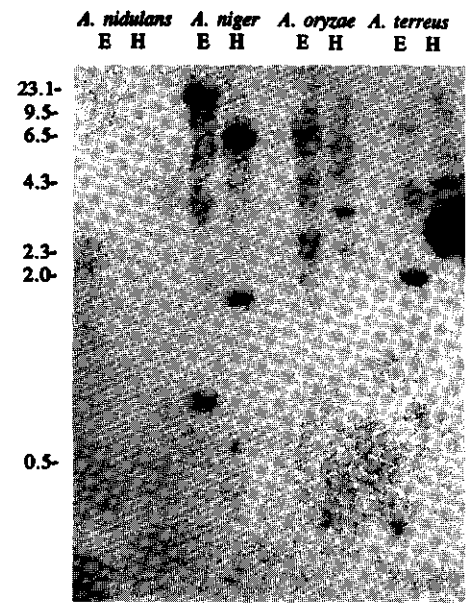

Fig. 2. Southern blot analysis of *Eco*RI (E) or *Hin*dIII (H) digested genomic DNAs from different *Aspergillus* species (*A. nidulans*, *A. niger*, *A. oryzae* and *A. terreus*). Samples of 10 μg of DNA were digested with the restriction enzymes indicated and loaded on a 0.8 % agarose gel. After electrophoresis, DNA fragments were transferred to nylon. The blot was probed with a fragment of the *A. niger* α-L-arabinofuranosidase A gene. The *A. terreus* lane of the *Hin*dII digest contains a ghost spot underneath the 4.3 kb fragment.

point and the same kinetic properties (results not shown). Regarding the endo-arabinase specific Western blot (Fig. 1, panel C), a strong crossreactive band corresponding to a protein with a molecular mass of 40 kDa is apparent. The purified *A. niger* endo-arabinase (43 kDa) in this control contains some degradation products (Fig. 1, Panel C, lane b).

It was not possible to detect any cross-reactivity with the *A. niger* α-L-arabinofuranosidase A antibodies, even using highly concentrated samples (Fig. 1, panel A), or other inducers (see below). To confirm the absence in *A. nidulans* of a protein homologous to the *A. niger* α-L-arabinofuranosidase A, a Southern analysis was carried out using the cloned *A. niger* α-L-arabinofuranosidase A gene as a probe against DNA from *A. nidulans*. The result of this experiment is shown in Fig. 2. The absence in *A. nidulans* of sequences related to the *A. niger abf*A gene is evident. In contrast, homologous signals do appear in other *Aspergillus* species (*A. oryzae* and

Table 1. PNAse and endo-arabinase activities of *A. nidulans* culture filtrates after growth on different carbon sources.

Carbon Source	PNPase activity (mIU/ml)	Endo-arabinase activity (mIU/ml)
Fructose	0	0
Glucose	0.5	0
Lactose	0	0
Apple pulp	16.1	28.4
Arabinogalactan	0	4.0
Guar gum	5.1	6.8
Pectin	0.2	0
Starch	0.2	0
Sugar beet pulp	30.5	48.4
Xylan	0.5	0

A. terreus). We can conclude therefore that in *A. nidulans* a gene encoding a protein similar to the *A. niger* α-L-arabinofuranosidase A lacks.

Induction of the arabinan degrading enzymes of *A. nidulans* by different substrates

To test the induction of arabinan degrading enzymes by *A.nidulans*, the fungus was grown in Erlenmeyer flasks for 24 h in minimal media containing different carbon sources. Then samples of the cultures were taken, filtered and the α-L-arabinofuranosidase and endo-arabinase activities were measured (Table 1). PNAse activity was present in almost all the media tested, but in the case of simple sugars (fructose, glucose and lactose) the detected values were zero or very low. In contrast to this, complex polysaccharides such as those present in apple pulp or sugar

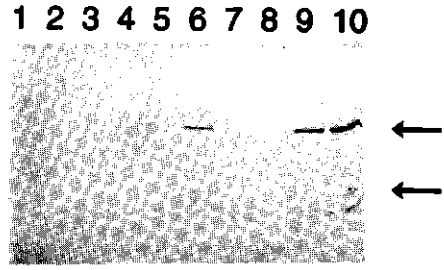

Fig. 3. Western blot of *A. nidulans* culture filtrates obtained after growth on different carbon sources. A mixture of polyclonal antibodies raised against *A. niger* α-L-arabinofuranosidase A, α-L-arabinofuranosidase B and endo-arabinase, were used. Arrows indicate α-L-arabinofuranosidase B (upper arrow) and endo-arabinase (lower arrow). 1. Glucose; 2. Fructose; 3. Lactose; 4. Starch; 5. Arabinogalactan; 6. Guar gum; 7. Xylan; 8. Pectin; 9. Apple pulp; 10. Beet pulp.

beet pulp induced high levels of PNAse activity. It is interesting to note that, in comparison with *A. niger*, *A. nidulans* produced lower amounts of PNAse activity when both fungi were grown under the same culture conditions (407 mIU/ml *vs.* 30.5 mIU/ml for *A. niger* and *A. nidulans* respectively).

No endo-arabinase production was detected in media containing fructose, glucose, lactose, pectin, starch or xylan. As in the case of PNAse activity, apple and sugar beet pulp were the best inducers. It was not possible to compare these results with the production levels of *A. niger* cited in the literature, since the endo-arabinase assays carried out in this microorganism were done using a method based on the release of reducing sugars, which measures both α-L-arabinofuranosidase and endo-arabinase activities (Veen et al. 1991).

To confirm these results, a Western blotting experiment was carried out using concentrated samples of all the culture media and a mixture of the three *A. niger* antibodies (Fig. 3). Surprisingly, it was not possible to correlate the activity values obtained with the presence or absence of both enzymes. Thus, in some of the media containing glucose, pectin, starch and xylan, in which a low PNAse activity was present, the α-L-

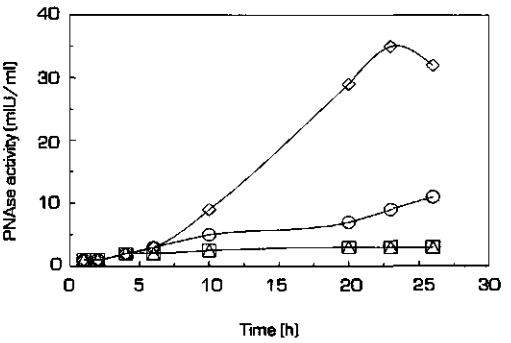

Fig. 4. *A. nidulans* PNAse activity after growth on different inducers. ○ L-arabinose; ◇ L-arabitol; □ D-xylose; ▵ Xylitol.

arabinofuranosidase B cross-reacting protein was not detected. An explanation for this paradox might be the existence in *A. nidulans* of other minor proteins which have PNAse activities, and which differ from the immunologically detected enzymes, or the concentrations of the proteins are too low to detect them even with concentrated samples.

Finally it was possible to compare the results of the Western blotting of culture filtrates of *A. nidulans* with the results of a similar analysis upon induction of the α-L-arabinofuranosidase B and the endo-arabinase of *A. niger* (Veen et al. 1991). The patterns of induction were very similar in both fungi, but there was no induction of the α-L-arabinofuranosidase B cross-reacting band by pectin in *A. nidulans*.

The natural inducer: relationship between the arabinan degrading enzymes and the arabinose catabolic pathway

Polysaccharides present in apple pulp or sugar beet pulp cannot directly act as the natural inducers of the synthesis of the arabinan degrading enzymes. However, some arabinan degradation products may play this role. The final product of arabinan degradation is L-arabinose. This compound can be transported to the cytoplasm of the fungal cell, to become

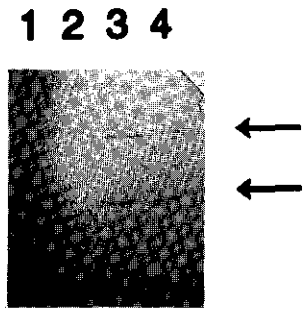

Fig. 5. A Western blot of *A. nidulans* culture filtrates obtained after 22 h of growth on the different media. A mixture of *A. niger* α-L-arabinofuranosidase A, α-L-arabinofuranosidase B and endo-arabinase antibodies were used. Arrows indicate the position of the *A. nidulans* cross-reacting proteins against the *A. niger* α-L-arabinofuranosidase B (upper arrow) or endo-arabinase (lower arrow).
1. D-Xylose; 2. Xylitol; 3. L-Arabinose; 4. L-Arabitol

reduced to L-arabitol. L-arabitol is then further metabolized via L-xylulose, xylitol, D-xylulose and xylulose 5-phosphate, respectively. The latter compound enters into the pentose phosphate pathway (Witteveen et al. 1989). In order to study whether L-arabinose, or other metabolites of the pentose catabolic pathway, act as inducer(s) of the synthesis of the arabinan degrading enzymes, we grew *A. nidulans* in minimal medium containing either L-arabinose, L-arabitol, D-xylose or D-xylitol as carbon sources. As shown in Fig. 4, no PNAse activity was detected when the fungus was grown in D-xylose or D-xylitol. On the other hand, L-arabinose or L-arabitol induced the appearance of PNAse activity. A similar situation has been observed in *A. niger* (Veen et al. 1993). Interestingly, in the case of L-arabitol the fungus produces seven times more activity than with L-arabinose (using 1 % w/v in both cases). Western blotting of these culture samples confirmed the results, indicating that L-arabitol strongly induces the synthesis of two proteins, one of which crossreacts with antibodies raised against the *A. niger* α-L-arabinofuranosidase B and the other with those against the *A. niger* endo-arabinase (Fig. 5). All these results indicate that L-arabitol could play an important role in the induction of the system. To confirm this it is necessary to perform further experi-

ments. For example, by synthesizing and testing inducer analogues or by isolating mutants impaired in the production of L-arabinose reductase or L-arabitol dehydrogenase.

Purification of the enzymes induced by L-arabitol

To purify the *A. nidulans* α-L-arabinofuranosidase B and endo-arabinase, *A. nidulans* was grown in 3 l of minimal medium with glucose as the only carbon source. After 16 h of growth at 37 °C we obtained 50 g of biomass that, after being washed with distilled water, was transferred to minimal medium with L-arabitol as the only carbon source. The culture was then incubated for another 16 h at 37 °C. Under these conditions the fungus produces only a limited number of extracellular proteins, whereas the PNAse and endo-arabinase activities (mU/ml) of the filtrate were only 5 times lower than those obtained with sugar beet pulp medium. In sugar beet pulp medium, however, a large number of other extracellular enzymes was also detected. In contrast, silver staining of the replacement culture filtrate without further concentration revealed the presence of only two bands with molecular weights corresponding with those of the α-L-arabinofuranosidase B and endo-arabinase cross-reacting proteins (results not shown). Taking all these results into account, we chose the strategy of replacement cultures as a preliminary step of purification.

The purification procedure was as follows. The culture broth was filtered and the resulting supernatant was diluted 2 times using distilled water. The pH was then brought to 7.0 with 0.1 N NaOH. Culture filtrate was passed through an equilibrated DEAE-Sephadex A-50 column, which retained all the arabinase activities. The column was then washed with sodium acetate buffer pH 5.0 and arabinase activity was eluted and fractionated with 200 ml sodium acetate buffer pH 5.0 + 0.5 M NaCl.

Table 2. Comparison of physico-chemical and kinetic properties of endo-arabinase and α-L-arabinofuranosidase B from *A. nidulans* and *A. niger*.

	Endo-arabinase		α-L-arabino-furanosidase	
Property	*A. nidulans*	*A. niger*	*A. nidulans*	*A. niger*[a]
Mr (Da)[b]	40000	43000	65000	67000
pI	3.25	3.0	3.3	3.5
Optimal pH	5.5	4.6	4.0	3.8
Optimal temperature (°C)	68	51	65	56
Km (10^{-4} M)[c]	3.36	0.52	6.79	5.2
Vmax (I.U.)[c]	1.53	0.36	9.94	23.5

[a]*A. niger* α-L-arabinofuranosidase B; [b]determined by SDS-PAGE; [c]determined on CM-linear 1,5-α-L-arabinan for endo-arabinase or on PNA for α-L-arabinofuranosidase.

Two different fractions were obtained, one with α-L-arabinofuranosidase activity and one with endo-arabinase activity. The fractions were concentrated and subsequently brought onto a Sephacryl S200 gel permeation. Final purification of endo-arabinase was achieved on a MONO Q column (0.02 M Piperazine-HCl pH 5.0; NaCl gradient from 0 to 0.05 M).

The α-L-arabinofuranosidase containing fraction was also brought first on a Sephacryl S200 column equilibrated with 0.01 M sodium phosphate pH 6.0 + 0.1 M NaCl, and then on a MONO Q column (0.02 M Piperazine-HCl pH 5.0; NaCl gradient from 0 to 0.05 M). These two steps resulted only in a slight purification. Therefore the PNA containing fractions were brought onto a Sepharose affinity column coupled with arabinan. For the last purification step, the α-L-arabinofuranosidase was brought on a chromatofocussing column (MONO P equilibrated with 0.025 M Bis Tris/HCl pH 6.3) and eluted with 10 % Polybuffer 74/HCl pH 3.5. The

two proteins each appeared as one band on SDS-PAGE and are considered to be pure.

The endo-arabinase cross-reactive protein degrades only linear arabinans, but the α-L-arabinofuranosidase B degrades both linear arabinans and PNA. We can conclude from these results that the *A. nidulans* proteins have the same enzymatic activities as the corresponding *A. niger* enzymes. The properties of the purified enzymes of *A. nidulans* are listed in Table 2. Comparison of the physico-chemical parameters of the *A. nidulans* enzymes with the corresponding *A. niger* activities (Veen et al. 1991) indicates differences in molecular weight and optimal pH and temperature. The molecular weights of the two *A. nidulans* enzymes were slightly smaller than of those from *A. niger*. The optimal pH of the *A. nidulans* α-L-arabinofuranosidase was higher than of the *A. niger* α-L-arabinofuranosidase B, but in the range of other fungal α-L-arabinofuranosidases (Fielding and Byrde 1969). The optimal pH for the *A. nidulans* endo-arabinase was slightly higher than that of the *A. niger* enzyme. As far as the temperature optimum of different fungal arabinases is concerned, both *A. nidulans* enzymes have the highest temperature optima described up to date. Finally, the K_m and V_{max} values of the *A. nidulans* enzymes also differ from those described for *A. niger*.

In summary, we can conclude that *A. nidulans* produces in response to the specific induction by L-arabitol, at least two enzymes involved in arabinan degradation. The advantage of the genetics of this fungus converts it into a perfect model to further study the regulation of the synthesis of these enzymes.

Acknowledgements

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Extracellular arabinases in *Aspergillus nidulans*: The effect of different *cre* mutations on enzyme levels

CHAPTER 5

EXTRACELLULAR ARABINASES IN *ASPERGILLUS NIDULANS*: THE EFFECT OF DIFFERENT *CRE* MUTATIONS ON ENZYME LEVELS[4]

Peter van der Veen, Herbert N. Arst, Jr, Michel J.A. Flipphi
and Jaap Visser

SUMMARY

The regulation of the syntheses of two arabinan degrading extracellular enzymes and several intracellular L-arabinose catabolic enzymes was examined in wild type and carbon catabolite derepressed mutants of *Aspergillus nidulans*. α-L-arabinofuranosidase B, endo-arabinase, L-arabinose reductase, L-arabitol dehydrogenase, xylitol dehydrogenase and L-xylulose reductase were all inducible to varying degrees by L-arabinose and L-arabitol and subject to carbon catabolite repression by D-glucose. With the exception of L-xylulose reductase all were clearly under the control of *cre*A, a negative acting wide domain regulatory gene mediating carbon catabolite repression. Measurements of intracellular enzyme activities and of intracellular concentrations of arabitol and xylitol in mycelia grown on D-glucose in the presence of inducer indicated that carbon catabolite repression diminishes but does not prevent uptake of inducer. Mutations in *cre*A resulted in an apparently, in some instances very marked, elevated inducibility, perhaps reflecting an element of 'self' catabolite repression by the inducing substrate. *cre*A mutations also

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resulted in carbon catabolite derepression to varying degrees. The regulatory effects of a mutation in *cre*B and *cre*C, two genes whose roles are unclear but likely to be indirect, were, when observable, more modest. As with previous data showing the effect of *cre*A mutations on structural gene expression, there were striking instances of phenotypic variation amongst *cre*A mutant alleles and this variation followed no discernible pattern, i.e. it was non-hierarchical. This further supports molecular data obtained elsewhere indicating a direct role for *cre*A in regulating structural gene expression and extends the range of activities under *cre*A control.

INTRODUCTION

L-arabinose is a major constituent of a variety of complex structural plant cell wall polysaccharides. In nature hyphal fungi play an important role in the degradation of these complex polysaccharides into monomeric sugars such as L-arabinose, which are then subsequently catabolized. A major form of gene regulation affecting catabolism of L-arabinose and other sugars released from polysaccharides is carbon catabolite repression (Arst et al 1990).

Amongst eukaryotes, carbon catabolite repression has been analyzed in detail in *Saccharomyces cerevisiae* and *Aspergillus nidulans*. In yeast the SUC2 gene encoding invertase and the GAL genes required for the utilisation of galactose have been particularly useful in identifying mutations resulting in altered responses to carbon catabolite repression, as reviewed by Carlson (1987) and Gancedo (1992). MIG1 (Nehlin and Ronne 1990) is one of the few genes involved in carbon catabolite repression in yeast that has been shown to have a direct regulatory

effect on gene expression. Strategies to obtain mutations in *A. nidulans* leading to carbon catabolite derepression have been devised by Arst and Cove (1973) and Bailey and Arst (1975), which resulted in the identification of several *cre* mutant strains. Of the *cre*A gene thus identified, a number of mutant alleles have been selected which, when tested, are recessive to wild type and display non-hierarchical heterogeneity of mutant phenotypes, indicative of a direct role in regulating gene expression (Arst and Bailey 1977). Arst and MacDonald (1975) and Arst and Bailey (1977) postulated the *cre*A product to be a negatively acting wide domain regulator. Dowzer and Kelly (1989;1991) and Drysdale et al. (1993) cloned *cre*A from both *A. nidulans* and *Aspergillus niger* and found it to encode a 'zinc finger' DNA binding protein of the Cys_2 His_2 type having considerable sequence similarity in the DNA binding region to MIG1. A fusion protein containing the 'zinc finger' of *A. nidulans* CREA shows sequence specific binding to DNA and experiments at the molecular level confirm the negative acting nature of this regulatory protein (Kulmburg et al. 1993; Cubero and Scazzocchio 1994). Hynes and Kelly (1977) using a similar selection strategy to Arst and Cove (1973), obtained some additional *cre*A alleles

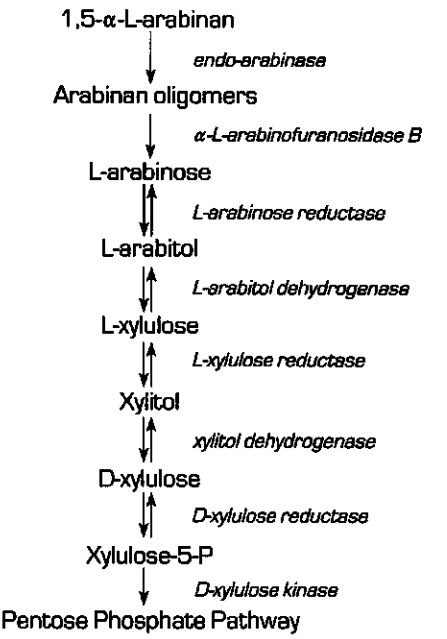

Fig 1 L-arabinose catabolic pathway in *Aspergillus nidulans*. (*cf* Witteveen et al. 1989).

as well as mutations in two other genes, designated *cre*B and *cre*C which probably do not have a direct role in carbon catabolite repression. Mutations in *cre*B had been obtained previously by selection for resistance to molybdate toxicity and designated *mol*B alleles (Arst et al. 1970; Arst and Cove 1970; C.R. Bailey as cited in Arst (1981)). *cre*B (=*mol*B) and *cre*C mutations affect toxicities and/or utilisation of a wide range of compounds and probably play an indirect metabolic role, *e.g.* in affecting intracellular metabolite concentrations and/or their subcellular distribution (Arst 1981). Another *cre* mutation (*cre*-34) has been found as a spontaneous suppressor of the effects of the *cre*C mutation and is thought to encode a protein that interact with the *cre*A gene product (Kelly 1994).

The pathways in which catabolite repression in *A. nidulans* has been studied heretofore concern intracellular enzyme systems and permeases. Less is known about the regulation of carbon-regulated extracellular enzyme systems in *Aspergillus*. However, the regulatory mechanisms involved seem to follow the usual pattern of pathway-specific induction and carbon catabolite repression as observed, for example, with polygalacturonase and pectin esterase in *A. niger* (Maldonado et al. 1989) and cellulase in *A. nidulans* (Bagga et al. 1989). The low molecular weight inducers for these enzymes, however, await identification.

We are particulary interested in the arabinan degrading extracellular enzyme spectra of *A. niger* and *A. nidulans*. From *A. niger* cultures grown on complex carbon sources such as sugar beet pulp, three enzymes have been purified, *viz.* α-L-arabinofuranosidases A and -B and endo-arabinase (Rombouts et al. 1988; v.d. Veen et al. 1991). *A. nidulans* secretes two such enzymes, *viz.* α-L-arabinofuranosidase B and endo-arabinase (Ramón et al. 1993). The arabinan degrading enzymes in both fungi can also be induced by simple carbon sources such as L-arabinose, and particularly L-

arabitol, but only in the absence of D-glucose (v.d. Veen et al. 1993; Ramón et al. 1993). Since L-arabitol arises as an intermediate of L-arabinose catabolism (Fig. 1) the regulation of the biosynthesis of extracellular arabinan degrading enzymes also depends on the regulation of L-arabinose utilisation itself.

The availability of various carbon catabolite derepressed mutants in *A. nidulans* and of a well-defined set of co-inducers for extracellular arabinase biosynthesis enables analysis of the role of carbon catabolite repression in relation to the production of these enzymes.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical quality and obtained from Merck (Darmstadt, FRG). PNPA was obtained from Sigma (St. Louis, Mo., USA). Arabinazyme tablets were obtained from Megazyme (Sydney, Australia).

Strains and growth conditions

The *Aspergillus nidulans* strain WG096 (*yA2 paba*A1), originating from a strain of Glasgow origin (FGSC4), was used as wild-type strain.

The *A. nidulans cre*A mutant strains used carried: *cre*A^{d}-1 (*paba*A1); *cre*A^{d}-2 (*paba*A1); *cre*A^{d}-3 (*paba*A1); *cre*A^{d}-4 (*bi*A1); *cre*A^{d}-30 (*bi*A1) (Arst and Cove 1973; Bailey and Arst 1975; Arst et al. 1990). Strains carrying *cre*B35 (=*mol*B35) (*bi*A1) (Arst et al. 1970) or *cre*C302 (*paba*A1) (C.R. Bailey as cited in Arst (1981) were also used. See Clutterbuck (1993) for definition of gene symbols.

The strains were grown on minimal medium as described previously (Pontecorvo et al. 1953). The vitamin *p*-aminobenzoic acid was added to a final concentration of 2 mg/l and biotin to a final concentration of 4 µg/l to all cultures. In the transfer experiments all strains were grown before transfer at 37 °C in 1 l Erlenmeyer flasks containing 300 ml minimal medium, 2 % (w/v) D-glucose and 0.05% (w/v) yeast extract. After 24 h

the mycelia were harvested, washed with saline and aliquots of 1 g mycelium (wet weight) were transferred to 100 ml Erlenmeyer flasks containing 30 ml minimal medium and various carbon sources at a final concentration of 1% (w/v) and were incubated for another 6 h. Mixed carbon sources were each added at 1% (w/v). Mycelia were harvested, washed with saline and frozen using liquid nitrogen. Culture filtrates were used directly for enzyme assay.

Preparation of cell-free extracts and protein determination

The preparation of cell-free extracts was carried out as described by Witteveen et al. (1989). Protein concentrations were measured using the Biorad BCA method as described previously (Van der Veen et al. 1991).

Determination of enzyme activities

α-L-Arabinofuranosidase activities were determined as described previously (Van der Veen et al. 1991). Endo-arabinase was measured using Arabinazyme tablets according to the supplier's instructions. The activities of L-arabinose reductase, D-xylose reductase, L-arabitol dehydrogenase and xylitol dehydrogenase were measured as described by Witteveen et al. (1989). Both the arabinases were measured from two different cultures in duplicate; the intracellular enzymes were measured from one culture in duplicate. Within one experiment the activities found did not vary more than 5-8% (measured in duplicate). The variations found between different experiments were higher and varied between 15-25%. The tendency in activity changes between the various strains and growth conditions was found to be the same in the different experiments.

Polyol extraction and determination

The extraction of intracellular polyols was carried out as described by Witteveen et al. (1993). Polyol concentrations were measured on a HPAEC (Dionex) with a CarboPac MA1 column using isocratic elution with 0.48 M NaOH (Witteveen et al. 1993). The polyol concentrations were measured once but simultaneously with arabinase activities.

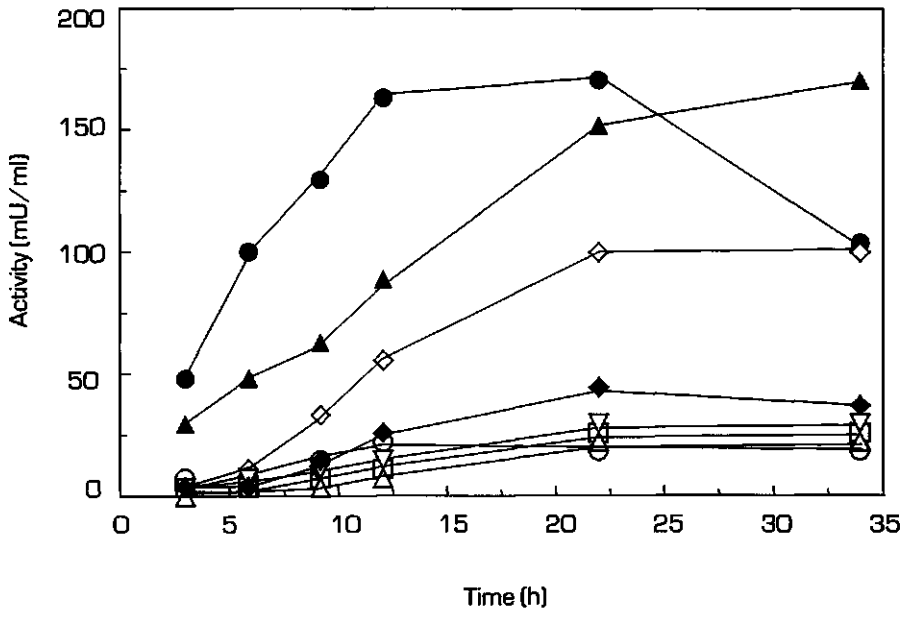

Fig. 2 PNPA hydrolysing activities of *A. nidulans* strains pregrown on 2% (w/v) D-glucose and transferred to fresh media containing 1% (w/v) L-arabitol as an inducer. Samples of the culture fluid were taken at various time intervals. Activities are expressed in mU/ml culture filtrate. □ WG096; Δ *creA*^{d}-1; ○ *creA*^{d}-2; ◆ *creA*^{d}-3; ▲ *creA*^{d}-4; ● *creA*^{d}-30; ∇ *creB35*; ◇ *creC302*

RESULTS

Induction characteristics of arabinase activity in various carbon catabolite derepressed mutant strains

Induction levels between some of the *cre*A mutant strains and the wild type differed markedly (Fig. 2). Although strains carrying *creA*^{d}-1, *creA*^{d}-2, *creA*^{d}-3 or *creB35* differed relatively little from the wild type, strains carrying *creA*^{d}-4, *creA*^{d}-30 and *creC302* showed much higher induction levels, the difference reaching nearly an order of magnitude.

Table 1. Activities of α-L-arabinofuranosidase B (upper part, I) and endo-arabinase (lower part, II) i culture filtrates of *Aspergillus nidulans* wild type and *cre*A, *cre*B35 and *cre*C302 mutant strains unde various growth conditions. All carbon sources were used at a concentration of 1 % (w/v). Activitie are expressed in mU/ml culture filtrate.

	Carbon Source	Strains							
		WG096	*creA*^{d}-1	*creA*^{d}-2	*creA*^{d}-3	*creA*^{d}-4	*creA*^{d}-30	*creB35*	*creC302*
I	D-glucose	2.6	3.0	2.0	1.8	2.5	10.6	2.7	10.2
	L-arabinose	8.2	10.1	8.5	9.0	70.1	147.3	14.2	24.2
	L-arabitol	15.2	26.6	11.7	25.9	119.5	157.2	11.7	31.7
	L-arabinose/ D-glucose	2.8	2.6	2.5	2.2	11.2	17.9	4.0	2.4
	L-arabitol/ D-glucose	2.0	2.4	2.2	2.2	29.9	27.4	5.0	8.1
	glycerol	2.9	2.3	1.8	1.8	2.8	4.2	3.4	2.4
II	D-glucose	3.1	1.5	4.3	0.1	1.4	6.7	4.4	4.6
	L-arabinose	6.5	9.4	17.3	15.8	43.8	63.4	16.3	8.9
	L-arabitol	15.2	9.7	23.6	30.3	86.3	90.9	35.4	20.7
	L-arabinose/ D-glucose	4.7	4.6	10.0	3.3	4.1	9.8	7.4	4.8
	L-arabitol/ D-glucose	4.7	4.5	7.3	7.9	3.6	7.6	6.3	4.8
	glycerol	5.1	3.5	3.4	4.0	1.2	4.2	6.3	3.9

*Cre*A^d-30 showed a particularly rapid induction of PNPA hydrolysing activity even after as little as 3 h. A late decline of activity in the *cre*A^d-30 mutant was not further investigated but might have resulted from degradation by proteases.

The most rapid induction was observed in most of the strains between 6 and 12 h after transfer. In further experiments an induction time of 6 h was chosen to ensure that the D-glucose concentration in mixed carbon source cultures remained sufficiently high (6-8 mg/ml) to exert full carbon catabolite repression.

Induction and carbon catabolite repression of arabinase activities

To investigate induction and carbon catabolite repression of arabinase activities independently the various *A. nidulans* strains were grown for 24 h on D-glucose and then transferred to fresh media containing different carbon sources. D-glucose is a strongly repressing carbon source and glycerol is derepressing (Arst and Cove, 1973; Bailey and Arst, 1975). L-arabinose and L-arabitol are carbon sources that induce the arabinases (v.d. Veen et al., 1993) and are carbon catabolite derepressing (Arst and Cove, 1973; Bailey and Arst, 1975), irrespective of *are*A mutations (v.d. Veen and Visser, unpublished results). The simultaneous effects of induction and repression were determined using a combination of D-glucose with L-arabinose or L-arabitol. All cultures were inoculated with mycelia (1 g wet weight) as stated in Materials and methods. We checked the biomass data for wild type and the *cre*A^{d}-30 strain that had the most outspoken growth problems. Within the 6 h transfer period, the 2 strains show an equal increase in biomass (dry weight) on the same carbon sources. The differences in biomass between the different carbon sources were found to vary within 10-20% (1.1-1.2 g wet weight). D-glucose is an exception as in both strains the biomass increased by 50% (1.5 g wet weight).

α-L-arabinofuranosidase B and endo-arabinase activities were measured in culture filtrates. As we did not measured the actual dry weights for all strains we present the activities in mU/ml culture filtrate (Table 1). In the wild type activities of ABFB (Table 1) were low and comparable to repressing D-glucose and derepressing glycerol. L-arabinose and L-arabitol induce, but repression by D-glucose negated the effects of induction.

Table 2 Intracellular specific activities of L-arabinose reductase under various growth conditions *Aspergillus nidulans* wild type and *cre*A, *cre*B35 and *cre*C302 mutant strains. All carbon sources we used at a concentration of 1 % (w/v). Activities are expressed in mU/mg soluble protein in extracts.

Carbon source	Strains							
	WG096	*creA*^{d}-1	*creA*^{d}-2	*creA*^{d}-3	*creA*^{d}-4	*creA*^{d}-30	*cre*B35	*cre*C3
D-glucose	40	27	71	75	97	28	35	75
L-arabinose	301	384	616	378	557	479	362	322
L-arabitol	252	283	431	465	919	545	161	354
L-arabinose/ D-glucose	34	184	210	93	197	131	51	126
L-arabitol/ D-glucose	90	198	161	152	96	107	100	88
glycerol	54	10	58	39	100	47	25	71

All *cre* mutants had levels of ABFB activity on D-glucose similar to the wild type except for *creA*^{d}-30 and *cre*C302 which had somewhat higher activities. Under inducing conditions the *creA*^{d}-30 mutant had strikingly more activity than the wild type (eighteen times on L-arabinose; ten times on L-arabitol). Of the other *cre*A mutants only the *creA*^{d}-4 mutant showed a markedly higher induced level of ABFB than the wild type (8.5 and 7.9 times, respectively). The other *cre*A mutants as well as *cre*B35 and *cre*C302 had only slightly elevated ABFB activity under inducing conditions.

In the presence of D-glucose and an inducing carbon source, most *cre*A mutants as well as *cre*B35 and *cre*C302 had ABFB activities comparable to that of the wild type. Only the *creA*^{d}-30 and *creA*^{d}-4 strains showed markedly higher levels, albeit not nearly so high as in the absence of glucose. Under derepressing conditions (i.e. glycerol) low activities were found for all strains. With respect to these enzymes one needs induction

at the same time to observe differences between effects of various carbon sources.

For the endo-arabinase (Table 1), a similar induction pattern was seen. Once again, L-arabitol was a more effective inducer than L-arabinose. In the presence of D-glucose and an inducing carbon source, ABNA activities were only slightly derepressed in some *cre* mutants, *viz.* *cre*A^{d}-30, *cre*A^{d}-2 and *cre*B35. In this case, *cre*A^{d}-4 had no marked effect.

Pentose catabolism and carbon catabolite repression

We established previously that in *A. niger* the catabolic pathway for L-arabinose plays an important role in the induction of the arabinases (v.d. Veen et al. 1993). Therefore the intracellular activities of the relevant enzymes of this pathway were also measured.

L-arabinose reductase (*cf* Fig. 1) is the first enzyme involved in the degradation of L-arabinose. In the *cre*A^{d}-2, *cre*A^{d}-3, *cre*A^{d}-4 and *cre*C302 strains higher activities than in the wild type were found after growth on D-glucose (Table 2). Under derepressing conditions (i.e. glycerol as carbon source) the activities of these strains were comparable to those of the wild type except for the *cre*A^{d}-1, *cre*A^{d}-3 and *cre*B35 strains which had lower activities and for *cre*A^{d}-4 which had a 2-fold higher activity. These enzyme data clearly illustrate a *cre*A allele-specific response of L-arabinose reductase expression both on D-glucose and on glycerol. To explain these observations in a quantitative context would require further molecular analysis of the interactions between the different CREA mutant proteins and the target gene. The inducing carbon sources L-arabinose and L-arabitol resulted in a strong increase in L-arabinose reductase activity in the wild type strain (6- to 7-fold) compared to D-glucose. All

Table 3 Intracellular specific activities of L-arabitol dehydrogenase (upper part, I) and of NAD dependent xylitol dehydrogenase (lower part, II) under various growth conditions in *Aspergill nidulans* wild type and *cre*A, *cre*B35 and *cre*C302 mutant strains. All carbon sources were used at concentration of 1 % (w/v). Activities are expressed in mU/mg soluble protein in extracts.

	Carbon Source	Strains							
		WG096	*creA*^{d}-1	*creA*^{d}-2	*creA*^{d}-3	*creA*^{d}-4	*creA*^{d}-30	*creB35*	*creC302*
I	D-glucose	10	1	13	10	11	6	2	33
	L-arabinose	70	171	335	188	392	308	131	122
	L-arabitol	89	107	364	257	689	272	63	183
	L-arabinose/ D-glucose	24	88	270	109	78	70	10	73
	L-arabitol/ D-glucose	16	70	140	104	57	68	54	92
	glycerol	11	15	11	27	39	75	20	27
II	D-glucose	5	11	10	27	21	9	9	19
	L-arabinose	111	182	348	218	188	308	149	131
	L-arabitol	86	126	296	330	360	191	92	209
	L-arabinose/ D-glucose	17	84	100	100	50	62	17	61
	L-arabitol/ D-glucose	9	90	101	143	49	61	54	63
	glycerol	22	24	24	45	55	50	30	51

*cre*A strains had elevated induced levels to varying degrees. The *cre*A alleles that on L-arabinose led to the highest levels of L-arabinose reductase were *creA*^{d}-2 and *creA*^{d}-4 whereas on L-arabitol these were *creA*^{d}-4 and *creA*^{d}-30. It is furthermore worth noting that L-arabitol was a more effective inducer in *creA*^{d}-30 and *creA*^{d}-3 strains, and particularly in *creA*^{d}-4, whereas in the wild type and other *cre*A mutants L-arabinose was the more effective inducer. When induced with L-arabinose or L-arabitol in the presence of D-glucose as a repressing carbon source, levels

decreased but tended to be higher than in the wild type strain, particularly with L-arabinose as inducer.

For L-arabitol dehydrogenase, the second enzyme in the pathway (*cf* Fig. 1), the activities of the wild type grown on D-glucose and glycerol were the same (Table 3, upper part). Amongst *cre* strains *cre*C302 showed elevated activity and *cre*A^{d}-1 and *cre*B35 reduced activities on D-glucose. With growth on glycerol both *cre*A^{d}-30 and *cre*A^{d}-4 had elevated L-arabitol dehydrogenase levels as did slightly *cre*A^{d}-3 and *cre*C302. With L-arabinose or L-arabitol as the carbon source, a 7- to 9-fold increase in L-arabitol dehydrogenase was found in the wild type. All *cre* mutants, with exception of *cre*B35, exhibited increased inducibility of L-arabitol dehydrogenase if grown on L-arabinose or L-arabitol. Relative to the wild type, carbon catabolite derepression was evident in nearly every case of a *cre* strain grown with an inducer, particularly for *cre*A^{d}-2 and *cre*A^{d}-3. For both L-arabinose reductase (Table 2) and L-arabitol dehydrogenase (Table 3), carbon catabolite repression was observed in the *cre*B35 strain when L-arabinose but not L-arabitol served as inducer.

The regulation of the synthesis of the fourth enzyme of the pathway, NAD^{+}-dependent xylitol dehydrogenase (Table 3, lower part), was broadly similar to that of L-arabitol dehydrogenase. Activities were low when the strains were grown on D-glucose and somewhat higher on glycerol. Strong induction occurred with L-arabinose or L-arabitol in all cases. The *cre*A mutants had elevated induced levels compared to the wild type. The highest induced levels were obtained with *cre*A^{d}-2 and *cre*A^{d}-30 on L-arabinose and with *cre*A^{d}-4 and *cre*A^{d}-3 on L-arabitol. When D-glucose was present together with one of the inducers, xylitol dehydrogenase activity was strongly repressed in the wild type. The same *cre*A allele specificity towards carbon catabolite repression found for L-arabitol

Table 4 Intracellular specific activities of NADPH-dependent L-xylulose reductase under various growth conditions in *Aspergillus nidulans* wild type and *creA*^{d}-30. All carbon sources were used at a concentration of 1 % (w/v). Activities are expressed in mU/mg soluble protein in extracts.

	Strains	
Carbon source	WG096	*creA*^{d}-30
D-glucose	5	2
L-arabinose	79	151
L-arabitol	172	179
L-arabinose/ D-glucose	30	34
L-arabitol/ D-glucose	18	22
glycerol	4	4

dehydrogenase was apparent with *creA*^{d}-3 and *creA*^{d}-2 being the most derepressed.

L-xylulose reductase (Table 4), the third enzyme of the pathway, might not be subject to *creA* regulation as even the usually phenotypically extreme *creA*^{d}-30 mutation (Arst et al. 1990) scarcely affected enzyme levels apart from a possible modest effect on L-arabinose.

Intracellular arabitol and xylitol concentrations

When analyzing the intracellular concentrations of xylitol and arabitol (Table 5), it is necessary to note that HPAEC cannot distinguish L-arabitol and D-arabitol. However, only L-arabitol acts as inducer for arabinase synthesis (v.d. Veen et al. 1993). Both isomers arise but differently. The arabitol observed in all strains grown on D-glucose consists of D-arabitol which is formed from the pentose phosphate

Table 5. Intracellular xylitol and arabitol concentrations in wild type *A. nidulans* and *cre*A strains under various growth conditions. All carbon sources are used at a concentration of 1 % (w/v). Concentrations are expressed in μmol/g dry weight.

arbon ource	Strains									
	WG096		*creA*^{d}-1		*creA*^{d}-2		*creA*^{d}-4		*creA*^{d}-30	
	xylitol	arabitol	xylitol	arabitol	xylitol	arabitol	xylitol	arabitol	xylitol	arabitol
)-Glucose	0	44	0	154	0	73	1	67	1	41
-arabinose	35	168	28	155	26	146	23	128	15	77
-arabinose/)-glucose	4	59	5	143	13	78	3	60	4	42

pathway intermediate D-xylulose-5-phosphate. However, L-arabitol is formed as an intermediate of L-arabinose catabolism. In this case the simultaneous appearance of xylitol (*cf* Fig. 1) is diagnostic for the presence of L-arabitol. When wild type and the various *cre*A mutants were grown on D-glucose no appreciable xylitol was present (Table 5), whereas considerable arabitol was detected. Under inducing conditions (L-arabinose), large amounts of arabitol were detected in all strains. Xylitol was also present but in lower amounts. On a mixture of D-glucose and L-arabinose total arabitol was present at the same level as upon growth on D-glucose. However, in all strains low levels of xylitol were also present indicative of some L-arabinose uptake and subsequent catabolism under these conditions, even in the wild type.

DISCUSSION

Carbon catabolite repression by glucose and other favoured carbon sources prevents the synthesis of a large number of enzymes and permeases involved in the utilisation of less preferred substrates including

polysaccharides. In *Aspergillus nidulans* carbon catabolite repression of many genes is mediated by the negative acting wide domain regulatory gene *cre*A (Arst and MacDonald 1975; Bailey and Arst 1975). Arst and Bailey (1977) observed that various *cre*A mutant alleles show non-hierarchical heterogeneity in their effects on expression of different structural genes indicating that *cre*A would encode a regulatory DNA binding protein. The prediction has been confirmed by the presence of two 'zinc fingers' of the Cys_2His_2 class in the derived CREA sequence (Dowzer and Kelly 1991) and by binding studies with a CREA fusion protein and wild type and mutant cognate receptor sites having a hexanucleotide consensus 5'-G/CPyGGPuG-3' (Kulmburg et al. 1993; Cubero and Scazzocchio 1994). Two other genes *cre*B35 and *cre*C302 in which mutations affect carbon catabolite repression are more likely to act indirectly as their highly pleiotropic mutant phenotypes extend well beyond the realm of carbon catabolite repression (Hynes and Kelly 1977; Arst et al. 1970; Arst and Cove 1970; C.R. Bailey as cited in Arst (1981)).

In this study we used five different *cre*A mutant alleles and a mutation in *cre*B35 and in *cre*C302 to investigate how the syntheses of α-L-arabinofuranosidase B and endo-arabinase and of the inducer L-arabitol are influenced by carbon catabolite repression. Although a number of modest regulatory effects resulting from the *cre*B35 and *cre*C302 mutations were apparent, much more striking elevations of enzyme levels were seen with *cre*A mutations, perhaps in keeping with the more direct role of this gene.

The data indicated a strong influence of *cre*A on the biosynthesis of both α-L-arabinofuranosidase B and endo-arabinase which were shown to be coordinately regulated (Ramón et al. 1993). It is possibly surprising that the effect of *cre*A mutations is most marked under inducing conditions. The induced enzyme levels in the *cre*A^d-30 and *cre*A^d-4 strains are

6- to 10-fold higher than those reached in the wild type. This suggests that there is considerable 'self' repression in the wild type, even under conditions of optimal induction. In the presence of D-glucose plus an inducer, certain *cre*A mutations, particularly *cre*A^{d}-30, result in carbon catabolite derepression. The *cre*A^{d}-30 mutant allele is the result of an inversion breakpoint within *cre*A that severs the region encoding the DNA binding domain from most of the rest of the gene and results in a generally more extreme phenotype than seen in other fully viable *cre*A mutants characyerised (Arst et al. 1990; Dowzer and Kelly 1991). The degrees of inducibility of ABFB and ABNA in the various *cre*A mutants give further indication of the non-hierarchical response of different activities in different mutants. For example, whereas *cre*A^{d}-2 exhibited elevated inducibility for ABNA and *cre*A^{d}-1 did not, the reverse was true for ABFB. This indicates a direct response of the two structural genes encoding ABFB and ABNA towards *cre*A. The *abf*B gene and *abn*A genes of *Aspergillus niger* (but not *A. nidulans*) have been cloned and sequenced (Flipphi et al. 1993a,b) and their promoter regions contain several putative CREA binding sites (Flipphi et al. unpublished data).

The effects of various *cre* mutations on levels of the specific activities of enzymes involved in L-arabinose catabolism were also investigated. These levels determine the flux through this pathway and hence the intracellular levels of the inducers L-arabinose and L-arabitol (for arabinases as well as arabinose catabolic enzymes). Wild type activity levels of L-arabinose reductase (Table 2), the first enzyme of the pathway, were higher than those of L-arabitol dehydrogenase (Table 3), xylitol dehydrogenase (Table 3) and L-xylulose reductase (Table 4) which leads to the formation of L-arabitol.

In contrast to the regulatory behaviour of the other three enzymes of arabinose catabolism, L-xylulose reductase levels were largely unaffected by *cre*A^{d}-30. It is therefore possible that the synthesis of this enzyme is not under *cre*A control thus avoiding the risk of accumulating L-xylulose which is more toxic to the cell than xylitol (Witteveen et al 1994).

Further non-hierarchical behaviour is evident from the effects of the various *cre*A mutations on the regulation of the arabinose catabolic enzymes. For example, whereas L-arabitol was a better inducer than L-arabinose of L-arabinose reductase in the *cre*A^{d}-4 strain, the reverse was true for the *cre*A^{d}-2 strain. Also, whereas *cre*A^{d}-30 resulted in strong carbon catabolite derepression of ABFB and *cre*A^{d}-1 resulted in none, *cre*A^{d}-1 resulted on average in stronger carbon catabolite derepression than *cre*A^{d}-30 for all three arabinose catabolic enzymes examined.

When comparing the levels of the various enzymes measured, it should be noted that both L-arabitol dehydrogenase and xylitol dehydrogenase were assayed near their pH optima (pH 9.6). These activities at a physiologically more relevant pH value (*e.g.* pH 6.5) are probably at least five-fold lower, as shown for *A. niger* (Witteveen et al. 1994). The thermodynamic equilibrium of these alternating reduction and oxidation steps in L-arabinose catabolism leads towards polyol formation. This was also demonstrated in *A. niger* by ^{13}C NMR spectroscopy leading to xylitol and especially L-arabitol accumulation to a ratio of approx. 1:3 (Witteveen el al. 1989).

From intracellular concentrations of arabitol and xylitol (Table 5), it is clear that under inducing conditions substantial amounts of xylitol and thus of the isomer L-arabitol are present. On a mixed carbon source (D-glucose/L-arabinose) xylitol was also be detected in all the strains including the wild type. Therefore we conclude that L-arabitol is also present

and that uptake of L-arabinose takes place even in the presence of D-glucose as repressing carbon source.

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Physiological consequences of an extreme *Aspergillus nidulans* *cre*A mutation on D-glucose utilization

CHAPTER 6

PHYSIOLOGICAL CONSEQUENCES OF AN EXTREME *ASPERGILLUS NIDULANS CREA* MUTATION ON D-GLUCOSE UTILIZATION[5]

Peter van der Veen, George J.G. Ruijter and Jaap Visser

SUMMARY

Aspergillus nidulans wild type and the extreme carbon catabolite derepressed mutant *cre*A^{d}-30 were characterized with respect to enzyme activities, metabolite concentrations and polyol pools all related to glycolysis, after growth on D-glucose. In the *cre*A^{d}-30 strain the enzymes hexokinase and fructose-6-phosphate reductase showed a 2-fold and 3-fold increase in activity, whereas phosphofructokinase and pyruvate kinase activity were decreased by a factor 2 and 3 respectively, in comparison with the wild type strain. The most remarkable changes in metabolite concentrations were that fructose-2,6-diphosphate and fructose-1,6-diphosphate showed a 2.5-fold increase, whereas pyruvate and also citrate were decreased in the *cre*A^{d}-30.

Striking differences were found for the polyol concentrations measured for the two strains tested. Intracellular glycerol and arabitol concentrations were 10-fold higher and erythritol 5-fold higher in the *cre*A^{d}-30, whereas intracellular trehalose and mannitol were both decreased. The total internal polyol concentration appears to be constant at $\approx$700

[5]Submitted for publication in Microbiology

μmol/g dry weight. All polyols were also detected in high amounts in the culture filtrate of the *cre*A^{d}-30 mutant strain but no extracellular trehalose was found. The overall production of polyols in this strain was therefore much higher than in the wild type.

The high level of polyols produced and the changes in metabolite concentrations in the *cre*A^{d}-30 strain, suggest that the differences in enzyme activities result in a altered flow through glycolysis leading to a more rapid formation of polyols which are subsequently secreted.

INTRODUCTION

Carbon catabolite repression provides microbial cells with a means to adapt their metabolism so that favoured carbon sources such as D-glucose, D-fructose or D-xylose are utilized preferentially in the presence of other less preferred carbon sources. Mutations in the hyphal fungus *Aspergillus nidulans* resulting in altered responses to carbon catabolite repression have been known for approximately two decades (Arst and Cove 1973; Bailey and Arst 1975). The strategies devised by these authors to obtain carbon catabolite derepressed mutations were based on the selection of pseudorevertants using parental strains with a specific genetic background. Mutants able to grow on a mixture of glucose and L-proline were, for instance, selected from an ammonium requiring *are*A defective parental strain. Similarly, carbon catabolite derepressed mutants were obtained from a glucose non-utilizing *pdh*A mutant which itself was unable to utilize ethanol in the presence of glucose due to carbon catabolite repression of alcohol- and aldehyde dehydrogenase. Of the *cre*A gene thus identified, a number of mutant alleles have been selected which

are recessive to wild type and which typically display non-hierarchical heterogeneity of mutant phenotypes (Arst and Bailey 1977, Van der Veen et al. 1994). The CREA protein was postulated to be a negatively acting regulatory protein directly involved in regulating gene expression (Arst and Bailey 1977; Arst and MacDonald 1975). This was confirmed by Dowzer and Kelly (1989; 1991) who cloned *cre*A and found it to encode a 'zinc finger' DNA binding protein of the Cys_2His_2 type. A fusion protein containing the 'zinc finger' domain of *A. nidulans* CREA showed sequence specific binding to promotors of genes which are under carbon catabolite repression control like those of the ethanol regulon (Kulmburg et al. 1993) or the proline gene cluster (Cubero and Scazzocchio 1994). Although the direct role of the CREA protein as a repressor of gene expression has now been confirmed, the physiological consequences of this mutation have not yet been investigated. We therefore decided to investigate which physiological changes take place upon growth on D-glucose in a mutant *cre*A strain compared to wild type *A. nidulans*. In this paper we present data on enzyme activities and metabolite levels related to glycolysis and energy metabolism. In addition intracellular and extracellular polyol concentrations were studied.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical quality and obtained from Merck (Darmstadt, FRG). All enzymes and substrates were obtained from Boehringer (Mannheim, FRG) except for fructose-2,6-diphosphate and pyrophosphate-dependent fructose-6-phosphate kinase which were obtained from Sigma (St. Louis, USA).

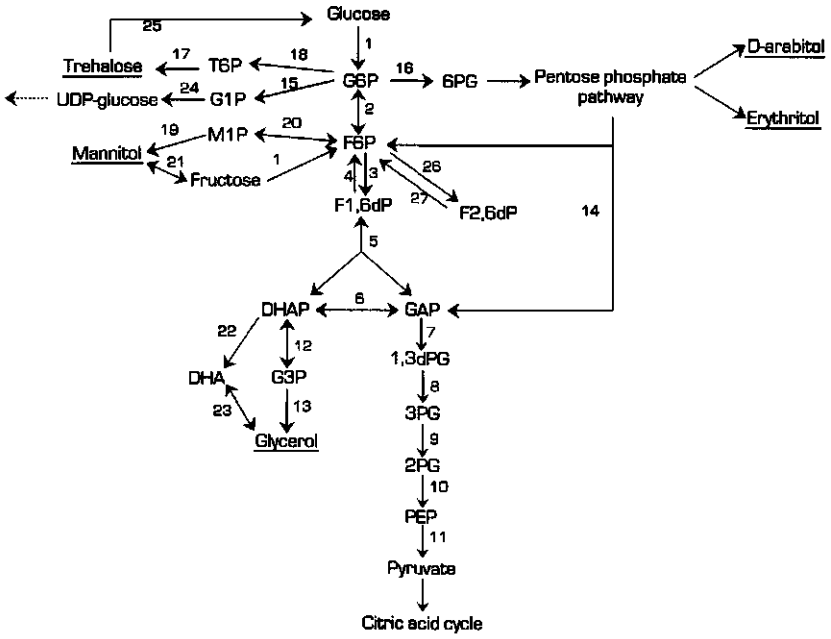

Fig. 1 Schematic representation of a part of the hexose monophosphate pathway and metabolic routes involved in polyol synthesis. 1: hexokinase; 2: phosphoglucose isomerase; 3: phosphofructokinase; 4: fructose-1,6-diphosphatase 5: aldolase; 6: triose phosphate isomerase; 7: glyceraldehyde-3-phosphate dehydrogenase; 8: phosphoglycerate kinase; 9: phosphoglyceromutase; 10: enolase; 11: pyruvate kinase; 12: glycerol-3-phosphate dehydrogenase; 13: glycerol-3-phosphate phosphatase; 14: transketolase; 15: phosphoglucomutase; 16: glucose-6-phosphate dehydrogenase; 17: trehalose-6-phosphate synthase; 18: trehalose-6-phosphate phosphatase; 19: mannitol-1-phosphate phosphatase; 20: fructose-6-phosphate dehydrogenase; 21: mannitol dehydrogenase; 22: dihydroxyacetonephosphate phosphatase; 23: glycerol dehydrogenase; 24: UDP-glucose pyrophosphorylase; 25: trehalase; 26: fructose-6-phosphate-2-kinase; 27: fructose-2,6-diphosphatase

Strains and growth conditions

The *Aspergillus nidulans* strain WG096 (*yA2 pabaA1*) was used as wild-type strain.

The *A. nidulans* carbon catabolite derepressed mutant strain used was *creA*^{d}-30 (*biA1*) (Arst et al. 1990). The strains were grown on minimal medium as described previously (Pontecorvo et al. 1953). *p*-Aminobenzoic acid was added to a final concentration of 2 mg/l and biotin to a final concentration of 4 μg/l to the cultures.

The two strains were grown at 37 °C for 16 h in 250-300 ml minimal medium, 0.05% (w/v) yeast extract and 1.5% (w/v) D-glucose in 1 l Erlenmeyer flasks using a New Brunswick orbital shaker at 250 rpm.

Preparation of cell-free extracts and protein determination

The preparation of cell-free extracts from mycelium was carried out as described by Witteveen et al. (1989). Protein concentrations were measured using the Biorad BCA method as described before (van der Veen et al. 1991).

Determinations of metabolites

The method to extract metabolites from mycelium as developed by Ruijter and Visser (unpublished results) for *Aspergillus niger* was used. It is a modification of the method described by de Koning and van Dam (1992). Concentrations of glycolytic metabolites were assayed enzymatically according to Bergmeyer (1985a; 1985b). Fructose-2,6-diphosphate (F2,6dP) was determined using a kinetic method by measuring the rate of PPi-dependent phosphofructokinase as described by Van Schaftingen et al. (1982).

All analyses were performed on a Cobas Bio Autoanalyser (Roche, Basel, Switzerland) that was connected with a MS/DOS computer for datalogging. Volumes in published methods were adapted to the working volume of the apparatus (0.25 ml). Analyses were performed at 30 °C.

Enzyme determinations

All enzyme determinations were carried out using an Aminco DW-2 UV/VIS double beam spectrophotometer (Silver Spring, MD, USA) with an Atari computer for datalogging and calculations. The enzyme activities were measured at 37 °C essentially as described by Bergmeyer (1985a; 1985b) but using 50 mM Pipes buffer pH 7.5 containing 100 mM KCl and 5 mM $MgCl_2$ in all cases.

Polyol extraction and -determination

The intracellular polyol determinations were carried out as described by Witteveen et al. (1993). The extracellular polyol determinations were done as follows. Just before harvesting of the mycelia, a sample was taken from the culture filtrate and spun down for 5 min in an Eppendorf centrifuge to remove mycelia and debris. The sample was diluted 5 times with deionized water obtained from a Millipore filtration unit, and the polyol concentrations were determined by HPAEC on a Dionex system (Sunnyvally,

California, USA) with a CarboPac MA1 column using isocratic elution with 0.48 M NaOH as described by Witteveen et al. (1993).

RESULTS

Enzyme levels

D-glucose is the most efficient carbon source to effect carbon catabolite repression in *Aspergillus nidulans*. The *cre*A gene known now to affect this phenomena was found to encode a 'zinc finger' DNA binding protein of the Cys_2His_2 type. Although the direct role of the CREA protein as a repressor of gene regulation has now been confirmed, the physiological consequences of a mutation in this gene have been overlooked. An analysis of glycolytic enzyme patterns and metabolite levels in wild type and a genotypically defined derepressed mutant was therefore initiated. Fig. 1 shows an overview of glycolysis with some of its branches.

Following growth on D-glucose of the *A. nidulans* strain WG096 and *cre*A^d-30 we found differences in the level of several glycolytic enzymes. The data are shown in Table 1. The first notable difference was the increased hexokinase activity in the *cre*A^d-30. The G6P pool formed by hexokinase is distributed to supply the glycolytic and pentose phosphate pathways as well as to synthesize UDPG. Moreover, G6P is required for the synthesis of trehalose. For none of the enzymes involved in G6P metabolism significant changes were found between the wild type and the *cre*A^d-30 strain. At the level of F6P metabolism however, we found two significant changes (*cf* Fig. 1). Phosphofructokinase, which is one of the key regulatory enzymes in glycolysis in many organisms, was decreased in the *cre*A^d-30 mutant strain. Another enzyme that converts

Table 1. Activities of various intracellular enzymes involved in glucose metabolism in *Aspergillus nidulans* extracts of wild type and *cre*A^{d}-30 mutant strain.[1]

	Strains	
Enzyme	WG096	*cre*A^{d}-30
Hexokinase	0.28	0.46
Glucose-6-P dehydrogenase	2.18	1.99
Phosphoglucomutase	1.22	1.56
Phosphoglucose isomerase	2.24	1.95
Fructose-6-P reductase	0.53	1.86
Phosphofructokinase	1.18	0.46
Fructose-1,6-diphosphatase	0.06	0.14
Aldolase	1.86	1.83
Triose phosphate isomerase	18.26	16.83
Pyruvate kinase	1.50	0.56
Citrate synthase	1.08	0.64

[1] The data given are the average of two independent experiments. Strains were grown for 16 h on 1.5% (w/v) D-glucose at 37 °C. Activities are expressed in U/mg protein.

F6P into mannitol-1P is fructose-6-phosphate reductase. The mannitol-1-phosphate formed is subsequently converted into mannitol and initially stored. Fructose-6-phosphate reductase levels were 3-fold higher in the *cre*A^{d}-30 mutant. Interestingly, this is opposite to the effect observed for phosphofructokinase. Another important enzyme activity in this connection is of course fructose-1,6-diphosphatase which catalyses the reverse reaction of phosphofructokinase (*cf* Fig. 1). This activity was low in both strains and although somewhat higher in the *cre*A^{d}-30 mutant, it is not likely that this increase can account for the significant physiological

Table 2. Metabolite determinations in mycelia of *Aspergillus nidulans* wild type and *creA*^{d}-30 strain.[1]

	Strains			
	WG096		*creA*^{d}-30	
Metabolite	conc	S.D.	conc	S.D.
Glucose-6-phosphate	1.23	± 0.34	1.02	± 0.21
Fructose-6-phosphate	0.20	± 0.05	0.22	± 0.04
Fructose-1,6-diphosphate	0.34	± 0.04	0.73	± 0.08
Fructose-2,6-diphosphate[2]	1.35	± 0.14	2.75	± 0.21
3-Phosphoglycerate	0.08	± 0.03	0.12	± 0.01
2-Phosphoglycerate	0.07	± 0.03	0.12	± 0.04
Phosphoenolpyruvate	0.18	± 0.11	0.29	± 0.15
Pyruvate	3.82	± 0.22	1.49	± 0.48
Citrate	7.04	± 0.67	2.42	± 0.23
ATP	1.37	± 0.28	1.49	± 0.44
ADP	1.88	± 0.43	1.66	± 0.38
AMP	0.75	± 0.12	0.67	± 0.18

[1] The data given are the average of 3 independent experiments. Concentrations are expressed in μmol/g dry weight.
[2] Concentration is expressed in nmol/g dry weight.

changes in the carbon catabolite derepressed mutant strain *creA*^{d}-30. The other glycolytic enzyme activities measured, *viz* triose phosphate isomerase and aldolase, were high and not altered in the *creA*^{d}-30 strain.

As for the other two enzymes measured, pyruvate kinase and citrate synthase, both differ in the wild type and the *creA*^{d}-30 strain. An almost 3-fold decrease in pyruvate kinase and a 1.7-fold decrease in citrate synthase activity was found in the *creA*^{d}-30 mutant.

Metabolite levels

Data on enzyme levels alone provides only limited information as the in vitro enzyme activities define only the capacity of a particular enzymatic step in the pathway. By measuring metabolite levels in addition, information is obtained about the actual cellular concentrations of the various reactants *viz* substrates, products and effectors from which actual metabolic rates can be calculated. The intracellular metabolite levels are presented in Table 2. The levels of G6P and F6P were quite similar in wild type and in the carbon catabolite derepressed strain *cre*A^{d}-30. However, in between the phosphofructokinase and pyruvate kinase reaction steps elevated levels of glycolytic intermediates F1,6dP, 2PG, 3PG and PEP were found. In contrast, levels of pyruvate and citrate, which depend on pyruvate kinase, were lower in the *cre*A^{d}-30 mutant.

Another striking change was seen at the level of F2,6dP which is a potent activator of phosphofructokinase. Similar to F1,6dP a 2- to 3-fold increase was found in the mutant. In order to determine whether the metabolic changes observed in glycolysis in the *cre*A^{d}-30 mutant strain would also have consequences for the energy state of the cells, we measured the levels of the total adenine nucleotide pools present. The values for the energy charge calculated on the basis of these measurements fluctuate between 0.5 and 0.6 and do not suggest drastic differences.

Polyol pools

A. nidulans forms several polyols from D-glucose. Mannitol and glycerol are derived from glycolytic intermediates, whereas arabitol and erythritol arise from pentose phosphate pathway intermediates. Initially the polyols accumulate inside the fungal cell, but finally they are also excreted.

Table 3. Intracellular and extracellular polyol concentrations in *Aspergillus nidulans* wild type and *creA*^{d}-30 mutant strain.[1]

	Strains			
	WG096	*creA*^{d}-30	WG096	*creA*^{d}-30
Polyol	Intracellular		Extracellular	
Trehalose	32.7	2.3	0	0
Mannitol	623.1	469.4	0.61	2.71
Glycerol	7.1	65.6	0.18	0.53
Arabitol	2.2	28.2	0	0.16
Erythritol	26.9	139.7	0.14	2.36
Total intracellular polyol concentration	692	705		

[1] The data given are the average of two independent experiments. Strains were grown for 16 h on 1.5% (w/v) D-glucose at 37 °C. Concentrations are expressed in μmol/g dry weight.

Re-utilisation of these polyols requires the induction of specific enzymes which are usually under carbon catabolite repression control. We therefore compared wild type *A. nidulans* and the *creA*^{d}-30 mutant with respect to the intracellular and extracellular polyols formed upon growth on D-glucose (Table 3). The major polyol present in the wild type upon growth on D-glucose was mannitol. In addition some trehalose and erythritol accumulated whereas minor amounts of glycerol and arabitol were found. In the *creA*^{d}-30 strain this pattern changed completely. Both mannitol and trehalose levels were much lower in *creA*^{d}-30 whereas the concentrations of arabitol (10-fold) and erythritol (15-fold) were strongly increased. The total intracellular concentration of these polyols including trehalose was

Table 4. Total amount of intracellular and extracellular polyols produced by *Aspergillus nidulans* wild type and *cre*A^{d}-30 mutant strain.[1]

	Strains	
Polyol	WG096	*cre*A^{d}-30
Glycerol	51	186
Erythritol	57	705
Arabitol	2	64
Trehalose	27	2
Mannitol	666	1063
Total polyol concentration	803	2020

[1] Concentrations are expressed in μmol per culture of 250 ml.

found to be similar in both strains (approximately 690-700 μmol/gram dry weight). In the culture filtrates of both the wild type and the *cre*A^{d}-30 strain polyols were found but no trehalose could be detected in either strain and arabitol was only present in the culture filtrate of the *cre*A^{d}-30 mutant. Extracellular polyols were present in much higher concentrations in the medium of the *cre*A^{d}-30 strain (*cf* Table 3).

Table 4 shows the total amounts of the various polyols produced by the wild type and *cre*A^{d}-30 mutant strain. Trehalose is the only polyol which is produced in higher amount in the wild type (25-fold), whereas the other polyol levels are higher in the *cre*A^{d}-30 strain (glycerol 3-fold; erythritol 12-fold; arabitol 28-fold; mannitol 1.5-fold). Also the total amount of the polyols formed in the *cre*A^{d}-30 mutant is almost 3 times higher than in the wild type strain.

DISCUSSION

Carbon catabolite repression can change the metabolism of cells in such a way that favoured carbon sources like D-glucose are utilized preferentially in the presence of other less preferred carbon sources. In the fungus *Aspergillus nidulans* a number of mutants having an altered response to carbon catabolite repression were selected (Arst and Cove 1973; Bailey and Arst 1975). The *cre*A gene thus identified encodes a 'zinc finger' DNA binding repressor protein (Dowzer and Kelly 1989, 1991; Kulmburg et al. 1993). The role of the CREA protein in carbon catabolite repression has been studied in both the ethanol regulon and the proline catabolism gene cluster (Kulmburg et al 1993; Cubero and Scazzocchio 1994). However, the role of the CREA protein in the total physiology of *A. nidulans* has never been part of any investigation. In this study we have therefore examined which physiological changes occur upon growth on D-glucose in *A. nidulans cre*A^{d}-30, a phenotypically extreme mutation.

The enzymatic data obtained suggest that a number of enzymes D-glucose metabolism are, directly or indirectly, influenced by the CREA protein, namely hexokinase, fructose-6-phosphate reductase, phosphofructokinase, pyruvate kinase and citrate synthase.

Hexokinase, the first enzyme in glycolysis (*cf* Fig. 1) was elevated in the *cre*A^{d}-30 strain (Table 1) whereas phosphofructokinase was decreased. However, these changes did not alter concentrations of G6P and F6P (Table 2) suggesting that overflow may occur via routes branching off from glycolysis (Fig. 1). Two possibilities are the conversion of fructose-6-phosphate to mannitol-1-phosphate by fructose-6-phosphate reductase which leads to the formation of mannitol and the conversion of glucose-6-phosphate to 6-phospho-glucono-δ-lactone by glucose-6-phos-

phate dehydrogenase which can lead, via the pentose phosphate pathway, to arabitol and erythritol formation. Fructose-6-phosphate reductase (Table 1) was strongly increased in the *cre*A^d-30 strain. Although the internal mannitol concentration (Table 3) is lower than in the wild type, the total amount of mannitol formed by the *cre*A^d-30 mutant is higher (Table 4). Furthermore, high internal and external concentrations of arabitol and erythritol (Table 3) were found in the *cre*A^d-30 strain, but it is not known whether these high internal concentrations can cause some toxic effect in the *cre*A^d-30 strain resulting in for instance compact growth. Because of the role polyols have in maintaining the osmotic balance (Beever and Laracy 1986; Van Laere 1989), it is likely that the total internal concentration of these polyols remains constant for a given set of growth conditions. Polyols which are overproduced are therefore likely to be secreted, as found in the *cre*A^d-30 mutant. This mutant had high extracellular levels of all polyols although the total internal polyol concentration was almost the same as in the wild type (Table 3).

The increased internal concentration of the metabolites F1,6dP, 2PG, 3PG and PEP in the *cre*A^d-30 mutant (Table 2) is likely to result from the decreased activity of pyruvate kinase in the *cre*A^d-30 mutant. The increased internal concentration of these metabolites might explain the formation of glycerol in the *cre*A^d-30 mutant. Reduced pyruvate kinase activity is also reflected by lower amounts of pyruvate and citrate in the mutant.

In yeast and fungi glycolysis is controlled mainly at the level of phosphofructokinase and pyruvate kinase (for a review see *e.g.* Gancedo and Serrano (1989)). In *A. niger* phosphofructokinase is also a key point in regulation of the glycolytic flux, which is activated by NH_4^+, AMP and fructose-2,6-diphosphate and inhibited by citrate and PEP (Habison et al.

1985; Arts et al. 1987). In the *cre*A^d-30 strain phosphofructokinase was lower than in the wild type strain. However, the concentration of fructose-2,6-diphosphate was higher. It is possible that this increase compensates for the lower amount of phosphofructokinase, but it is not known whether in the *cre*A^d-30 strain this results *in vivo* in a phosphofructokinase activity that is comparable with the wild type strain.

Another control point of glycolysis is the first step, the phosphorylation of glucose by hexokinase. In yeast a strong correlation was found between the catalytic activity of the gene *hxk*2, which encodes the hexokinase isoenzyme P-II, and its capacity to mediate catabolite repression (Ma et al. 1989; Rose et al. 1991). Furthermore *S. cerevisiae* hexokinase activity was recently found to be strongly inhibited by trehalose-6-phosphate (Blázquez et al. 1993). In wild type *A. nidulans* more trehalose was detected than in the *cre*A^d-30 strain, suggesting that the flux towards trehalose formation is higher in the wild type or, more likely, that trehalose turnover is higher in the *cre*A^d-30 strain. Recently it has been found that hexokinase in *A. nidulans* is also inhibited by trehalose-6-phosphate suggesting a similar regulation (Ruyter and Visser, unpublished results).

In summary, the *cre*A^d-30 mutation is responsible for radical changes in D-glucose degradation. These changes occur mainly at the level of those enzymes known to play a role in regulating glycolysis, namely hexokinase, phosphofructokinase and pyruvate kinase. The result of these changes is a higher flux through the side chains towards formation of polyols and a possible decreased flux through the TCA cycle due to lower activity of pyruvate kinase. The changes found did not disturb the energy charge in the *cre*A^d-30 mutant which is comparable to that of the wild type. Besides these effects found inD-glucose degradation it is likely that more enzyme

systems will be effected by the *cre*A mutation, which could have consequences when studying gene regulation of particular enzymes within this *cre*⁻ background

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Summary and concluding remarks

CHAPTER 7

SUMMARY AND CONCLUDING REMARKS

The first aim of this thesis was to get a better understanding of the properties and the induction features of arabinan degrading enzymes and enzymes involved in the intracellular L-arabinose catabolic pathway in *Aspergillus niger*. The second aim was to understand the which role carbon catabolite repression plays in the induction process and to inventarize what phusiological consequences of an extreme carbon catabolite derepressed mutant are. The first part of this thesis the deals with the induction and characterisation of the arabinolytic enzyme system in *Aspergillus niger*. In the second part results of detailed studies on carbon catabolite repression of arabinase biosynthesis in *Aspergillus nidulans* are reported.

In Chapter 2 several carbon sources were tested for their ability to induce arabinan degrading enzymes in *A. niger* N400. It was found that sugar beet pulp was the best inducing substrate for the induction of three arabinolytic enzymes. These three arabinan degrading enzymes were purified from the culture filtrate after growth on sugar beet pulp as the carbon source. The physico-chemical and kinetic properties of these three enzymes were characterised and found to correspond with those of the three arabinolytic enzymes purified from a commercial *A. niger* enzyme preparation by Rombouts et al. (1988). Also, the α-L-arabinofuranosidase B was found to correspond with an α-L-arabinofuranosidase B already characterised by Kaji and Tagawa (1970). Cloning and genetic analysis of the three genes by Flipphi et al (1993a; 1993b; 1993c) has shown that there are some discrepancies between the amino acid composition found by HPLC analysis and the one derived from the sequence data. This could be due to inaccuracies in HPLC analysis.

Because of the finding in Chapter 2 that the monomeric sugar L-arabinose induces arabinolytic activity, this sugar together with L-arabitol as an intermediate of the metabolic pathway of L-arabinose, was tested for inducing capacity in Chapter 3. In this case also an *A. niger* N572 xylulose kinase negative mutant strain was used which is disturbed in the last step of the metabolic pathway of L-arabinose, the conversion of D-xylulose to xylulose-5-phosphate (for a Scheme see Chapter 1 Fig. 2). This strain was characterised by Witteveen et al. (1989) by ^{13}C-NMR analysis. Xylitol and L-arabitol were found to accumulate upon mycelial transfer to L-arabinose or D-xylose containing media.

Using the sugars present in the metabolic pathway of L-arabinose and D-xylose it was found that within the wild type strain, L-arabinose induces α-L-arabinofuranosidase B but L-arabitol induces all three known arabinolytic enzymes. However, using the xylulose kinase negative mutant the level of induction on these two substrates was remarkable higher (up to 2 times). Moreover, in this mutant induction was also found using D-xylose and xylitol as a carbon source in transfer experiments. Of these two strains also the enzymes present in the catabolic pathway of L-arabinose were investigated. It was found in all cases that the activities in the xylulose kinase mutant were higher. The finding that L-arabitol dehydrogenase activity was almost 3 to 4 times higher on D-xylose and xylitol in comparison with the wild type was remarkable. Also induction of PNPA hydrolysing activity could already be detected using low amounts of L-arabitol (1.5 mM). Furthermore the induction of arabinases was found to be under the control of carbon catabolite repression. Induction on L-arabitol in the presence of 0.2% (w/v) D-glucose could only be detected when the concentration of D-glucose in the media became very low.

These experiments lead to the conclusion that L-arabitol plays an important role in the induction of the arabinases in *A. niger* and that α-L-arabinofuranosidase B may trigger the biosynthesis of the other arabinases. It is known that the arabinolytic complex releases monomeric L-arabinose (Rombouts et al. 1988). This release of L-arabinose and its subsequent uptake by *A. niger* will convert the monomeric substrate into L-arabitol and thus induce all three arabinan degrading enzymes.

Chapter 2 and 3 gave us information about the arabinan degrading enzyme system existing in *A. niger* and its induction, which could be activated by using simple low molecular weight substrates like L-arabitol an intracellular intermediate of the L-arabinose catabolic pathway. This induction system which involves both extra- and intracellular enzymes provides an excellent tool to study carbon catabolite repression of these enzymes in more detail. Chapter 4 and 5 describe experiments using *Aspergillus nidulans*, a fungus which has become a model organism for molecular genetic studies also because of the availability of a large collection of well defined mutant strains. In this case *A. nidulans* WG096, which is used as the wild type strain, was first investigated for the presence of arabinolytic enzymes (Chapter 4). It was found that *A. nidulans* WG096 only produces 2 of the known arabinases, namely α-L-arabinofuranosidase B and endo-arabinase. α-L-arabinofuranosidase A could not be detected by Western blotting and also a genomic blot probed with the *A. niger abf*A gene did not give any positive signal. Biosynthesis of both arabinases was also found to depend on pathway-specific induction and carbon catabolite repression. Like in *A. niger* L-arabitol was also found to act as an efficient inducer for both arabinases present in *A. nidulans*.

The observations made by De Vries et al. (1994) confirm the postulated role of L-arabitol in the induction mechanism of arabinan degrading enzymes. They have characterised an *A. nidulans* L-arabinose non-utilising mutant strain, which was first isolated by Roberts (1963) and characterised by Clutterbuck (1981) as an L-arabinose non-utilising mutant. It was found to be a L-arabitol dehydrogenase negative mutant strain. They also investigated the formation of polyols when transferred to L-arabinose containing media and found a strong accumulation of intracellular L-arabitol. This accumulation of L-arabitol resulted in this strain in a strong induction of the two *A. nidulans* arabinases, which was confirmed both by enzymatic analysis as by Western blotting.

The existence of some genetically well defined carbon catabolite derepressed mutants (Arst and MacDonald 1975; Arst and Bailey 1977; Dowzer and Kelly 1989;1991) gave us an excellent tool to investigate the carbon catabolite repressing phenomena in more detail with respect to the induction of the two arabinases present in *A. nidulans*. In this case also the response of the intracellular L-arabinose degrading enzymes to carbon catabolite repression was investigated together with intracellular xylitol and L-arabitol measurements (Chapter 5). The data found indicate a strong influence of *cre*A on the biosynthesis of both α-L-arabinofuranosidase B and endo-arabinase. The effect of the *cre*A mutations was found to be most marked under inducing conditions. Both the more extreme carbon catabolite derepressed mutants *cre*A^{d}-30 and *cre*A^{d}-4 show a remarkable induction of arabinase activities up to 6- to 10 fold the level reached in the wild type suggesting a considerable 'self' repression in the wild type. In the presence of D-glucose plus an inducer the *cre*A mutations, particularly *cre*A^{d}-30, result in carbon catabolite derepression although the level of activity found did not reach the level which was

found under inducing conditions. The intracellular enzymes present in the L-arabinose catabolic pathway show a more non-hierarchical behaviour. For example, whereas L-arabitol is a better inducer than L-arabinose of L-arabinose reductase in the *cre*A^{d}-4 strain, the reverse is true for the *cre*A^{d}-2 strain. Furthermore, from the finding that intracellular xylitol and thus L-arabitol could be detected on a mixed carbon source (D-glucose/L-arabinose) we can conclude that uptake of L-arabinose and conversion takes places even in the presence of D-glucose as a repressing carbon source.

Although the direct role of the CREA protein in carbon catabolite repression by acting as a repressor of gene expression has now been elucidated (Kulmburg et al 1993; Cubero and Scazzocchio 1994), the physiological consequences of this mutation have not yet been investigated. Therefore a comparison was made on the level of enzyme activities and metabolite concentrations present in the metabolic pathway of D-glucose and polyol concentrations which can be derived from this route (Chapter 6). The enzymatic data obtained suggest that only a few enzymes are, directly or indirectly, influenced by the CREA protein. These are hexokinase and fructose-6-phosphate reductase, which were found to have elevated activities and phosphofructokinase and pyruvate kinase, which were found to show decreased activities within the *cre*A^{d}-30 mutant. Both the metabolites fructose-2,6-diphosphate and fructose-1,6-diphosphate, which are the respective activators for the last two enzymes, were found to have increased internal concentrations. The result of these changes in activity is that this leads to a higher flux through the side chains towards the formation of polyols and possibly to a decreased flux through the TCA cycle due to the decreased activity of pyruvate kinase. The accumulation of polyols in the *cre*A^{d}-30 mutant strain is

remarkable. Although the amount of polyols produced intracellularly remains at a constant level in both strains, the total amount (intra- and extracellular) produced by the *cre*A^{d}-30 mutant strain is almost up to 3 times the total polyol concentration formed by the wild type strain. However the nature of the repressing signal remains unclear.

The induction of the arabinolytic enzyme system in *A. nidulans* was studied together with its response to carbon catabolite repression by using different carbon catabolite derepressed mutant strains. A study of the carbon catabolite repression on extracellular and intracellular enzymes together with an an easy induction system of these enzymes is scarce. The high induction of arabinase activity on L-arabitol in the extreme mutant strain *cre*A^{d}-30 has almost the same level as found in wild type *A. niger* under normal induction conditions. In this case it would be interestingly which level of arabinase activity could be reached if carbon catabolite derepressed mutants of *A. niger* become available.

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Samenvatting

Het in dit proefschrift beschreven onderzoek richtte zich op de inductie en karakterisatie van het arabinolytische enzym systeem in de filamenteuze schimmels *Aspergillus niger* en *Aspergillus nidulans*. Tevens is in de schimmel *A. nidulans* onderzoek verricht naar het fenomeen koolstof kataboliet repressie en het effect daarvan op de inductie van de arabinases. Hoofdstuk 1 geeft een algemene inleiding over dit onderwerp. In Hoofdstuk 2 zijn verschillende koolstof bronnen getest op hun vermogen arabinases te induceren. Daarbij werd gevonden dat suikerbieten pulp in *A. niger* drie verschillende arabinases induceerde. Deze drie arabinases zijn gezuiverd en gekarakteriseerd voor wat betreft hun physico-chemische eigenschappen en kinetische constanten. De gevonden enzymen bleken te corresponderen met drie arabinases die gezuiverd werden uit een commercieel enzym preparaat.

Doordat in Hoofdstuk 2 was aangetoond dat ook de monomere suiker L-arabinose arabinolytische activiteit induceerde is in Hoofdstuk 3 onderzoek verricht naar het vermogen van L-arabinose, en intermediairen uit de afbraak route van L-arabinose, om arabinases te induceren. Daarbij is ook gebruik gemaakt van een xylulose kinase mutant die gestoord is in de laatste stap van de afbraak route van L-arabinose. Van deze mutant is bekend dat de polyolen L-arabitol en xylitol intracellulair opgehoopt worden vanuit L-arabinose en D-xylose. Gebruik makend van immunochemische technieken bleek dat L-arabinose α-L-arabinofuranosidase B induceerde, maar dat L-arabitol alle drie de arabinases induceerde. In de mutant bleek L-arabinose dit ook te doen. Tevens trad in deze mutant inductie van arabinases in aanwezigheid van D-xylose en xylitol. De inductie van de arabinolytische activiteit bleek afhankelijk te zijn van de aanwezigheid van D-glucose in het medium. Was D-glucose samen met

een inducerende koolstof bron (L-arabitol) aanwezig dan werd geen inductie gevonden. Daalde de D-glucose concentratie in het medium tot zeer lage waarden dan kon arabinolytische activiteit aangetoond worden. Deze experimenten leiden tot de conclusie dat L-arabitol een belangrijke rol speelt in het inductie mechanisme van de arabinases en dat het systeem onder koolstof kataboliet repressie staat.

Terwijl *A. niger* een industrieel belangrijk organisme is, met name voor de produktie van extracellulaire enzymen is *A. nidulans* een belangrijk model organisme voor moleculair genetisch en fysiologisch onderzoek. Daarom is in Hoofdstuk 4 onderzocht welke arabinases aanwezig zijn in *A. nidulans*. Daarbij is gevonden dat *A. nidulans* α-L-arabinofuranosidase B en endo-arabinase produceerde. α-L-arabinofuranosidase A kon niet worden aangetoond. Ook het gen kon niet met behulp van een heterologe hybridisatie worden aangetoond. De controle van het inductie mechanisme van de twee arabinases was hetzelfde als voordien gevonden in *A. niger*.

De aanwezigheid van enkele goed gedefinieerde koolstof kataboliet gederepresseerde stammen van *A. nidulans* gaf een goede gelegenheid dit fenomeen met betrekking tot de inductie van de arabinases nader te bestuderen. Dit deel van het onderzoek is beschreven in Hoofdstuk 5. Ook de enzymen aanwezig in de intracellulaire afbraak route van L-arabinose zijn daarbij meegenomen samen met de bepaling van intracellulaire concentraties van de polyolen xylitol and L-arabitol. Het bleek dat er een sterke invloed is van *cre*A op de biosynthese van α-L-arabinofuranosidase B en endo-arabinase, waarbij het effect het sterkst was onder inducerende condities. De twee extreme mutanten *cre*A^{d}-30 en *cre*A^{d}-4 gaven een 6- tot 10-voudig hogere inductie te zien dan het wild type. Dit suggereert een zeer sterke 'zelf' repressie van deze enzymen in het wild type. Onder represserende/inducerende condities werd vooral in de *cre*A^{d}-30 stam derepressie gevonden alhoewel het niet het niveau bereikte dat onder inducerende condities bereikt werd. De resultaten van de intracellulaire

enzymen gaven een meer niet-hiërarchisch beeld te zien. Bijvoorbeeld daar waar L-arabitol een betere inducer is voor L-arabinose reductase dan L-arabinose in de *cre*A^{d}-4 stam is het tegengestelde waar voor de *cre*A^{d}-2 stam. Tevens kon xylitol en arabitol aangetoond worden onder groei condities met gemengde koolstof bronnen, wat suggereert dat ook onder represserende omstandigheden L-arabinose opgenomen en omgezet word.

Hoewel de directe rol van het CREA eiwit als repressor opgehelderd is, is het mechanisme waarbij de cel de aanwezigheid van glucose of een andere represserende koolstof bron detecteert en het koolstof kataboliet repressie systeem activeert niet bekend. Ook de consequencies van mutaties in het koolstof kataboliet repressie systeem voor de gehele fysiologie van de schimmel is onbekend. Om hier meer inzicht in te krijgen is in Hoofdstuk 6 onderzoek gedaan naar de fysiologische veranderingen die optreden in de *cre*A^{d}-30 mutant bij groei op glucose vergeleken met het wild type. Hierbij is een vergelijking gemaakt op het niveau van enzymen aanwezig in de afbraak route van glucose, de concentraties van de metabolieten in deze afbraak route en de concentratie van polyolen welke uit deze route kunnen ontstaan. De enzym gegevens suggereren dat maar enkele enzymen, direct of indirect, door het CREA eiwit beïnvloed worden. De enzymen hexokinase en fructose-6-P reductase werden in verhoogde activiteit aangetroffen. Daarnaast vertoonden de enzymen phospfofructokinase en pyruvate kinase echter een verlaagde activiteit in de *cre*A^{d}-30 mutant. De beide metabolieten fructose-2,6-diphosphaat en fructose-1,6-diphosphaat, welke de respectievelijke activatoren van de laatste twee enzymen zijn, werden in verhoogde intracellulaire concentraties gevonden. Het resultaat van deze veranderingen is een hogere flux door de zij-paden die aftakken van de glycolyse en betrokken zijn bij de formatie van polyolen. De hoeveelheid aan polyolen aangemaakt door de *cre*A^{d}-30 stam was ook sterk verhoogd. Intracellulair waren de totale concentraties tussen de *cre*A^{d}-30 stam en het wild type weliswaar

ongeveer gelijk, maar de totale hoeveelheid gevormde polyolen (intra- en extracellulair) in de *creA*^{d}-30 stam was ongeveer 3 maal verhoogd.

Curriculum vitae

Peter van der Veen werd op 2 juni 1961 geboren te Groningen. In 1978 behaalde hij het HAVO diploma aan de Christelijke Scholengemeenschap te Leek. In datzelfde jaar begon hij te studeren aan de Nieuwe Leraren Opleiding "Ubbo Emmius" te Groningen met als vakken Biologie en Scheikunde. In 1985 behaalde hij daar zijn 2e graads bevoegdheid, waarna hij in september verder studeerde aan de subfaculteit Biologie van de Rijks-Universiteit te Groningen. Hij studeerde in 1988 af met als hoofdvak Microbiële Fysiologie en bijvak Fysiologische Chemie/Moleculaire Genetica en 1e graads lesbevoegdheid. In september van datzelfde jaar werd hij aangesteld als A.I.O bij de Sectie Moleculaire Genetica van Industriële Micro-organismen van de Landbouwuniversiteit Wageningen op een project dat gefinacieerd werd door de Programma Commissie Landbouw Biotechnologie (PcLB) wat resulteerde in dit proefschrift.

Het Nawoord

Zoals dit boekje het einde betekent van mijn promotie onderzoek, zo is Het Nawoord vaak het einde van het proefschrift. Daarbij is Het Nawoord toch wel de meest gelezen, en mischien ook wel de eerste en enige gelezen bladzijde van een proefschrift. Men wil blijkbaar graag weten of men met naam genoemd worden en wat er evt. over hen gezegd wordt. Toch zal ik niet iedereen bij name noemen doch dat wil niet zeggen dat ze niet op een of andere manier een bijdrage, hoe klein ook, geleverd hebben al was het alleen maar het mee bepalen van de leuke sfeer op het lab.
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En natuurlijk niet te vergeten mijn ouders, broers en schoonzussen die ieder op hun manier een motiverende werking hadden om dit onderzoek goed af te ronden.


WAT IS SUCCES? VOLGENS SOMMIGEN ROEM EN RIJKDOM... VOLGENS ANDEREN GEWOON EEN GEVOEL VAN EIGEN WAARDE, MAAR VOLGENS MIJ IS HET EEN BIJDRAGE AAN HET WELZIJN VAN DE MENSHEID.
WAT IS JOUW DEFINITIE VAN SUCCES, GARFIELD?
JIM DAVIS 7-7
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TWINTIG PIZZA'S ETEN ZONDER OVER TE GEVEN.
WAT JE OOK ZEI, WE VOELDEN HET AL AANKOMEN.