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The active component in the flax-retting system of the zygomycete *Rhizopus oryzae* sb is a family 28 polygalacturonase

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Abstract The zygomycete *Rhizopus oryzae* sb is a very efficient organism for retting of flax, the initial microbiological step in the process of making linen. An extracellular polygalacturonase, when isolated could perform retting, and therefore probably is the key component in the retting system of *R. oryzae*. This was purified and characterized. The purified enzyme has a molecular mass of 37,436 Da from mass spectrometric determination, an isoelectric point of 8.4, and has non-methylated polygalacturonic acid as its preferred substrate. Peptide sequences indicate that the enzyme belongs to family 28, in similarity with other polygalacturonases (EC. 3.2.1.15). It contains, however an *N*-terminal sequence absent in other fungal pectinases, but present in an enzyme from the phytopathogenic bacterium *Ralstonia solanacearum*. The biochemical background for the superior retting efficiency of *R. oryzae* sb is discussed.

Keywords Enzymatic retting · *P*olygalacturonase · *Rhizopus oryzae* · *P*ectin biodegradation · *Ralstonia solanacearum*

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Introduction

Bast fibres from plants like flax (*Linum usitatissimum*) and hemp (*Cannabis sativa*) have excellent qualities for use in textiles, high quality papers and composites. However, for most applications a processing is necessary, which separates the useful bast fibres from other plant tissues with fibres of lower quality. Retting is such a biotechnological treatment that degrades the pectin-rich middle lamella connecting adjacent fibre cells to release the bast fibre from the epidermis/cuticle and lignified core fractions of the flax stems, producing free fibre-bundles and, ultimately, free elementary fibres (cells) [28]. In the past, flax stems were submerged in rivers and lakes to be retted by anaerobic bacteria (water-retting). However, this practice was discontinued in most western countries several decades ago, because of the pollution from fermentation products and the high cost of drying. The method in use instead is *dew-retting*, where the harvested straws are spread on fields for fungal degradation of the middle lamella. The disadvantages of this method lie both in weather dependency and risk of fibre damage by cellulolytic fungi [2, 9], and the quality in general is also considered to be lower than that of the water-retted material [23]. Retting is one of the greatest problems in flax/linen fibre production [23], and therefore, there is a need for more rational retting procedures. Alternative ideas for retting have been developed, such as chemical retting using chelating agents and/or high pH [6, 10, 22], and enzymatic retting using suitable enzyme mixtures [28]. The latter technique results in cleaner fibres of higher and more consistent quality [27]. The enzyme mixtures (normally fungal culture filtrates) applied for enzymatic retting, like *Flaxyme*, contained a large number of different enzymes, including different types of pectinases, hemicellulases and cellulalses. Pectin is a highly complex polysaccharide with a main chain of partly methanol-esterified galacturonic acid residues and some rhamnose, and side

chains rich in arabinose and galactose. Therefore there are several types of pectinases, as enzymes directed on methylesterified or unesterified pectin, respectively [21]. It was claimed that in addition to the different types of pectinases *hemicellulases* and maybe *cellulases* were also necessary components for efficient retting, since pectinase activity on intact pectin structures were not proportional to retting efficiency. There are even examples of enzyme mixtures with very high pectinase activity that lack retting efficiency [30]. In contrast, we have found that the retting abilities of different enzyme mixtures are strongly correlated only to polygalacturonase activity, i.e., hydrolysis of glycosidic bonds in non-esterified polygalacturonan, and that a pure polygalacturonase can perform efficient retting [30].

In an earlier study, several organisms were isolated from dew-retted flax at various locations and re-inoculated on flax straws. The retting efficiency varied considerably between the different organisms [9], but *Rhizopus oryzae* sb (misidentified as *Rhizomucor pusillus* earlier) was superior. It appeared to remove middle lamella material and separate bast fibre at a relatively long distance from the mycelium [2], suggesting that *R. oryzae* sb produced extracellular enzymes that could independently degrade the middle lamella. Furthermore, cellfree *R. oryzae* sb culture filtrate cultivated on citrus pectin was very efficient in separating bast-fibres from flax straws [11]. Therefore, this zygomycete is an ideal model system for studying the mechanisms of dew-retting and enzymatic retting of flax. Interestingly, no hemicellulase activity was detected in this culture filtrate indicating that removal of hemicellulose is not important in the retting process. Pectinases are the only enzymes identified as active components so far [11], and a purified pectinase was shown to perform retting [3].

In this study, a polygalacturonase from *R. oryzae* was purified. The isolated enzyme was characterized in terms of molecular mass, isoelectric point, amino acid composition and peptide sequence. The ability of the enzyme to degrade different pectins, such as citrus pectin, low-esterified pectin, high-esterified pectin and orange peel that represent the native protopectine—structure with rhamnose residues and arabinose and galactose-rich side chains [21]—was tested, and its retting ability was compared to that of the culture filtrate. The sequence data allowed family assignment for the enzyme.

Materials and methods

Materials

Bio-Gel P-100 and Bio-Gel P-150 were purchased from Bio-Rad. Hiload TM 26/10 SP sepharose high performance columns were obtained from GE Healthcare. BR-14 fermentor was from Belach Bioteknik AB, Sweden. Polygalacturonic acid sodium salt, pectin and esterified pectin were obtained from Sigma, St Louis, USA. Bovine trypsin, sequencing grade, was from Roche, Switzerland. The orange peel powder was prepared as described previously [29]. All other reagents were of analytical grade.

Microorganism

R. oryzae strain sb isolated from flax dew-retted in South Carolina, USA [9] was used. The strain is deposited at the American collection of microorganisms (NRRL 29086).

Cultivation

The organism was cultured in Vogel's medium [28] containing per litre: 2.5 g sodium citrate, 5 g KH_2PO_4, 2 g NH_4NO_3, 0.4 g $MgSO_4{\cdot}7H_2O$, 0.2 g $CaCl_2{\cdot}H_2O$, 2.5 mg $FeSO_4$, 0.98 mg $MnSO_4{\cdot}H_2O$, 0.83 mg $ZnCl_2$, 1.0 mg $CoCl_2$. The pH was adjusted to 5 using concentrated HCl. All cultivations were done at 30 °C. A 300 mL shaken flask starting culture with 10 g/L citrus pectin (Sigma) was used to inoculate 7 L of Vogel's medium containing 2 g/L citrus pectin in a BR-14 fermenter with 10 L working capacity. During cultivation, pH was regulated at 5 adding H_3PO_4 or NaOH solution, pressure at 0.5 atm overpressure, and aeration was 3 L/min. The stirring rate of the 15 cm diameter flat bed impeller was kept as low as 200 RPM to avoid damage of the mycelium by sharing forces. About 3 L of 30 g/L citrus pectin in Vogel's medium was fed to avoid a possible carbon catabolite repression like during cellulase production [24]. Samples were withdrawn regularly. The culture was harvested after 114 h.

Pectinase assay

The enzyme solutions were incubated in a final volume of 750 µL 50 mM sodium acetate buffer, pH 5, with 1% substrate for 10 min at 37 °C. The reaction was terminated by addition of 750 µL dinitrosalicylic acid (DNS) reagent [17]. Insoluble material was removed by centrifugation and the samples were boiled for 5 mins. The concentration of released reducing sugars was determined by measuring absorbance at 640 nm, using a glucose standard treated in parallel. Background was compensated with control samples without enzyme and substrate.

Protein purification

The enzyme was purified from 10 L culture filtrate of *R. oryzae* sb. After cultivation, insoluble material was removed from the liquid by centrifugation and the supernatant was filtered. Sodium azide (0.05%) was added to prevent microbial growth. The resulting culture filtrate was concentrated 100-fold at 4 °C by ultrafil-

tration. All other purification steps were at room temperature. The concentrated culture filtrate was applied to a Bio-Gel P-100 column (3×80 cm) equilibrated with 50 mM ammonium acetate, pH 5.0, at a flow rate of 10 mL/h. The fractions with pectinase activity were pooled, diluted five-fold with Milli-Q water and applied to a High load-S column (2.6×10 cm) equilibrated with 10 mM ammonium acetate, pH 5.0. The adsorbed material was eluted by a linear gradient of 10–50 mM sodium phosphate, pH 6.0 at a flow rate of 0.5 ml/min. The fractions containing pectinase activity were pooled and applied to a Bio-Gel P-150 gel filtration column (1.5×90 cm) equilibrated with 50 mM ammonium acetate, pH 5.0, at a flow rate of 5 mL/h. The fractions with pectinase activity were pooled and applied to a Mono-S cation exchange column. The adsorbed material was eluted by a linear gradient of 10–50 mM sodium phosphate, pH 6.0, at a flow rate of 0.4 mL/min.

Analytical electrophoresis

Enzyme purity was controlled by SDS-PAGE in a MIDGET electrophoresis unit as described by Maizel [15]. The separation gel contained 10% (w/v) polyacrylamide, and the protein bands were stained with Coomassie Brilliant Blue G-250 or silver staining [16]. Analytical isoelectric focusing was performed with Phast System (GE Healthcare) according to the instructions of the supplier, using precast polyacrylamide gel in the pH range 3–9.

Amino acid analysis

Amino acid analysis was carried out in an LKB alpha plus analyser after hydrolysis for 24 h at 110 °C in 6 M HCl. Tryptophan content was estimated spectrophotometrically.

Protein identification by mass spectrometry

The two purified fractions containing pectinase activity were desalted to 50% acetonitrile containing 0.1% fomic acid using C4-ZipTip unit (Millipore) before being introduced to the mass spectrometric analysis.

The SDS-PAGE gel bands (Coomassie Brilliant Blue stained) corresponding to the purified pectinase and an unknown protein with molecular weight of 66 kDa were cut out and subjected to digestion by trypsin solution as described earlier [7, 19]. The samples were desalted to spraying solution (1:1 acetonitrile/water containing 0.1% formic acid) using C-18 ZipTip unit (Millipore).

The protein and peptide samples were analysed on an ESI-Q-Tof II mass spectrometer fitted with a nano Z spray source (Waters Corporation, Micromass MS Technologies, Manchester, UK). The protein was introduced into the mass spectrometer by infusion through the spray source with a syringe pump, 200 nL/min (source voltage 3.3 kV). The tryptic peptides were introduced into the source by off-line needle spraying (capillary voltage 1.5 kV). Tof-MS data of the peptide mixtures were acquired over the m/z range 500–2,000, and Tof-MS/MS data of the individual peptides were acquired over the mass range m/z 50–2,000, with various collision potential of 30–55 V. De novo sequencing was performed using the MassLynx Software's PepSeq to achieve the peptide sequence tags.

Identification of sugars released from protopectin (orange peel powder)

About 200 mg of orange peel powder were washed three times by suspending in water and incubating at 4 °C for 2 days, in order to reduce background. The washed samples were incubated with and without 0.005 absorbance units of purified enzyme in 10 ml of 10 mM ammonium acetate buffer at 37 °C overnight. Sample aliquots were then withdrawn to quantify the released reducing sugar by the DNS method [17]. The remaining samples were centrifuged and the supernatants were freeze-dried. In the enzyme-treated sample, 70 µmol of reducing sugar was released after 48 h of incubation. The sugar composition of the freeze-dried samples were analysed by capillary electrophoresis in the sugar analysis laboratory of Swedish Pulp and Paper Research Laboratory (STFI, Stockholm, Sweden) as described by Dahlman et al. [5].

Retting experiment (modified Fried test)

About 10 cm long flax straws were incubated in 10 mL of 50 mM ammonium acetate buffer, pH 5.0 containing 0.01 M EDTA [10] and 0.005 absorbance (280 nm) units of purified enzyme. Flax straws and the retting formulations including controls were then incubated for 23 h at room temperature with end-over-end agitation. A modified version of the Fried test [6, 10, 18] was used to monitor the fibre separation [30, 31].

To avoid bias, all samples were given random numbers and the examination was done by an examiner without knowledge of the exact treatment of the individual sample. The experiments were always done in triplicates and their average score was regarded as the degree of fibre separation.

Results

Purification and characterization of the enzyme

The procedure and yields are summarized in Table 1. Isoelectric focusing and SDS PAGE (Fig. 1) of the final enzyme preparation gave single bands corresponding to pI of 8.4 and apparent molecular mass of around

Table 1 Purification of polygalacturonase from *R. oryzae* sb

Step	Volume ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Crude filtrate	60	133	18.3	0.138	–	100
Bio-Gel-P-100	130	27.78	2.12	0.076	0.55	11.6
Hiload Sp	3.2	1.467	0.86	0.586	4.25	4.7
Bio-Gel-P-150	3	0.1975	0.645	3.266	23.67	3.5

40 kDa. The normalized amino acid composition is found in Table 2. The final Mono-S chromatography step did occasionally provide two separate peaks with virtually identical functional and chemical properties.

Mass spectrometric characterization of the enzyme

Mass spectrometric analysis of the purified enzyme gave an accurate mass determination of the purified protein as 37,436 Da (Fig. 2), with very small contribution of side bands. Mass spectrometric analysis of tryptic peptides of the protein by de novo sequencing gave the peptide sequences in Table 6. Homology search in the Swissprot database indicated that peptides (i) and (ii) were clearly homologous to polygalacturonase sequences in glycosyl hydrolase family 28. Figure 3 shows alignments to five pectinases that represent different phylogenetic groups. The peptide tag NTDAIDVSSSS-GIIFK contains a conserved region in family 28. Two other peptides showed homology to a polygalacturonase from the bacterium *R. solanacearum*.

Substrate specificity

The purified enzyme showed the highest activity towards non-methyl esterified polygalacturonic acid (Table 4), but could also degrade crude citrus pectin, esterified pectin and protopectin (orange peel powder), although with lower activity than the non-esterified pectin. When the sugars released from the latter substrate were analysed, only galacturonic acid concentration was significantly higher than that in the control (Table 3).

Retting experiment

Retting experiment was performed with concentrated culture filtrate and the pectinase purified as shown earlier above. The purified polygalacturonase could clearly perform retting (Table 5).

Discussion

A pectin-degrading enzyme was purified from culture filtrate of *R. oryzae* sb cultivated on citrus pectin to a single band on SDS PAGE (Fig. 1) and isoelectric focusing. This enzyme was the only protein present in the culture filtrate that released reducing sugar from Sigma citrus pectin, which is a complex pectin structure with both esterified and non-esterified domains. The final Mono-S chromatography did occasionally give two active peaks. The components in the two peaks were, however, identical with respect to not only isoelectric points and SDS-PAGE migration (Fig. 1) but also in mass spectrometry and enzyme specificity (Table 4).

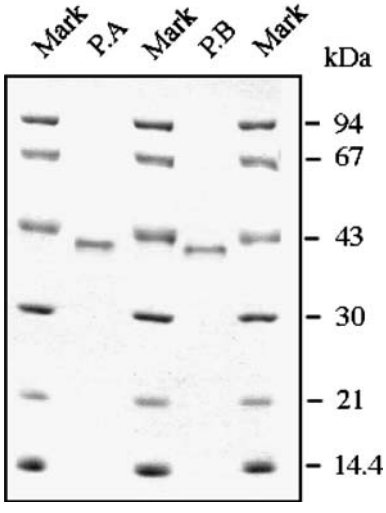


Fig. 1 SDS-PAGE of purified enzyme fraction A and B Mark: molecular weight marker. *PA* Purified enzyme fraction A, *PB* purified enzyme fraction B

Table 2 Amino acid composition of the purified polygalacturonase

Amino acid residue	Mole percent
Aspartic acid and asparagine	14
Threonine	9.4
Serine	12.5
Glutamic acid and glutamine	11
Proline	3.1
Glycine	9.2
Alanine	7.9
Valine	7.4
Methionine	0.5
Isoleucine	4.6
Leucine	5.3
Tyrosine	2.0
Phenylalanine	2.0
Histidine	1.3
Lysine	4.8
Argenine	1.3
Tryptophan	4.6
Cysteine	Present

The tryptophan content was estimated spectrophotometrically

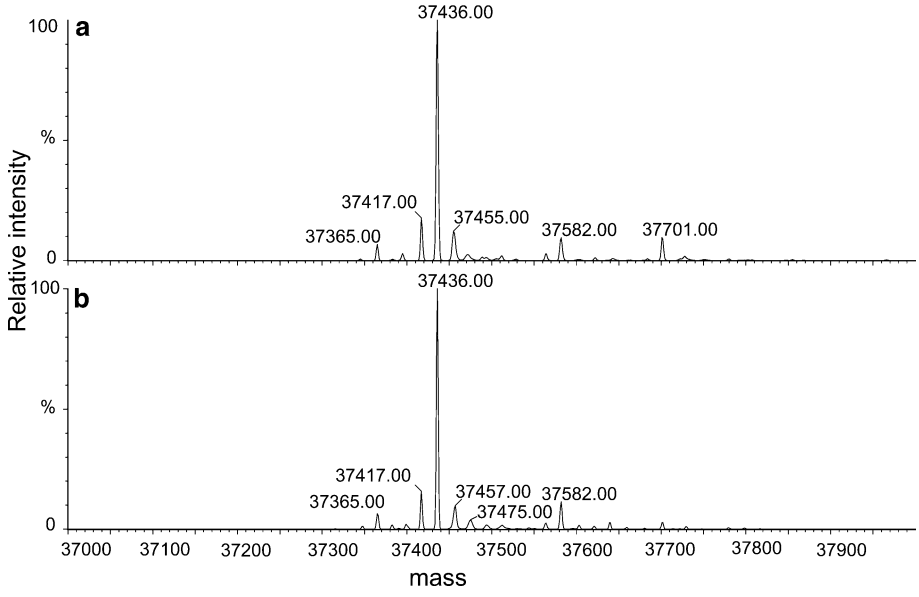


Fig. 2 Mass determination of the purified protein

Table 3 Sugars released from orange peel protopectin by purified polygalacturonase

Sugar released	Control (μg/mg)	Enzyme incubation (μg/mg)
Glucose	10	3
Arabinose	14	11
Galactose	42	40
Galacturonic acid	0	40

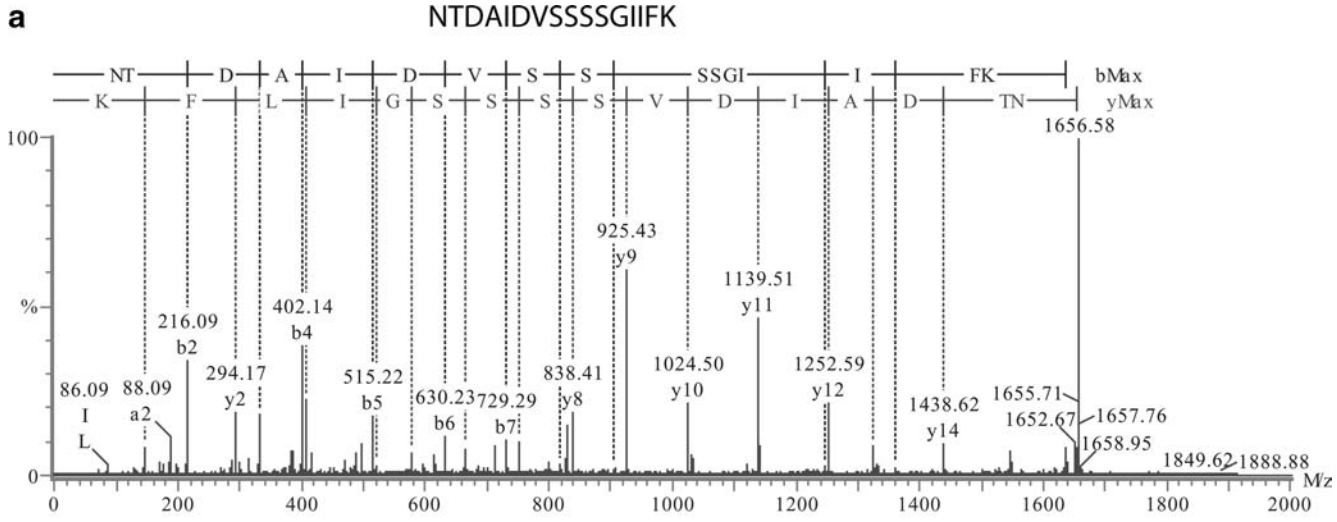
Table 4 Substrate specificity of purified enzyme fractions

Fraction	Substrate mM/(min Absorbance unit)			
	Pectin	Polygalact-uronic acid	Esterified pectin	Proto pectin (orange peel)
A	56.4 ± 14.6	77.9 ± 25.8	42.8 ± 14.3	17.2 ± 13.9
B	69.8 ± 14.5	76.2 ± 13.9	35.5 ± 13.8	17.4 ± 14.5

Therefore, it is not likely that they represent transcription products from different genes, or even different glycosylation of the same enzyme. One explanation is that the two peaks represent a slow equilibrium between two alternative conformations, which sometimes can lead to such observations [26]. The preferred substrate is unesterified polygalacturonan and thus the enzyme can be classified as a polygalacturonase. Some peptide sequences were obtained by masspectrometry sequencing of tryptic digests (Table 5), and one of the peptides ("i" in Fig. 3) corresponded to a conserved region in family 28 of glycosyl hydrolases [12]—the family where most of the known polygalacturonases belong to—strongly indicating that the *R. oryzae* sb enzyme also belongs to this family. This is further supported by the homology of another peptide ("ii" in Fig. 3). The mass of 37,436 Da is considerably higher than that of, e.g., the polygalacturonase purified by Saito et al. [19] from *R. oryzae* NBRC 4707, and larger than that of most other fungal polygalacturonases as well. Interestingly, two peptide sequences ("iii" in Fig. 3) suggest that this polygalacturonase carries an *N*-terminal sequence that is not present in other fungal family 28 pectinases, but present in a bacterial polygalacturonase from *Ralstonia solanacearum* (former name *Pseudomonas solanacearum*) (Fig. 3) and also in some other proteins including a fungal (*Aspergillus chavachii*) endoglucanase. *R. solanacearum* is a plant parasite that causes severe damages (brown rot) among others such as potato and ginger. The polygalacturonase is one of the components in the extracellular machinery of this pathogen, which loosens the structure of the plant cell wall during the pathogenic attack [13]—a process not totally unlike retting. It is unclear if this N-terminal sequence plays an important role in the degradation of the pectins, and this may be a subject for further investigations.

The isolated enzyme, without involvement of any other protein, can perform efficient retting of flax (Table 5). This is in accordance with the report by Akin et al. [3] that an isolated pectinase from *R. oryzae*, probably identical to the protein described here, can perform enzymatic retting of flax, and that addition of hemicellulases and other enzymes did not improve the retting. It thus seems that *R. oryzae* sb relies on one single enzyme to attack the middle lamella pectin in flax and thereby perform the retting, possibly supported by oxalic acid or any other chelating agents that enhance the degradation ability [4, 7, 10, 30]. Sakai et al. [21] have suggested that insoluble pectin is converted to soluble pectin by special enzymes, *proto-pectinases*, before polygalacturonases can start the degradation. This model for pectin degradation, however, seems not to be relevant for *R. oryzae* sb, where obviously a single enzyme can attack the native pectin structure (Table 6).

a



b

i)

Cryptomeria japonica	231	P**NTD**GIDIFASKNFHLQKNTIGITDDCYAIGTGS
Lycopersicon esculentum	246	P**NTD**GVHVSNTQYIQISDTIIGTGDDDISIVSGS
Ralstonia solanacearum	281	K**NTD**GFDPGQSTNVVLAYSYINTGDDHVAVKASS
Aspergillus niger	183	H**NTD**GFDISESTGVYISGATVKNQDDCIAINSG-
Penicillium olsonii	185	H**NTD**AFDVGESTYITISNANIKNQDDCLAINSG-
Tryptic peptide from this work		**NTD**AIDVSSSSGI-IFK
(mass 1653.57 Da)		

ii)

Cryptomeria japonica	309	**NG**L**RIK**TWQGGS**G**MASHIIYENEMINSENP
Lycopersicon esculentum	324	**NG**V**RIK**TWQGGS**G**QASNIKFLNEMQDVKYP
Ralstonia solanacearum	362	**NG**L**RIK**SDASRG**G**KVTNIVYDGCMRNVKEP
Aspergillus niger	259	**NG**V**RIK**TIYKET**G**DVSEITYSNQLSGITDY
Penicillium olsonii	261	**NG**I**RIK**TIYKAK**G**EVADVTFSNELSNIAKY
Tryptic peptide from this work		NSITGGS**G**FPGPFTILN-L--VK
(mass 2019.01 Da)		

iii)

1) *Ralstonia solanacearum*

2) Tryptic peptides from this work (masses 1668.80 Da and 1251.74 Da)

1)	1 MNHRYT**LLAL** AA**AA**LS**AG**AH **A**TGTSVTAPW GEVAEPSLPA DSA**VC**KT**LS**A **S**ITPIKGSVD	60
2)	C**LLAL** DV**AA**VG**AG**LG **A**VR CP**VC**VN**LS**G **S**SK	

Fig. 3 Identification of the protein with pectinase activity by mass spectrometry analysis. **a** De novo sequencing of tryptic peptide sequence tag (mass 1653.57 Da). **b** Sequence alignment of the tryptic peptide sequence tags obtained from the MS/MS analysis with five pectinases found in Swiss-prot database. Tryptic peptides (protonated masses 1653.57 and 2019.01 Da respectively) were found in the consensus regions among all five selected pectinases (i and ii). Numbering used are from full length sequences of the five pectinases. *Bold letters* indicate the conserved amino acid residues

As discussed above, pectin has a complex structure with smooth regions of unesterified polygalacturonan and esterified galacturonan, and hairy regions rich in side chains of mainly galactose and arabinose residues, connected to rhamnose residues in the main chain [1, 21, 25]. Although this enzyme can attack esterified polygalacturan to some extent (Table 4), its dominating activity is towards the unesterified regions, and none or little of the side chains of the protopectin seem to be degraded (Table 3). This preference might explain the superior potential for retting of *R. oryzae* sb. As unesterified polygalacturan forms complexes known as *egg boxes* with Ca^{2+} . These egg boxes cross-link two pectin chains and their degradation most likely leads to a general weakening of the structure of the pectin-rich middle lamella, i.e., retting [30] (Fig. 4). Supporting this,

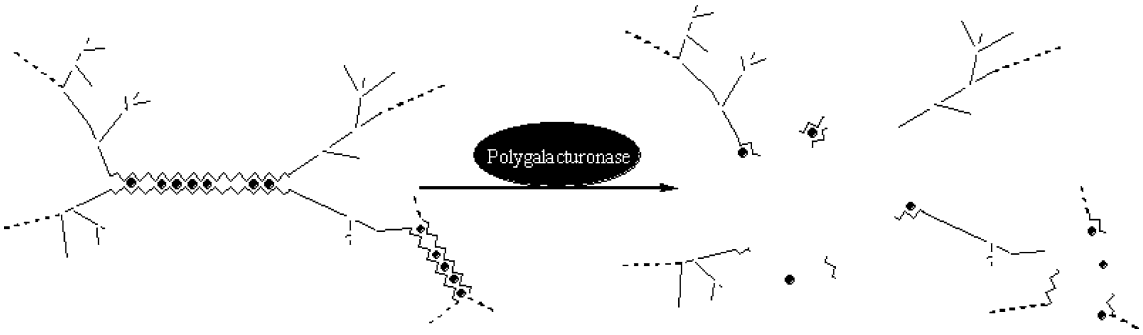


Fig. 4 Hypothesis for the mechanism of retting by *R. oryzae* sb. The protopectin structure contains branched "hairy" regions alternating with "smooth" regions rich in unesterified galacturonic acid that form crosslinking complexes, "egg boxes", with Ca^{2+}.

Table 5 Mass spectrometric analysis of the tryptic digested protein with pectinase activity

Observed tryptic peptide protonated mass (Da)	Sequence	Scores
1031.69	PGGEGWSDVK	434
1164.69	LSSTGALTRMK	86
1251.74	CPVCVNLSGSSK	152
1634.8	PDDSDGSNNCGSPNAGK	222
1653.57[a]	NTDAIDVSSSSGIIFK	368
1668.69	CLLALDVAAVGAGLGAVR	258
1677.6	NNADTPNGHNYSVFK	410
1,693	CTSENLNVTVNELEK	172
1996.8	CPNTNSNVGYFTLLGGSPR	342
2019.1[a]	NSLTGGSGFPGPFTILNIVK	1307

[a]These sequence tags are homologous to the conserved amino acid residues of pectinases from other organisms (see Fig. 3)

Table 6 Retting efficiency for a total protein load of 0.005 absorbance unit[a]

Retting agent	Retting efficiency (modified Fried test)
None (control)	0.25 (1, 0, 0, 0)
Culture filtrate	1.25 (1, 1, 1, 2)
Purified enzyme	4.75 (5, 5, 5, 4)

[a]1 millilitre of protein solution with absorbance equal to 1 at 280 nm contains one absorbance unit

Henriksson et al. [10] found that removal of Ca^{2+} from egg boxes enhances enzymatic retting. A microorganism with an advanced pectinolytic enzyme system for degrading all pectin structures should possibly be less good in retting, since the organism could more easily get soluble sugar from the whole pectin structure. *R. oryzae* sb, however, does not utilize the complete pectin structure and can only get sugar from the egg-box structures, and this glueing structure must therefore be quickly degraded to provide the organism with sufficient carbon source and energy. The pectinolytic culture filtrate of this strain did contain large amounts of acidic oligosaccharides that made an initial ion exchange step impossible (data not shown), and this material may be the residual part of the pectin chains after degradation, i.e., the section of the polymer chain with side chain oligosaccharides connected to rhamnose residues [25]. Taken together, this may lead to the surprising conclusion that the superior properties of *R. oryzae* sb for practical retting are a consequence of a very simple pectinolytic system. This insight might be important for design of improved biotechnical processes for separation of bast fibres, and may also be for better understanding of plant parasites.

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