Amino acids involved on binding and catalysis of *Aspergillus niger* pectin lyase A.

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Introduction. Pectin is a polysaccharide widespread in nature, and it is probably the most complex carbohydrate in plants. The pectin molecule consists of two main regions commonly referred to as "smooth" and "hairy" regions. The first of these two is more or less homogeneous and consist of α-1,4 linked D-galacturonic acid residues, usually methylesterified at C6. The degree of esterification is variable but is commonly up 70%. The second, "hairy", region is the more complex part of pectin and consists of D-galacturonate residues alternating with α-1,2 linked L-rhamnose. L-rhamnose usually carries side chains of galactans and arabinans. Due to the complex structure of pectin molecule, a great variety of enzymes is needed for its complete degradation. *Aspergillus niger* pectinases identified thus far include a family of six pectin lyases (PL), a family of seven endo-polygalacturonases (PG), one pectin esterase (PME), one exo-polygalacturonase (PGX), a pectate lyase (PLY), two rhamnogalacturonases (RHG), one rhamnogalacturonan lyase (RGL) and one rhamnogalacturonan acetylesterase (RGAE). Many pectinases from Aspergillus spp. amongst which pectin lyases are extensively used in the food industry for fruit juice extraction and clarification. Pectin lyases depolymerize pectin by a β-elimination mechanism resulting in the formation of a double bond between C-4 and C-5 of galacturonate at the non-reducing end. Lately for several pectinases the 3-D structure has been elucidated. This includes PL, PLY, endoPG, RHG and PME. For all these enzymes with their different reactions it was found that they all have the same topology. The main chains all have the same basic right-handed parallel β-helix architecture. However, the side chains at the active sites and the loops forming the substrate binding cleft have diverged extensively. All structures show a typical cleft in which the substrate is bound to the enzyme and catalysis takes place. Pectin-lyase A produced by *Aspergillus niger* has been cloned and sequenced and the structure elucidated (1). Structure-based studies between several pectin and pectate lyases reveal that two amino acids are conserved at the active site, these are Asp154 and Arg 236. For PLA a number of mutations have been prepared and the effect of each mutation on the catalytic properties of the enzyme have been studied. The results clearly demonstrate that Asp154 and Arg236 play a role in catalysis, respectively. Here we present some results of our attempts to access by molecular modeling approach combined with site-directed mutagenesis and kinetic measurements, the binding mode of pectin lyase A as well as the amino acids directly involved in binding and catalysis.

Materials and methods. Site directed mutagenesis was carried out on the promoter gene fusion pki-pelA (pIM3500) using the QuickChange site direcetd mutagenesis kit (Statagene, La Jolla, CA USA) and synthetic oligonucleotides (Isogen, Maarsen, The Netherlands) as we described previously (2). The promoter gene fusion consists of the strong promoter of the pyruvate kinase gene and the pelA gene allowing expression of PLA (mutants) during growth on glucose. Plasmid DNA was isolated and sequenced to confirm the desired mutation. Those plasmids showing the correct sequence were used to transform *A. niger* strain NW188. Mutant PLA producing transformants were selected by growing individual transformants in minimal medium (3). Wild type and mutant PLA were purified from culture medium of *A. niger pki-pelA* multicopy transformants, using anion exchange columns. The purity of enzyme was confirmed by SDS-PAGE. Standard PLA assays were carried out at pH 6.0 and 30°C using pectin with different degree of esterification. The activity was determined by measuring the increase of absorbance at 235 nm (ε_{235nm} = 5200 $M^{-1}cm^{-1}$) as a result of the double bond formation. Km and Vmax values were determined from triplicate initial rate measurements in the same way described for the standard assays in which the pectin concentration was varied from 0.3 to 5 $mg.ml^{-1}$.

Results and Discussion. A fully esterified galacturonic acid decamer was constructed *in silico* to be used for substrate modelling and to assess where methyl groups might be bound to the enzyme. To evaluate the validity of the structure, mutagenesis was carried out at subsites –2 , Val180Ser and Asp217His, and +3, Ala204Ser and Tyr211His. These two subsites accommodate a methyl group in the modelled complex. The effect of mutation on catalysis and substrate specificity, random versus blockwise demethylated substrates was assessed.

The biochemical characterisation of each mutant enzyme was done and the kinetic parameters determined. It appears that Val180 and Ala204 are involved in binding and Tyr211 is apparently necessary for catalysis rather that binding since Vmax was reduced drastically. The role of Asp217 is still not clear and we can not explain its effect at this time.

Conclusions. Molecular modelling, site-directed mutagenesis and kinetic measurements have allowed to

propose a detailed picture of the amino acids directly involved in binding and catalysis for the pectin lyase A β-elimination cleavage of methylesterified polygalacturonic acid.Our data are not conclusive with respect to accommodation of a methyl group at subsite –2 and / or +3.

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