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HEMICELLULOSE-PECTIN LINKAGE AND EXOPECTATE HYDROLASES INVOLVED IN HOMOGALACTURONAN DEGRADATION

PEKTIN-HEMICELULÓZOVÝ KOMPLEX A EXOPEKTÁT HYDROLÁZY DEGRADUJÍCÍ HOMOGALAKTURONAN

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ABSTRAKT

Tato práce se zaobírá tématikou polysacharidů rostlinné buněčné stěny potenciálně zahrnutých do tvorby pektin-hemicelulózového komplexu a vybraných enzymů degradující homogalakturonanovou část pektinu tohoto konjugátu.

V první části práce byl izolován a částečně purifikován pektin-hemicelulózový komplex z buněčných kultur petržele. S pomocí analytických metod jako FT-IR spektroskopie a NMR bylo naznačeno, že izolovaný vzorek obsahuje jednu z hemicelulóz (arabinoxylan) a jednu ze součástí pektinu, pravděpodobně homogalakturonan. Na základě získaných výsledků byl prokázán hetero-transfer mezi těmito polysacharidy s užitím hrubého proteinového extraktu z kořene petržele, sulforhodaminem značenými oligosacharidy galakturonové kyseliny (fluorescenčně značený akceptorový substrát) a arabinoxylanem hrající roli substrát donoru. Výsledky naznačují, že extrahovaný precipitát rostlinných proteinů obsahuje neznámé enzymy, s velkou pravděpodobností některou z transglykosyláz, umožňující katalýzu tohoto přenosu mezi oligosacharidy galakturonové kyseliny a arabinoxylanem. Tento druh hetero-transferu nebyl u rostlinných druhů dosud popsán.

Cílem druhé části práce byla izolace, purifikace a charakterizace pektát hydroláz štěpících polygalakturonovou kyselinu (homogalakturonan). Je popsáno celkem pět forem exo-pektát hydroláz izolovaných z kořene petržele. Hlavním rozdílem mezi těmito enzymy je substrátová preference související s délkou řetězce. Pouze jeden z enzymů preferující dekamer jako substrát byl striktně vázán na buněčnou stěnu. V dalších buněčných strukturách byly nalezeny další tři formy preferující hexamer. Dále byla identifikována pouze jedna z forem enzymu s preferencí k polymernímu substrátu (typická exopolygakturonáza), která se nacházela částečně vázána na buněčnou stěnu a částečně volně v cytosolu. Oligogalacturonát hydroláza s jedinečnou preferencí k dekameru (tato preference nebyla dosud u rostlin popsána), může hrát důležitou roli při stanovení poločasu rozpadu oligogalakturonové kyseliny u infikovaných rostlin.

KLÍČOVÁ SLOVA

Pektin-hemicelulózový komplex, *Petroselinum crispum*, exo-pektát hydrolázy

ABSTRACT

This work is focused on the plant cell wall polysaccharides potentially involved in forming of the proposed hemicellulose-pectin complex and chosen enzymes involved in degradation of the pectic part (homogalacturonan) of this conjugate.

In the first part of the work, the hemicellulose-pectin complex was isolated from parsley suspension cells and partially purified. Further analyses (using FT-IR and NMR) indicated that sample contained the hemicellulose (arabinoxylan) as well as pectin part (probably homogalacturonan). In terms of obtained results, the hetero-transfer between these polysaccharides was checked-out using the crude proteins extract from parsley roots, fluorescently, with Sulforhodamine labelled oligosaccharides derived from galacturonic acids (OGA-SR) used as an acceptor substrate and arabinoxylan (AX) used as a donor substrate.

Obtained positive results indicate that plant protein precipitates contain unknown type of enzymes, probably some kind of transglycosylase, enabling the catalysis of this hetero-transfer between OGAs and AX. This type of hetero-transfer has not been described in plant yet.

Aim of the second part was to isolate, purify and characterise pectate hydrolases operating on homogalacturonan. Five forms of exopectate hydrolases obtained from parsley roots are described in this work. The main difference between these enzymes is the substrate preference in relation to the chain length. One enzyme only with preference to decameric substrate (OGH10) was strictly bound to the cell wall. Other three forms, with preference to hexagalacturonate (OGHs6), were found in other cell structures. Only one typical form of exopolygalacturonase (exoPG) with preference to polymeric substrate was identified. The OGH10 enzyme with unique substrate preference to decamer (not described in plant yet) could be important in determining the half-life of oligogalacturonic acids in the infected plant tissue.

KEYWORDS

Hemicellulose-pectin komplex, *Petroselinum crispum*, exopectate hydrolases

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PROHLÁŠENÍ

Prohlašuji, že jsem disertační práci vypracovala samostatně a že všechny použité literární zdroje jsem správně a úplně citovala. Disertační práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího disertační práce a děkana FCH VUT.

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1. INTRODUCTION

The plant cell wall can play a key role in cell expansion control, differentiation, size and structure. Among others, plant cell wall performs structural and mechanical support for intracellular components, can also act as a medium regulating the circulation and distribution of water, minerals and nutrients. In addition, the wall constitutes the source of biological active signal molecules, which could influence intracellular processes, interaction and regulation of external factors.[1-3]

The cell wall structure is formed by a complex of various components, including polysaccharides, lignin and proteins, whereas its composition and representation can vary during the cell growth and evolution. There are many studies focused on plant cell wall changes, which occur within the cell division, growth and differentiation or during reaction to stress conditions.[4]

The cell wall of higher plants has been in question for many studies involving methods of glycochemistry, biochemistry or cell biology. Results represent the plant cell wall as a dynamic complex structure of polysaccharides and structural proteins.[5] Currently, plant research targets the proteins participating in wall loosening and degradation as well as the explanation of the polysaccharides biosynthesis and their integration into the fibril network. The understanding of plant cell growth process requires the clarification of the cell wall structure and metabolism. [6-7]

This work is focused in part on the presence of complex hemicellulose-pectin, which has been in the hub of interest for many plant cell wall investigations in recent years.[8] Although the existence of this complex has been confirmed, the origin, localization or the type of linkage require a further research. In 2003, Vincken et al. performed a new pectin model, where the homogalacturonan, xylogalacturonan and arabinan occur as a side chains to the rhamnogalacturonan type I.[9] These results invoke numbers of "old-new "questions about the structure of pectin complex and as well as provide for the comeback of enzymes operating on the pectin structure, degrading the homogalacturonan as a pectin side chain, respectively.

Accordingly, the second part of this work targets the characterization, identification and purification of chosen exopectate hydrolases, including exopolygalacturonases (exoPGs) and oligogalacturonate hydrolases (OGHs) that cleave homogalacturonan chains and thus loosen the pectic network. These pectin-acting enzymes have not yet been fully characterized in terms of developmental roles but could clearly have significant involvement in cell expansion processes.

Another important role of the pectate hydrolases in plant cell wall is the release of oligogalacturonides. These oligogalacturonides (OGAs) are known as the biologically active oligosaccharide fragments and could have a signalling role in a receptor-based mechanism or they may bind to other homogalacturonan domains and directly modify the properties of the pectin structure.[10,11]

2. LITERATURE REVIEW

2.1 Plant cell wall

The plant cell wall includes the complex of polysaccharides that constitutes the raw material used to manufacture textiles, paper, lumber, films, thickeners and other products. The plant cell wall is also the primary source of cellulose the most abundant and useful biopolymer in the nature. The cell wall represents not only the mechanical support of the cell, but has also a key role in plant growth, cell differentiation, intercellular communication, water movement and defence.[4]

Plant cell wall consists of three layers; middle lamella, primary cell wall and secondary cell wall (Fig. 1). The middle lamella is formed during the cell division and can serve as a binding site for adjacent cells connecting each other together. Middle lamella is essentially composed of pectic material.[2, 12] During cell expansion the flexible primary cell wall is deposited. The primary cell wall represents a glycoproteinaceus layer composed of pectin, cellulose, hemicellulose and proteins.[12] When cell expansion ceases, a secondary wall is sometimes laid down inside the primary wall. Secondary wall is composed of cellulose, hemicellulose as well as lignin making this matrix stronger and relative hydrophobic. [13]

In plants, as much as 35 different cell types were found, each of this differs in its shape, size and location within the plant body and wall characteristics. In the growing plant cell wall dominate complex polysaccharides whereas the amount of structural proteins is rather low.[4]

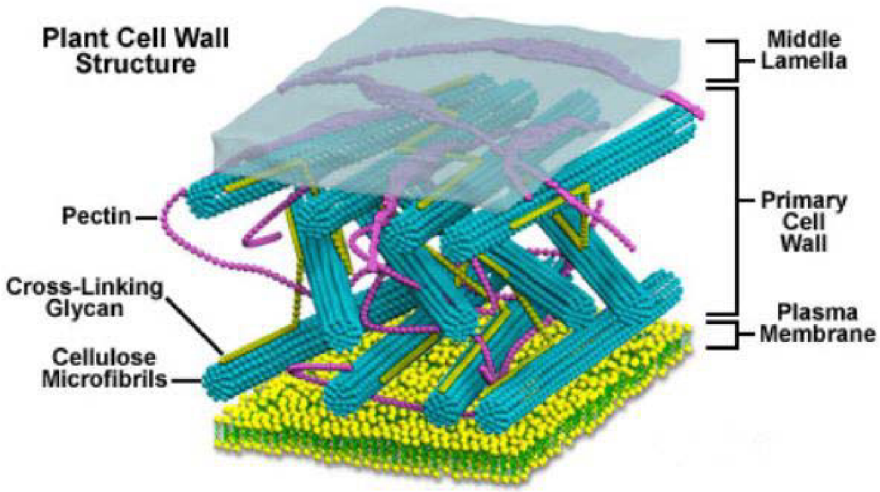

Fig. 1: Primary cell wall structure

(http://micro.magnet.fsu.edu/cells/plants/cellwall.html)

2.2 Primary cell wall structure

Primary cell wall is the flexible extracellular matrix deposited during the cell expansion. Primary walls isolated from higher plant tissues and cells are composed predominantly of polysaccharides (cellulose, hemicellulose, pectin) [14,15] together with lesser amounts of structural glycoproteins (hydroxyproline-rich expansins), phenolic esters (ferulic and coumaric acids), ionically and covalently bound minerals (e.g. calcium and boron) and enzymes. In addition, wall proteins (expansins) are believed to play key role in regulation of wall expansion.[4,16,17]

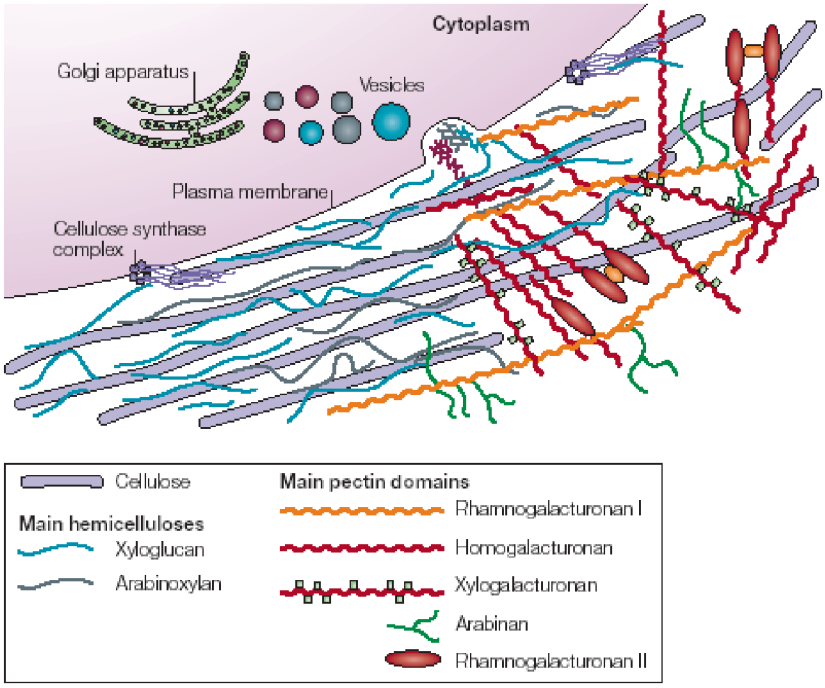

Fig. 2: Structure of the primary cell wall [4]

Cellulose microfibrils are synthesized by large hexameric complexes in the plasma membrane, whereas hemicelluloses and pectins, which compose the matrix polysaccharides, are synthesized in the Golgi apparatus and are deposited to the wall surface by vesicles (Fig. 2). In the most plant species the main hemicellulose is xyloglucan, while hemicelluloses such as arabinoxylans and mannans are found in lesser amounts.[4] The main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan, with smaller amounts of xylogalacturonan, arabinan, arabinogalactan I and rhamnogalacturonan II. Pectin domains are believed to be covalently linked together and to bind to xyloglucan by covalent and non-covalent bonds.[4,18]

2.2.1 Cellulose

Cellulose, one of the most abundant polymers in nature, consists of flat chains of poly-1,4-β-glucose. Most of the cellulose in nature is found in the plant cell walls, usually as the major component. Cellulose occurs as a composite of many chains, termed microfibrils. These microfibrils size can vary from 5-15 nm wide and are spaced 20-40 nm from each other.[19] The long synthesized polymeric glucose chains are attached via a network of hydrogen and Van der Waals bonds. The highly organized crystalline domains are spaced by amorphous regions. The crystalline areas form tight arrays, which shield many of the glycosidic bonds from enzymatic attack. [19,20]

In addition, the presence of other components, such as hemicelluloses, pectin and xyloglucans together with cellulose make the cell wall a very compact and inaccessible substrate. The complete degradation of cellulose to glucose units requires the action of at least three different types of enzymes, such as endo-1,4-β-glucanase, exo-1,4-β-glucanase and β-glycosidase.[4]

2.2.2 Hemicelluloses

Hemicelluloses are another important group of plant cell wall polysaccharides. Hemicelluloses bind to cellulose, but branches and other modifications in their structure prevent them from forming microfibrils by themselves.[2,21] Xyloglucan and arabinoxylan are two of the most abundant hemicelluloses. Details of their structure vary slightly among plant species. Xyloglucan has a backbone that is similar to cellulose, but it is decorated with xylose branches on 3 out of 4 glucose residues. The xylose can also be serially appended with galactose (Gal) and fucose (Fuc) residues. Arabinoxylan consists of a (1,4)-linked β-D-xylan backbone substituted with arabinose branches. [4,9]

Other residues, such as glucuronic acid and ferulic acid esters (FAE), are also attached in arabinoxylans that are particularly abundant in cereal grasses. Mannans are also found in primary cell walls and probably function in the same way as xyloglucan and arabinoxylan.[9,22,23]

2.2.3 Pectins

Pectins are defined as a group of complex acidic polysaccharides that consist of 1,4-linked α-D-galacturonic acid (GalA) residues. Currently, it is supposed that there are three major groups of pectic polysaccharides – homogalacturonan (HG), rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II). This complex and heterogeneous group of polysaccharides consists of distinctive domains, which are believed to be covalently linked together.[24,25,26]

Homogalacturonan (HGA) – "polygalacturonic acid"

Homogalacturonan performs the most abundant pectic domain. The backbone is composed of a linear polysaccharide of repeating (1→4)-linked α-D-GalA residues and it is supposed that contains about 100 – 200 GalA units. The homogalacturonan chain seems to consist of blocks of methyl-esterified (neutral) GalA residues alternating with blocks of non-esterified

(negatively charged) GalA residues. The charged blocks can be cross-linked by Ca^{2+} ions. Some GalA residues could be also *O*-acetylated or substituted with xylose units forming xylogalacturonan (XGA).[27,28]

Rhamnogalacturonan I

Rhamnogalacturonan I (RGI) is highly variable complex of pectic polymers that can be isolated during the polygalacturonases treatment of cell wall. This fact could indicate an attachment by glycosidic bonds to HGA polysaccharides. [27]

The backbone of RG I is composed of repeating unit: α-D-GalA-(1→2)-α-L-Rha-(1→4). As well as in homogalacturonan, some of the GalA residues in RG-I are O-acetylated. There are many different side-chains rich in (1→4) linked β-D-galactan and (1→5)-linked α-L-arabinan. The term rhamnogalacturonan I is typically used to refer to this pectic polysaccharide as shown in Fig. 3: [2, 22,25]

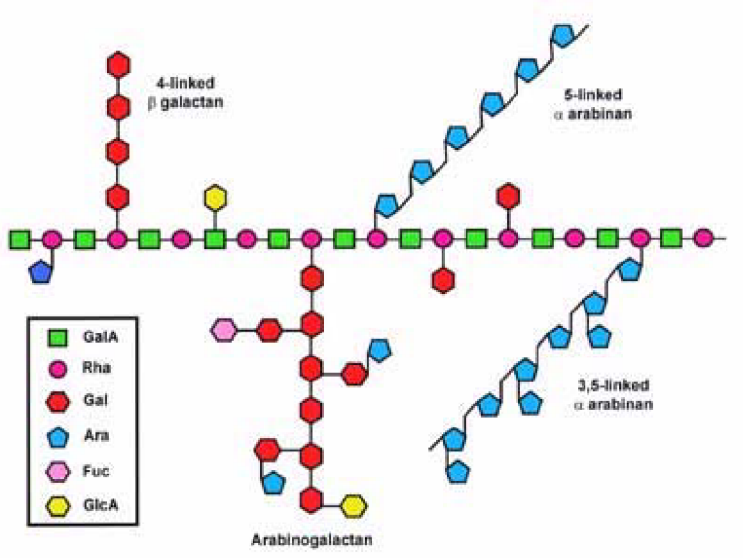

Fig. 3: Structure of RG-I

(*http://www.ccrc.uga.edu/~mao/rg1/rg1.htm*)

Rhamnogalacturonan II

Rhamnogalacturonan-II is a highly complex structure (Fig. 4). It has not been reported to occur in free form in plants, but has been found in red wine [15], suggesting that it can be released by the action of yeast enzyme. RG-II has a backbone rich in GalA, to which several different side-chains with unusual structure are attached. The backbone of RG-II contains at least 8 residues of 1,4-linked α-D-GalA. Two structurally distinct disaccharides (C and D) are attached to C-3 of the backbone and two structurally distinct oligosaccharides (A and B) are attached to C-2 of the backbone. The side chains could contain 11 different sugar residues such as apiose (Api), aceric acid (AceA) or 2-keto-3-deoxy-D-*manno*-octulosonic acid (KDO).[15,29]

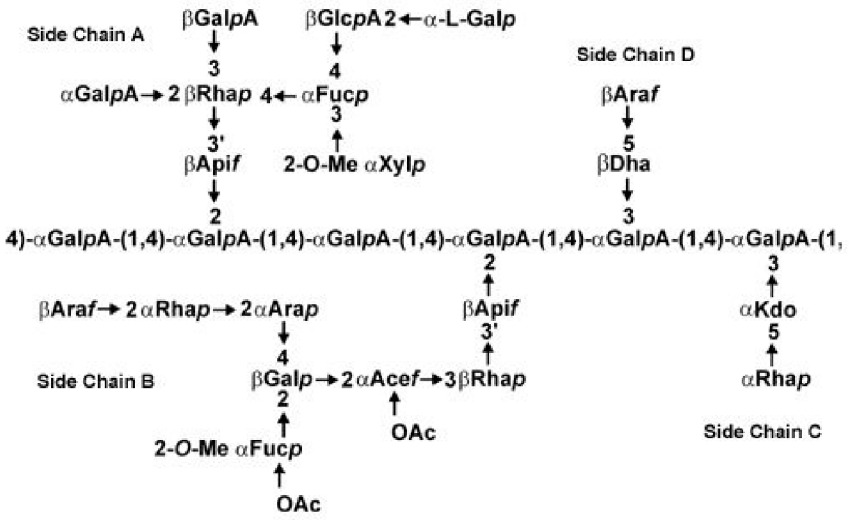

Fig. 4: Structure of RG-II
(http://www.ccrc.uga.edu/~mao/rg2/intro.htm)

For many years RG-II remained an enigmatic polysaccharide with unknown function. However, in 1996 Matoh and colleagues demonstrated conclusively that RG-II exists in the primary wall as a dimer that is cross linked by a 1:2 borate-diol diester (Fig. 5). [29,30]

Subsequently, research at the CCRC showed that the dimer contains a single borate diester cross link and that the ester is formed between the apiosyl residue in side chain A of each monomer subunit. The borate atom is chiral and thus two diesteroisomers can form. It is not known which of the two isomers is present in naturally occurring RG-II.[30]

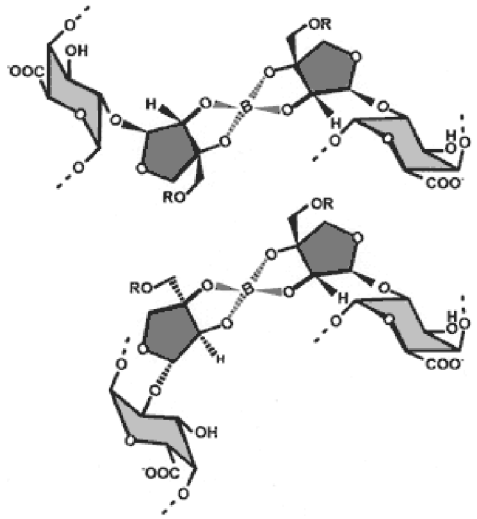

Fig. 5: RG-II dimer structure
(http://www.ccrc.uga.edu/~mao/rg2/intro.htm)

2.2.4 Expansins

Expansins are plant cell wall proteins first discovered in studies of plant cell enlargement. They have unique "loosening" effects on plant cell wall and are believed to function in plant cell growth, cell wall disassembly or cell separation.[5,16]

Sequence analyses indicate that expansins consist of two domains, an N-terminal domain (~15 kDa) with distant sequence similarity to the catalytic domain of the GH family 45 endoglucanases, and C-terminal domain (~10 kDa) that is related to a family of grass pollen allergens of unknown function. Expansins are thought to disrupt non-covalent bonds of wall polysaccharides, e.g. by inducing of cellulase activity. [31]

2.3 Synthesis of matrix polysaccharides

Cellulose (β-1,4-glucan) and callose (β-1,3-glucan) are synthesized in plasmatic membrane by cellulose-synthase and callose-synthase (Fig. 6). These enzymes are known as integral membrane glycosyltransferases (GTs) that possess several membrane spanning regions and both the NH_2 and COOH termini are predicted to be oriented toward the cytosol.[32]

The non-cellulosic polymers, hemicelluloses and pectins, are synthesized by GTs presumably located in the different compartments of the Golgi apparatus. These GTs are believed to be type II membrane-bound proteins with the catalytic domain (C-terminal) facing the lumen of the Golgi apparatus. [33, 34]

Matrix polysaccharides possess a more diverse set of glycosidic linkages and sugar residues than cellulose. Specific genes that encode the relevant glycosyltransferases belong to the CSL (Cellulose synthase-like) superfamily. This superfamily includes eight other families called CSLA, CSLB and so on up to CSLH, as well as the CESA family.[35] CSL proteins are proposed to synthesize the β-D-glycan backbone of hemicelluloses and are localized in the Golgi apparatus; The CSLA proteins synthesize the mannans and glucomannans of the growing cell wall. [36, 37] Other glycosyltransferases are needed to add the branches to the β-D-glycan backbone. [4]

Enzymes involved in plant cell wall synthesis could be splitted into two groups:

- glycosyltransferases localized either in the plasmatic membrane (involved in synthesizing of cellulose and callose) or in the Golgi apparatus (participating in synthesis of hemicelluloses, pectins, mannans and other glycoproteins).[35]

- transglycosylases, in CAZy database classified as glycoside hydrolases (GHs), localized in the cell wall, partake in modification of wall-matrix polysaccharides and in their embedding into the cell wall structure.[35]

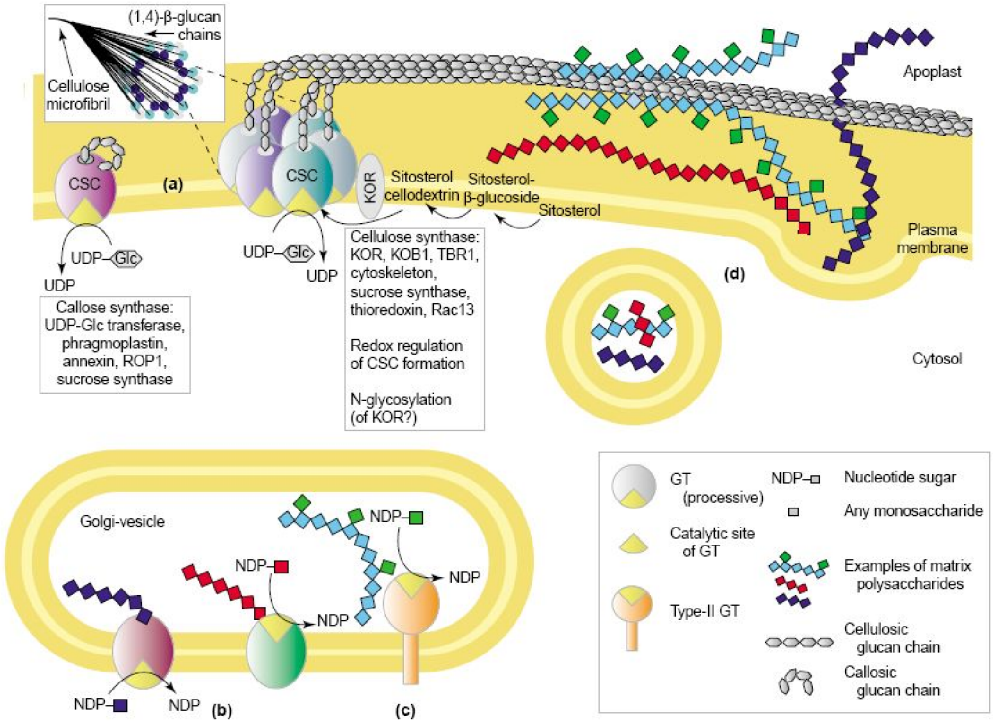

Fig. 6: Synthesis of cell wall polysaccharides [38]

Glycosyltransferases (GTs) utilize nucleotide sugars as donor substrates to generate polysaccharides.

a) Cellulose and callose are synthesised at the plasma membrane. Different CESA isoforms aggregate into higher-order rosettes (box in top left corner) to produce (1,4)-β-glucan chains that coalesce into cellulose microfibrils. Proteins that are known or suspected to interact with the CSC, and mechanisms that are known to be important for cellulose synthesis and CSC activity, are shown in the central text box in Fig. 6. The proposed pathway of primer synthesis for cellulose synthesis, which starts with sitosterol and requires KOR endoglucanase activity, is also depicted. CSLA and proteins reported to interact with it are schematized on the left in Fig. 6. [35,38]
b) Matrix polysaccharides are synthesized within the Golgi apparatus. Depending on the topology of the catalytic sites, nucleotide sugars can be employed from the cytosol or from the inside of the cisternae.
c) Type-II GTs, which consist of a transmembrane, stem and a catalytic domain, play a major role in decorating polysaccharides with side-chains.
d) The synthesised matrix polysaccharides are secreted by exocytosis into the apoplast, where they form highly ordered networks with cellulose microfibrils. [38]

2.4 Forming networks of polysaccharides

After secreting of the matrix polysaccharides into the wall, they become associated with newly synthesized cellulose microfibrils, as well as with the pre-existing wall polymers forming a network (Fig. 7). This network formation involves spontaneous physical-chemical interactions between the wall polysaccharides and their enzymatic cross linking. From the polymer-polymer interaction point of view, the precise structure of the cell wall has not yet been explained in detail and still represents the key task for true understanding of the cell wall expansion.[4, 39]

Primary cell wall includes three structurally independent domains. Firs domain, cellulose-xyloglucan complex is embedded into the second domain of pectic polysaccharides. The third independent domain is represented by structural glycoproteins. [4]

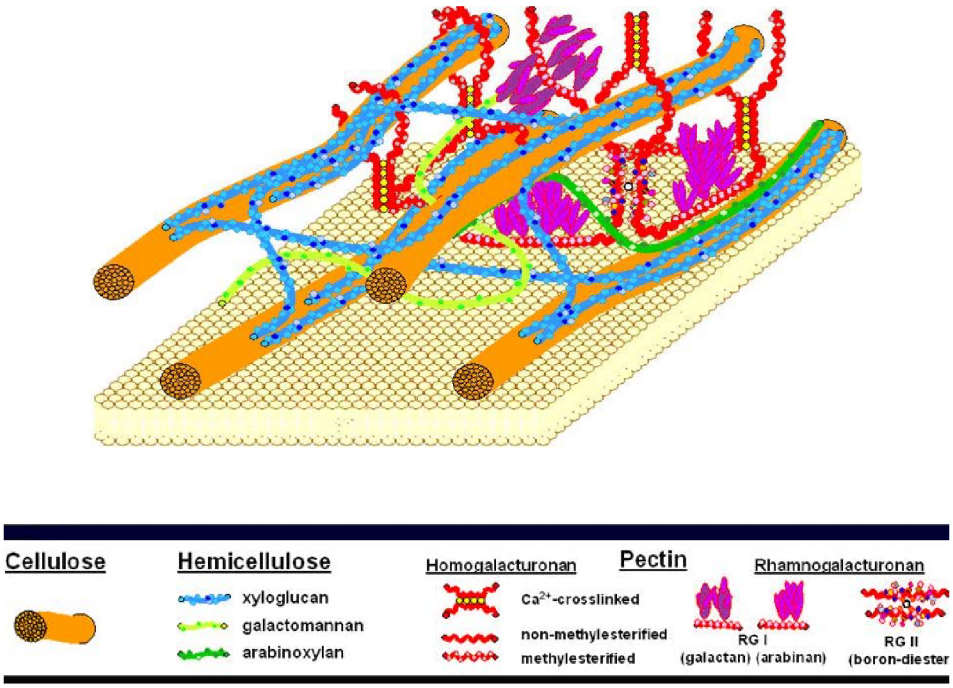

Fig. 7: Polysaccharides complex – primary cell wall [40]

2.4.1 Pectin network

The pectin domains are thought to be covalently cross-linked to each other but the origin of this linking has not been determined yet. In addition, within the pectin structure forming there are two other types of linkage mechanisms involving boron and calcium ions.[4]

The pectin domain can be covalently linked together forming a large macromolecular pectin network. Recent pectin model by Vincken at al. supposes that rhamnogalacturonan I serves as the backbone and the other pectin domains are attached as a side chains (Fig. 8a).[4,9] Homogalacturonan is ionically cross-linked by calcium ions whereas boron is bound to rhamnogalacturonan II via diester linkages. Rhamnogalacturonan II forms dimmers through a

borate ester bond (Fig. 8b). This cross-linking plays an important role for normal wall formation as well as for control of wall porosity. [4]

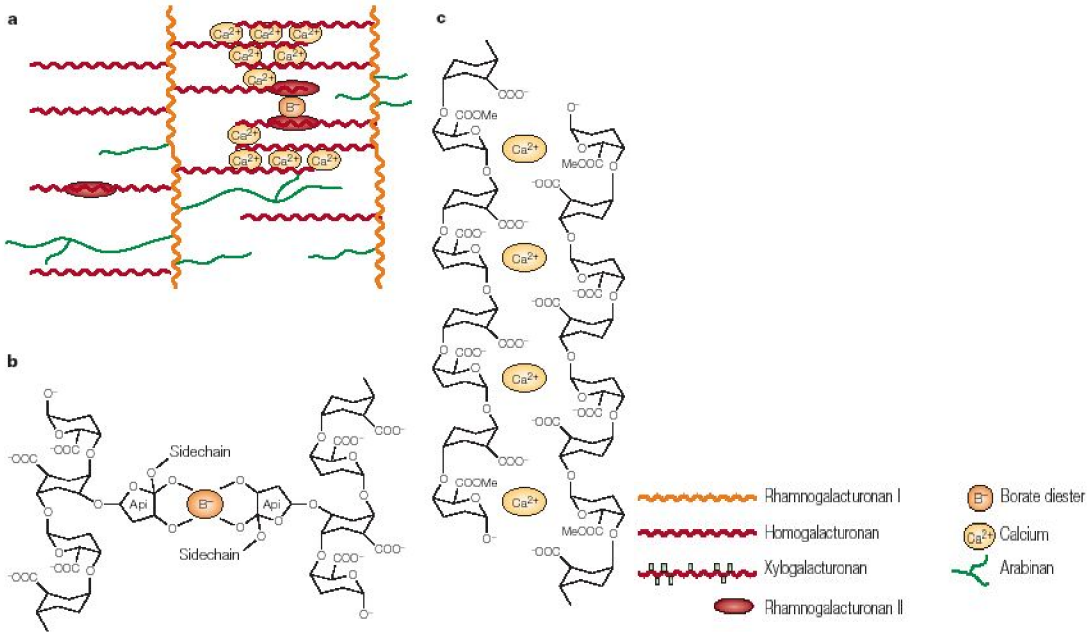

Fig. 8: Pectin domain structure [4]

Homogalacturonan ("polygalacturonic acid") is able to form a gel-like structure through calcium ions involving the cross-linking of its carboxyl groups (Fig. 8c). The growing cells synthesize homogalacturonan in which 75 % of the carboxyl groups are methyl esterified. This modification removes the negative charge of carboxyl ions and locks its ability to undergo Ca^{2+} cross-linking. High esterified homogalacturonans do not form gels and their secretion can maintain the cell flexible and elastic. After the cell growth is terminated, the action of pectin methylesterases is activated. Methylesterases are secreted by cells into plasma membrane space where they hydrolyse methylesters. Free carboxyl groups are available for forming of gel structures through Ca^{2+} ions. [4]

2.4.2 Cellulose-hemicellulose network

The binding of xyloglucan to cellulose microfibrils may be of considerable significance in the modelling of primary cell walls of plants as well as in the process of cell wall assembly. Xyloglucan, like cellulose, has β-(1,4)-linked glycosyl residues backbone, although this backbone is substituted with mono-, di- and trisaccharide side-chains that modify the physical properties of the polymer. [41] Early observation suggested that xyloglucan polymers and cellulose associate non-covalently by hydrogen bonding between their backbones. However, further analyses indicate that the oligosaccharide side-chains of xyloglucan can moderate its binding to the microfibrils. [42]

There are at least five ideas how hemicelluloses can form a network with cellulose microfibrils (Fig. 9). [4] The ideas involve the direct or indirect linkages between cellulose

microfibrils and hemicelluloses. Hemicelluloses may form a primary network with cellulose and may also be linked to acidic pectins. Additionally, neutral pectin polysaccharides, such as arabinans, are also able to bind to the cellulose surface: [39]

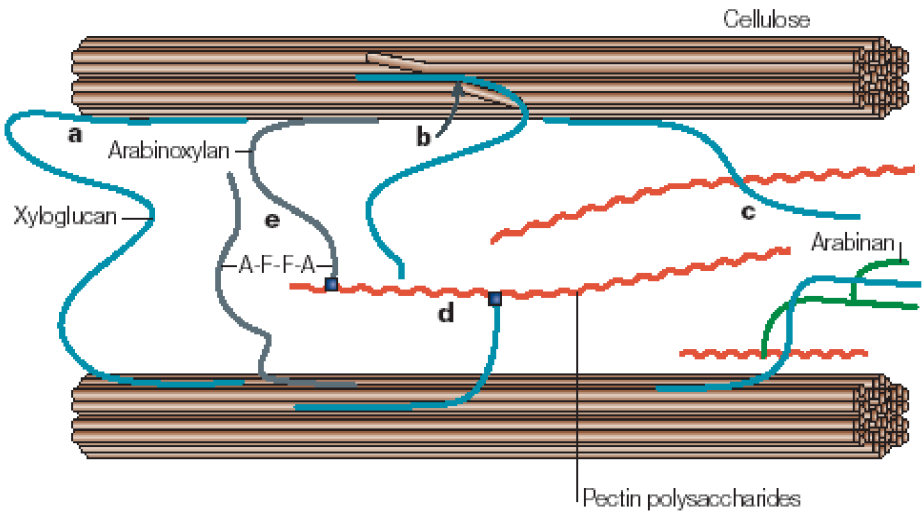

Fig. 9: Network of cellulose and hemicelluloses [4]

- Hemicelluloses might spontaneously bind to the surfaces of cellulose microfibrils and tether adjacent microfibrils together (part a in Fig. 9). [43,44]

- Xyloglucan might become entrapped during formation of the ordered microfibrils (part b in Fig. 9). The entrapped remainder of the xyloglucan would be free to bind to other cellulose surfaces or to other matrix polymers, thereby anchoring the microfibril firmly to its neighbors.[41,45]

- Cellulose microfibrils might be simply coated with xyloglucans, which adhere to other matrix polysaccharides without direct linkage between microfibrils (part c in Fig. 9). [46]

- Xyloglucans can be also covalently attached to pectin polysaccharides, forming a macromolecule that anchors the microfibrils by sticking xyloglucan to cellulose surfaces (part d in Fig. 9). Probably, xyloglucan is attached to homogalacturonan. [47,48] The nature of the hypothetical xyloglucan-pectin linkage has not been established yet.

- Arabinoxylans might adhere to cellulose and be cross-linked by ferulic acid esters (A-F-F-A) (part e in Fig. 9). This type of phenolic crosslink might also crosslink other hemicelluloses and pectins, particularly in gross/rough cell walls.[4]

2.4.3 Hemicellulose-pectin network

A covalent linkage between xyloglucan and pectin is thought to make a major contribution to cell wall structure and metabolism, although the mechanism has not been established yet.

The occurrence of covalent xyloglucan-pectin binding within the dicot primary cell wall was first proposed in the Albersheim model. [49] This model proposed glycosidic linkage between the reducing end of xyloglucan and pectin side-chain. It was based on assumption that uronic acid and xylose residues were uniquely characteristic for pectins and xyloglucans, respectively. The enzymatic dissection of wall polysaccharides to yield oligomeric products containing both uronic acid residues and xylose residues was taken as evidence of covalent xyloglucan-pectin complex. Later, it has become recognised that some hemicelluloses (glucuronoarabinoxylans) in dicot cell wall contain uronic acid residues [50] and some pectins contain xylose and 2-*O*-methylxylose residues [51,52] and therefore the earlier model was insufficient.

The other favour model proposes two polymer networks – a cellulose-xyloglucan network held together by hydrogen-bonds and a network of pectic polysaccharides held together partly by Ca^{2+} bridges [53,2]. This model lacks any interactions between both networks by hydrogen-bonds, ionic interactions or covalent bonds.

However, Femenia (1999) [54] and Thomson and Fry (2000) [47] provided strong evidence that covalently-linked pectin-xyloglucan complexes are present in cell walls of cauliflower stems and rose suspension-cultured cells, respectively. Results presents evidence that up to 12% of the xyloglucan in the walls of suspension-cultured rose cells is attached covalently (probably via an arabinan/galactan domain) to homogalacturonan. In all, about 30% of the xyloglucan in these walls is linked to acidic polymers.

Accordingly, for a long time there was the compromise between two prevalent wall models: about 1/3 of xyloglucan is covalently attached to acidic pectins – Albersheim model [49] and about 2/3 of xyloglucan is bound to the cellulose microfibrils possibly connecting them [44] but without linkage to pectic network.[2]

Further evidence for xyloglucan-pectin complexes was provided by Abdel-Masih et al. (2003) [13], when particular enzyme preparations from etiolated pea shoots were able to incorporate ^{14}C from UDP-[^{14}C]Gal into pectic oligo-[^{14}C]galactan chains. The polysaccharides containing these oligogalactans showed a strong affinity for paper (cellulose). The paper affinity of the ^{14}C-labelled product was greatly reduced by treatment with endo-(1→4)-β-D-glucanase, which digests xyloglucan. [55] In addition, newly synthesised, intraprotoplasmic [^{3}H] xyloglucans were found in [^{3}H] arabinose-fed cultured maize cells, increasing greatly the Mr after the start (about 15-20 min) of radio-labelling. Part of this increase was supposed to be the result of post-synthetic bonding between [^{3}H] xyloglucans and additional polysaccharide chains. [56]

Xyloglucan-pectin covalent complexes, accounting for 30-70 % of the total xyloglucan, were found in a broad variety of angiosperm cell suspension cultures (Arabidopsis, sycamore, rose, tomato, spinach, maize and barley) despite wide variation in their xyloglucan structure and in their overall plant cell wall compositions. [57] This suggests that the xyloglucan-pectin linkage is evolutionarily conserved among angiosperms and could be required for effective plant cell wall structure and function.

Currently, there is little known about the sub-cellular site of the formation of the xyloglucan-pectin linkage *in vivo*. Nothing is known about its enzymatic mechanism. In 2008, Popper and Fry presented two quite distinct hypotheses explaining the mechanism of the formation of xyloglucan-pectin complexes (Fig. 10a,b). [58]

Hypothesis I

Hypothesis I proposes that xyloglucan-pectin linkages are formed by transglycosylation, with xyloglucan as a donor substrate and an RG-I (arabinan or galactan) side chain as an acceptor substrate. Heterotransglycosylation of xyloglucan has recently been reported with closely related β-glucans but not with pectic components.[59] In principle, xyloglucan-to-pectin transglycosylation could occur within the endomembrane system or after secretion into the plant cell wall (Fig. 10a).

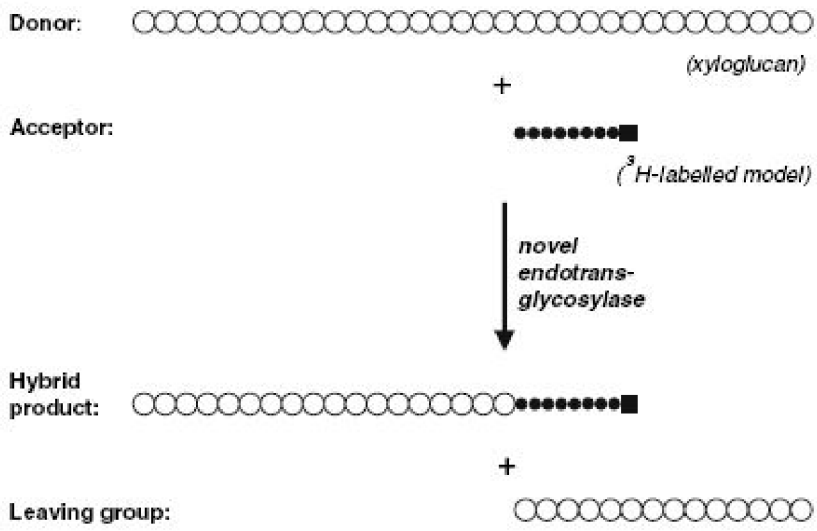

Fig. 10a: Hypothetical xyloglucan-to-pectin transglycosylation reaction: *a non-radiolabelled donor substrate (xyloglucan) is proposed to react with a radioactive model of a RG-I side chain. This hypothetical reaction would result in forming of a xyloglucan-* ^{3}*H-labelled RG-I covalent complexes detected in vivo. This reaction could theoretically occur either intra-protoplasmically or within the cell wall.* [58]

Hypothesis II

In contrast, hypothesis II, proposes that some xyloglucan molecules are synthesised de novo on a primer that consists of an RG-I side chain. According to this hypothesis, part of the RG-I would be synthesised first and then NDP-sugars would be used for building a xyloglucan chain on to a side chain of the RG-I. (Fig. 10b)

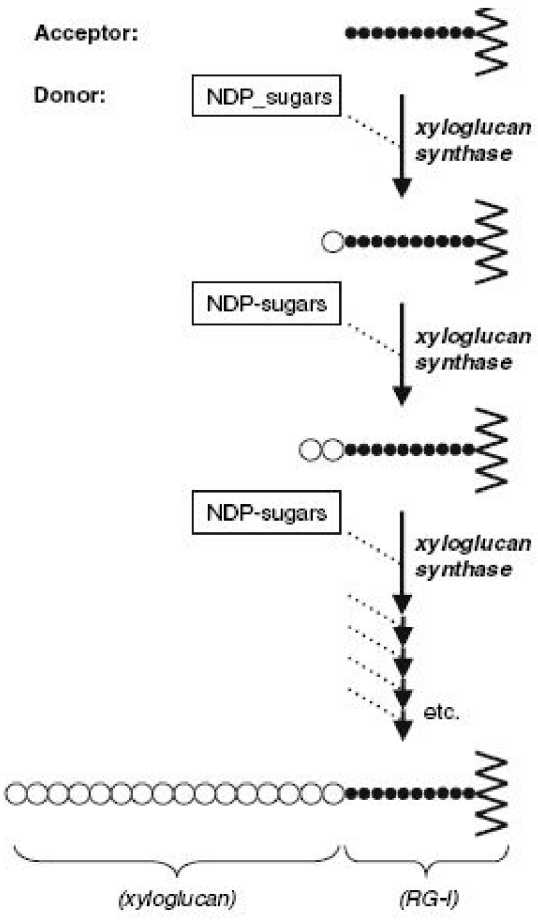

Fig. 10b: The favoured in vivo mechanism of formation of [^{3}H] xyloglucan-RG-I covalent bonds*: in this scheme part of the xyloglucan chains are built up de novo, using NDP-sugars as a donor substrates, on a RG-I side chain.* [58]

2.5 Enzymes involved in the degradation of cell wall polysaccharides

Cell wall polysaccharides can undergo to the action of numerous enzymes. Fry classified these enzymes into three groups: exopolysaccharidases, endopolysaccharidases and other hydrolases that do not belong under these two groups.[60] Exopolysaccharidases attack polymeric and oligomeric substrates from the non-reducing end, or substituted side chains, releasing monosaccharides and rarely disaccharides. Endopolysaccharidases attack the polymer backbone with random action pattern. This type of enzymes has a large impact on the molecular weight of polysaccharides. Hydrolases from the third class can cleave the substituted non-carbohydrate groups linked to wall polysaccharides such as *O*-acetyl, *O*-methyl, *O*-feruloyl and others.[60,61] Wall enzymes can be also divided into groups according to their substrate specificity, i.e. cellulases, hemicellulase, pectinases, ligninases *etc.*

Polysaccharidases are located in the cell wall or in the plasma membrane. These enzymes participate in regulation of cell wall expansion and alteration. After biosynthesis and deposition of polysaccharides they participate in their degradation. Modifications induce then

changes in the structure and composition of these polysaccharides. Plant cell wall polysaccharides present very heterogenous and complex polymers, and consequently the spectra of activities of polysaccharidases are very diverse.[4, 61]

Hydrolases of plant cell wall represent the most stable enzymes due to the content of cystine (S-S) bridges. On the other hand, some of them could be inactivated by reducing agents, e.g. ascorbate and glutathione – compounds that are often added to preparations of intracellular enzymes to stabilize them. Many wall enzymes have pH optimum in the range of 4-5, whereas intracellular enzymes tend to have optima around pH 8. [17, 60]

2.5.1 Pectinases

Due to the complex structure of pectin many types of enzymes are involved in its modification. Thus, the pectin degrading enzymes are actually a diverse group of enzymes. They can be classified according to the site of cleavage (exo or endo), preferred substrate (pectin or pectate) and the mode of cleavage (hydrolases or lyases).[17, 61] According to the cleavage site, pectinases can be further divided into two groups, those acting on the main chain (as polygalacturonases, pectin and pectate lyases, rhamnogalacturonases, rhamnogalacturonan acetyl esterases and methyl/acetyl esterases) and those acting on the side chains of the pectin hairy regions (arabinofuranosidases, endoarabinases, β-galactosidases, endogalactanases and feruloyl esterases). [62] Another division is found according to part of pectin which can be attacked – rhamnogalacturonases, rhamnogalacturonan lyases and rhamnogalacturonan acetyl esterases for the rhamnogalacturonic segment or polygalacturonases, methyl and acetyl esterases as well as pectin and pectate acetyl lyases for the smooth region.[17, 62]

2.5.1.1 Degradation of homogalacturonan

Degradation of homogalacturonan (HG) is schematically shown in Fig. 11.

Polygalacturonases (PGs) are enzymes catalyzing the hydrolytic cleavage of glycosidic linkages of deesterified HG [62, 63, 64] and can be of the exo- or endo-acting types. The *exo*PG (EC 3.2.1.67) removes single galacturonic acid units from the non-reducing end of polymers, whereas the *endo*PG (EC 3.2.1.15) cleaves such polygalacturonic acid at random.

Pectin (methyl) esterases (PME, EC 3.2.2.11) catalyze the hydrolysis of methyl ester groups of HG. This is accomplished by removing of methyl groups from C6 position of galacturonic acid residue. Demethylation of pectin results in changes of charges and the pH in the cell wall what allows the formation of a calcium-linked gel structure as well as makes the substrate susceptible to degradation by PGs.[61]

Pectate lyases and pectin lyases (EC 4.2.2.2, EC 4.2.2.10) are enzymes attacking glycosidic bonds of pectate or pectin by trans-elimination from C-4 into C-5 site of aglycon part of substrate. Both types of lyases differ in substrate specificity. Pectate lyases cleave pectate and pectin lyases prefer pectin as a substrate, whereas these enzymes can operate by endo- as well as by exo- mechanism.[61, 63]

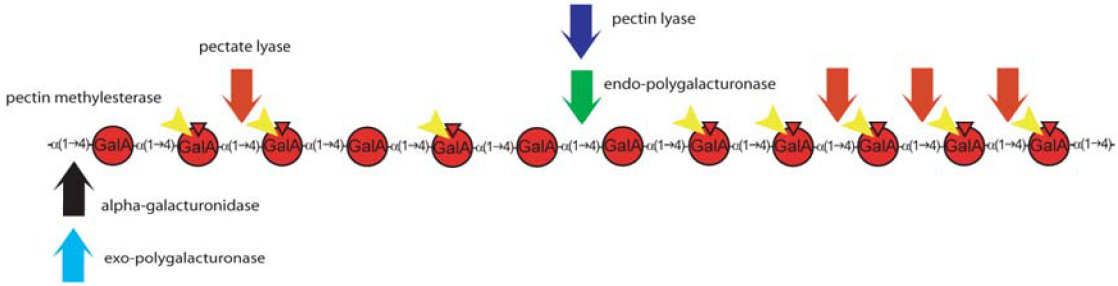

Fig. 11: Scheme of the HG degradation
(http://www.ccrc.uga.edu/~mao/rg2/intro.htm)

2.5.1.2 Biological activities of oligogalacturonides

The plant cell wall presents a source of regulatory molecules involved in control of defense and developmental processes. Currently, the best characterized class of plant cell wall-derived signals is the group of oligogalacturonides (OGAs), homopolymers of α-1,4-linked D-galacturonic acid. OGAs are degradation products of de-esterified homogalacturonan. Enzymes responsible for HG fragmentation and releasing of OGAs are mainly pectin methyl esterases and polygalacturonases. [10, 65, 66]

A number of different biological responses to OGAs have been reported, and the particular response observed depends on the plant species, the bioassay and the chemical structure of the OGAs used. A spectrum of modified and unmodified OGAs of various degree of polymerization is active in different systems. The biological responses of plants to OGAs can be divided into two categories: plant defense, and plant growth and development.[65]

In particular, oligogalacturonic acids derived from pectins show hormone-like effects, such as the inhibitor of auxin-induced stem elongation, stimulation of flowering and the inhibition of root formation.[65] In addition; OGAs can also induce defense-related changes, including the induction of protein inhibitor gene expression, expression of pathogenesis-related genes and phytoalexin production.[65, 66]

2.5.1.3 Degradation of rhamnogalacturonan I

The changes of RG-I backbone are catalyzed by rhamnogalacturonan lyases (A,B), and rhamnogalacturonan acetyl esterases (Fig. 12).

Currently, only two enzyme activities are known to participate in hydrolysis of RG-I side chains: bifunctional α-*N*-arabinofuranosidases/xylan-1,4-β-xylosidases and β-galactosidases that can be involved in degradation of arabinan and galactan, respectively (Fig. 12). [67, 68, 69]

α-N-arabinofuranosidases/xylan-1,4-β-xylosidases (EC 3.2.1.55/3.2.1.37) are able to release L-arabinose from arabinan and are associated with ripening processes, pectin solubility, cell wall porosity and other processes required for cell wall growth and development.[67]

β- galactosidases (EC 3.2.1.23) are believed to participate in the removal of galactose from galactans and other cell wall components.[61, 67]

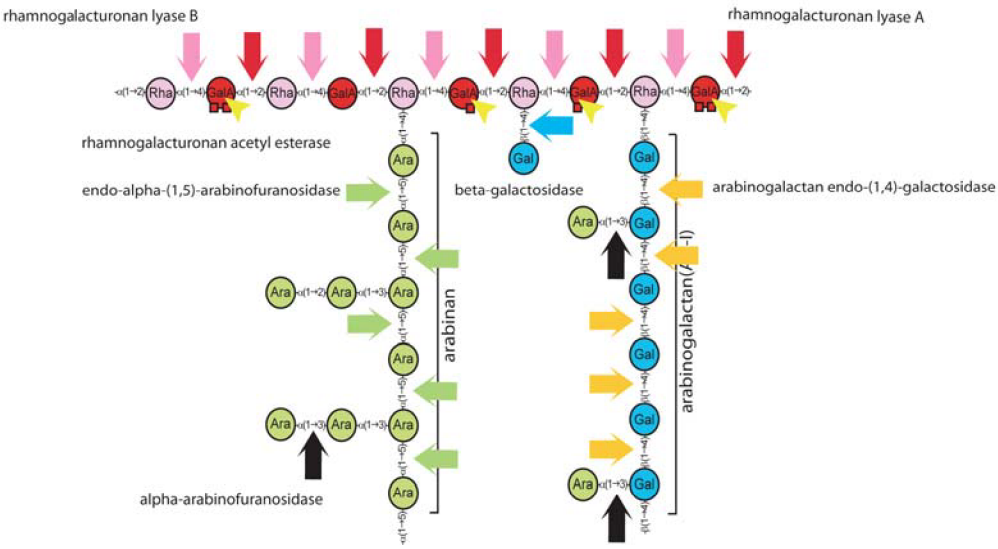

Fig. 12: Scheme of RG-I degradation
(http://www.ccrc.uga.edu/~mao/rg2/intro.htm)

2.5.2 Hemicellulases

Hemicelluloses are generally less complex than pectin and consist of hetero or homopolysaccharide main chains, often with short side chains.[17] On the other hand, there are very diverse types of hemicelluloses as xylans, glucomannans, xyloglucans etc. Therefore, hemicellulases consist of endo-acting enzymes attacking the main chain, debranching enzymes removing side chains, as well as enzymes involved in modifications such as deacetylation. Generally, hemicellulases present group of hydrolytic enzymes. [17, 61, 70]

2.5.2.1 Degradation of xyloglucan

Enzymes involved in the modification of xyloglucan structure are various xyloglucan specific hydrolases including xyloglucan specific endo-glucanases, xyloglucan endo-transglycosylases,[17,70] xyloglucan β-galactosidases,[71] xyloglucan α-fucosidases, specific cellobiohydrolases and xyloglucan oligosaccharide α-D-xylosidases (Fig. 13).[72]

Xyloglucan endo-transglycosylase (XET, EC 2.4.1.207) and *xyloglucan specific endo-β-1,4-glucanase* (EC 2.4.1.151) are postulated that play various functions, including wall loosening, wall strengthening, integrating new xyloglucans into the wall, trimming xyloglucan strands that are not tightly stuck to the cellulose surface and hydrolysing xyloglucans. XET carries out two reactions, first a scission of a glycosidic bond in the xyloglucan backbone, followed by the re-formation of the bond with other xyloglucan chain. [4, 17, 70]

β-galactosidases (EC 3.2.1.23) catalyze removal of the terminal non-reducing β-D-galactopyranosyl residues from xyloglucans. [71]

α-L-fucosidases (EC 3.2.1.51/3.2.1.63) hydrolyze the terminal α-(1→2)-fucosidig linkage of oligosaccharides. [72]

Xylan-1,4-α- xylosidases (EC 3.2.1.37) cleave specifically the α-xylosyl residue attached to the glucose residue of the xyloglucan-oligosaccharide. [72]

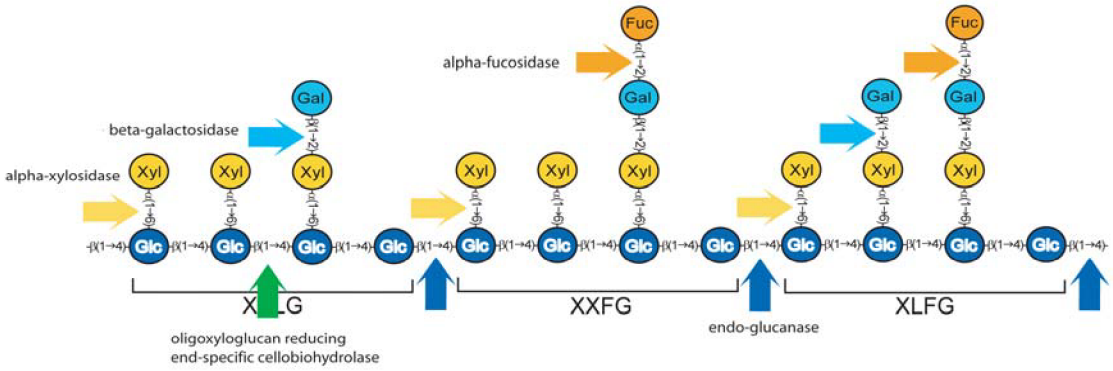

Fig. 13: Degradation of xyloglucan
(http://www.ccrc.uga.edu/~mao/rg2/intro.htm)

2.5.2.2 Degradation of xylan and arabinoxylan

Three classes of plant enzymes degrading xylan were identified: *endo-1,4-β-xylanases* (EC 3.2.1.8), *β-xylosidases* (EC 3.2.1.37) and *α-N-arabinofuranosidases* (EC 3.2.1.55). Endoxylanases and β-xylosidases are enzymes responsible for cleavage of xylan backbone while α-N-arabinofuranosidases remove side chain arabinose substituents from xylan or oligoxylan. In addition, β-xylosidases are a key enzyme for the complete degradation of xylan. [61, 73]

2.5.2.3 Degradation of galactomannan

Enzymes responsible for galactomannans degradation are generally endo-β-mannanases, β-D-mannosidases and α-D-galactosidases.

Mannan endo-1,4-β-mannosidases (EC 3.2.1.78) hydrolyse the mannan backbone releasing manno-oligosaccharides that are hydrolyzed further by *β-mannosidases* (EC 3.2.1.25) and *α-galactosidases* (EC 3.2.122) to remove the galactose unit on side chains. Enzymes α-galactosidases play the key role in complete degradation of galactomannan. [61]

2.5.3 Cellulases

Cellulases refer to a family of enzymes that hydrolyse β-1,4 glycosidic bonds in cellulose. These enzymes belong to the 0- and S-glycosyl hydrolases. Traditionally, cellulases can be divided into exoglucanases, *cellulose-1,4-β-cellobiosidase* (EC 3.2.1.91) and endoglucanases, *cellulase* (EC 3.2.1.4). [61, 62]

2.6 Plant tissue culture

Plant tissue culture presents the growth of plant cells outside an intact plant (*in vitro* cultivation). This technique is used in many areas of the plant sciences. Method is based on maintaining of plant cells in aseptic conditions on a suitable nutrient medium. The culture can be sustained as a mass of undifferentiated cells for an extended period of time or can be regenerated into whole plant (Fig. 14). This process is based on totipotency of cells – the basic characteristic of somatic cells. [74, 75]

Plant tissue culture has direct commercial applications as well as value in basic research in cell biology, genetics and biochemistry. These techniques include callus and suspension cells cultures, anthers, ovules and embryos on experimental to industrial scales, protoplast isolation and fusion, cell selection and meristem and bud culture. [75, 76]

Applications include:

- micropropagation using meristem and shoot culture to produce large numbers of identical individuals
- screening programmes of cells
- large-scale growth of plant cells in liquid culture as a source of secondary products
- crossing distantly related species by protoplast fusion and regeneration of the novel hybrid
- as a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants

Culture is generally initiated from sterile pieces of a whole plant. These pieces are termed „explants“ and may consists of pieces of organs, such as leaves or roots, or may be specific cell types, such as pollen or endosperm. Many features of the explants are known to affect the efficiency of culture initiation. Generally, younger, more rapidly growing tissue or tissue at an early stage of development is most effective. [75, 77]

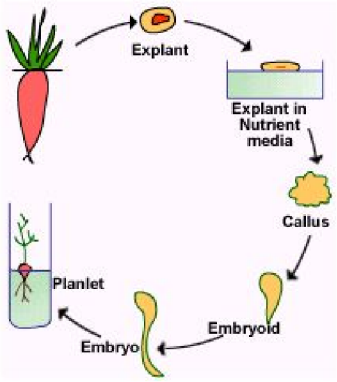

Fig. 14: Induction of cultures in vitro from plant explants [77]

2.6.1 Callus culture

Explants, when are cultured on the appropriate medium, can give rise to an unorganised, growing and dividing mass of cells. It is thought that any plant tissue can be used as explants,

if the correct conditions are found. In culture, this proliferation can be maintained more or less indefinitely, provided that the callus is subcultured on to fresh medium periodically. During callus formation there is some degree of differentiation, both in morphology and metabolism.

Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokine ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can be subsequently produced. [75, 77]

Three developmental stages are used to describe the growth of callus cultures: induction, division and differentiation.

Induction

Cells are placed in conditions that induce them to begin dividing. The actual time spent in this phase depends on the type of cells in the explants and the culture conditions. Induction often starts with wounding and is enhanced by the adding of hormones to the medium. [77, 78]

Division

Cellular metabolism is activated and cells begin to divide. Explants cells dedifferentiate, they are becoming unorganized in relationship to each other. The increase in numbers of cells produced during this phase can be very rapid (>1000% per week).

Cell divisions occur first in the outer layers of the explant. The callus can grow as a mass of tightly connected cells or it may be friable, where the cells are more loosely connected and fall apart easily. [78]

Differentiation

Under the proper conditions, cells in the culture begin to differentiate. They form meristematic centres that will form shoots, roots or embryos, depending on genotype and conditions. [78]

2.6.2 Cell - suspension cultures

Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies. Callus can be converted to a cell-suspension culture by aseptic transfer into conical flask containing autoclaved liquid medium (usually the same as for callus without agar). Cell suspensions are continually cultured by repeated subculturing into fresh medium. [77, 78]

Suspension cultures have a characteristic growth curve (Fig. 15). The culture passes first through a lag phase with a small growth, next through the exponential phase, until the stationary phase when some nutrients in the medium become limiting and growth starts to slow down. Therefore, cells should be transferred in the exponential or linear phase. Both callus and suspension cultures can be induced to undergo cell differentiation, organogenesis or embryogenesis. This can be achieved by empirical manipulation of the composition of the medium and can provide a useful system for study of cell wall changes associated with these developmental processes. [78]

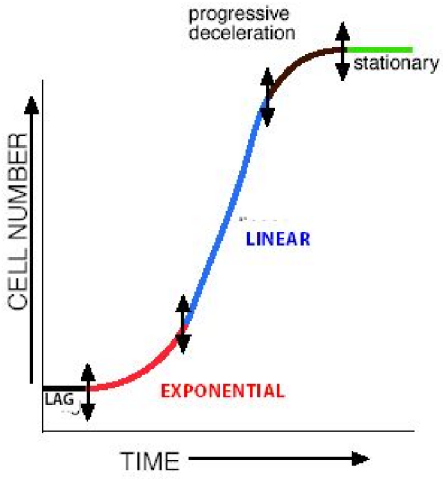

Fig. 15: Induction of cultures from plant explants in vitro [77]

3. EXPERIMENTAL

3.1 Plant material

The parsley roots (*Petroselinum crispum* cv. Olomoucká dlouhá) in an amount of 20 kg from October crop were collected from the same field. Roots were treated immediately after sampling and were used for study of enzymes as well as for plant tissue cultivation *in vitro*.

For enzyme extraction, the plant material was mechanically disturbed using juice extractor. For cell cultivation in vitro, thin transverse slices of roots were used.

3.2 Plant tissue culture

3.2.1 The initiation and maintenance of callus cultures

The plant material surface was sterilized using 10 % SAVO and washed 3 times in 500 ml sterile tap water for 30 minutes. Roots were cut into 1 mm thick transverse slices and transferred to a sterile Erlenmeyer flask (100 ml) containing Murashige and Skoog (MS) agar medium. [79] The composition of cultivation medium is shown in Table 1.

Table 1: Composition of cultivation medium of parsley roots

Component	Concentration
MS medium	4,9 $g.l^{-1}$
Sucrose	30 $g.l^{-1}$
2,4-D -auxin	1 $mg.l^{-1}$

Explants were incubated at room temperature in the dark and the cultivation process was controlled until first callus formation (Fig. 16). After 21 days the best callusing explants were selected and, after cutting the callusing regions, transferred into fresh medium. Callus cultures were still maintained under the same conditions and sub-cultured at 5 weeks interval.

Callus cultures were used as an initial material for initiation of cell suspension cultures as well as material for continuous sub-cultures.

Fig. 16: Callus culture obtained from parley root after 14 days of cultivation

3.2.2 The initiation and maintenance of plant cell suspension cultures

The friable, light-grown callus cultures were aseptically transferred from agar medium into Erlenmeyer flasks (250 ml) containing the liquid MS medium (without agar). Cultures were incubated under shaking to obtain sufficient aeration. Interval of sub-cultivation was cut short to 14 days and the growth of cells was controlled. The PCV method (Packed Cell Volume) was used for monitoring of biomass growth.[78] This method is based on volume of cells (sediment of biomass). Suspension culture was observed microscopically (Fig. 17) and the samples for next processing were collected during the exponential phase of cells growing.

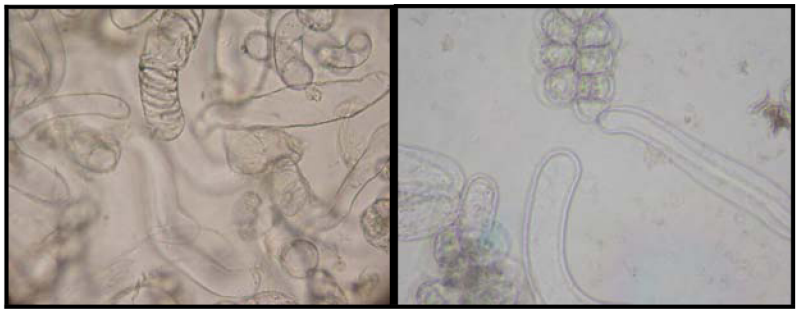

Fig. 17: Suspension cultures of parsley in liquid MS medium after 14 days of cultivation: *The presence of cells of different size and shape in culture is shown.. In the suspension the occurrence of isodiametric divisive cells as well as elongated and tracheary elements can be seen. The appearance of elongated cells could be due to utilization of auxin.*

3.3 Extraction methods

3.3.1 Extraction of polysaccharides

Extraction of cell wall polysaccharides can be evaluated using two different ways; sequential extraction with minimal degree of degradation and extraction based on partial degradation of polymer. [60] In this work the sequential extraction was used. Polysaccharides were extracted from suspension cell cultures.

Cells in exponential phase were collected, centrifuged (15, 000 x g, 20 min, 4 °C), washed with sterile water and centrifuged again. Sucrose free cell walls were filtrated using the very fine nylon cloth and processed according extraction pathway described below (Fig. 18):

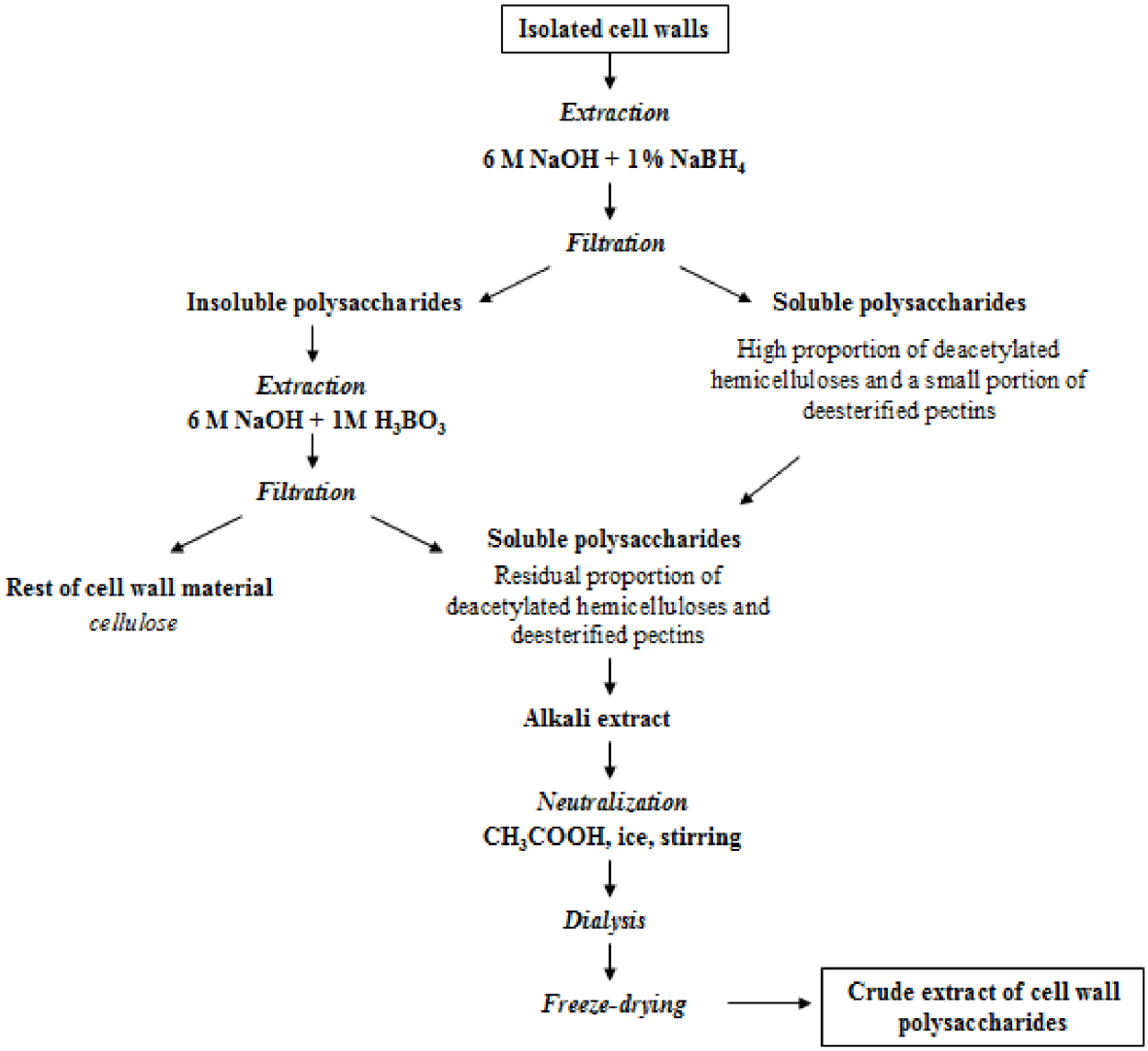

Fig. 18: Sequential extraction of cell wall polysaccharides [60]

3.3.2 Extraction of proteins

3.3.2.1 Protein extraction and isolation from parsley roots

The first step of protein extraction was performed using a juice extractor. The obtained juice was filtered, precipitated with ammonium sulphate until 90% of saturation as determined by refraction method. After 24 h at 4 °C the sample was filtered again, dissolved in a small amount of water, dialyzed against distilled water and freeze-dried (Fig. 19).

The proteins retained in pulp were extracted for 12 h with 0.1 M imidazole solution, pH 6.0 containing 1 M NaCl. After centrifugation (24, 000 x *g*, 20 min, 4 °C), the obtained juice was handled as described for juice previously (Fig. 19).

3.3.2.2 Protein extraction and isolation from cell cultures

The release of proteins from disrupted cells (callus or suspension cell cultures) was provided in 0.1 M acetate buffer, pH 5.0, with 1 M NaCl. After centrifugation (24, 000 x *g*, 20 min, 4 °C), the obtained material was handled as described for extraction from intact parsley roots (Fig. 19).

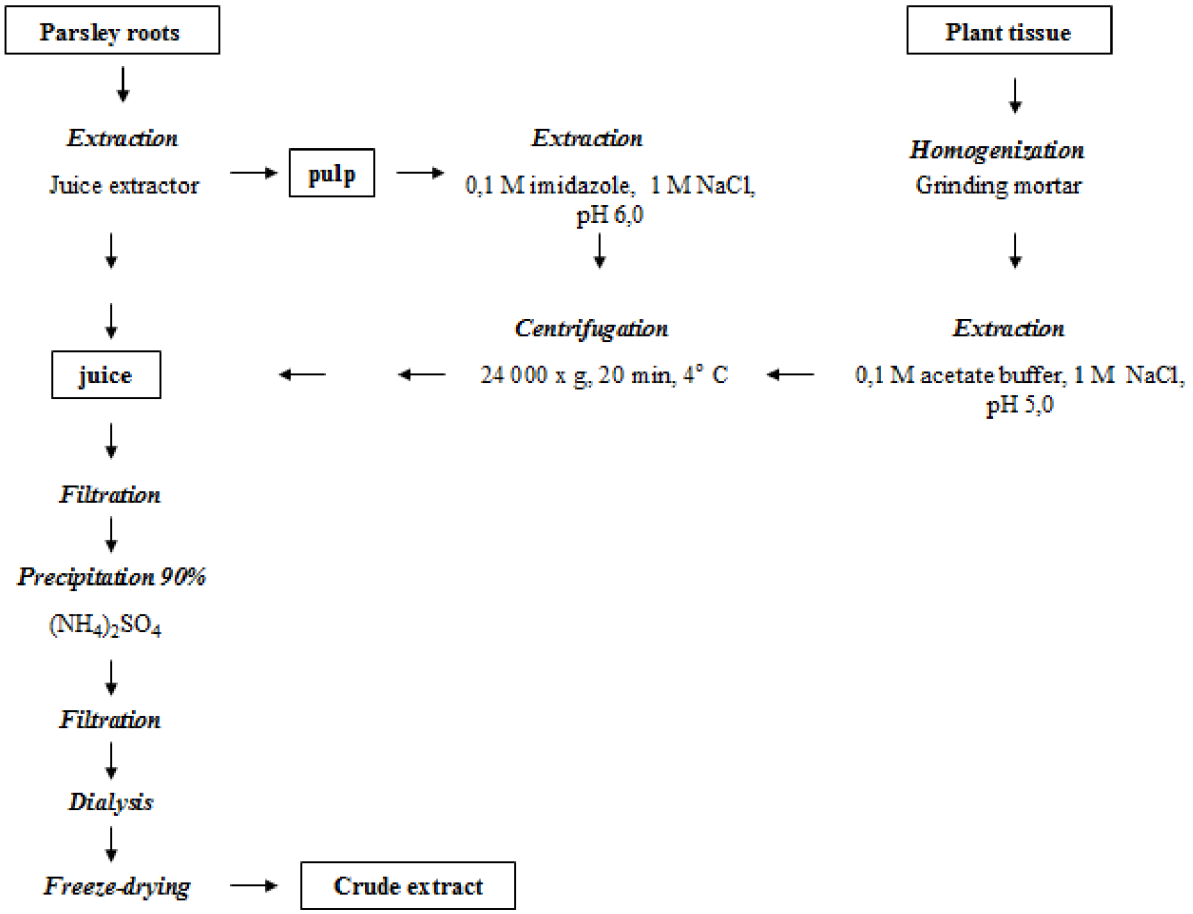

Fig. 19: Pathway of protein isolation from parsley roots and plant tissue

3.3.2.3 Differential ultracentrifugation

For localization of exopolygalacturonase forms in individual cell structures the method of differential ultracentrifugation was used. [80] The sample (parsley root) was first homogenized and suspended in 0.1 M acetate buffer, pH 5.0 containing 0.03 M mannitol. Suspension of broken cells was then centrifuged (800 x g, 10 min, Optima L-90K preparative ultracentrifuge, Beckman Coulter, Fullerton, California) and further processed according the scheme below (Fig. 20). The individual fractions were collected and the proteins of interest were isolated and in-between compared. Individual forms of enzyme were identified on the basis of different pH optima and substrate preferences.

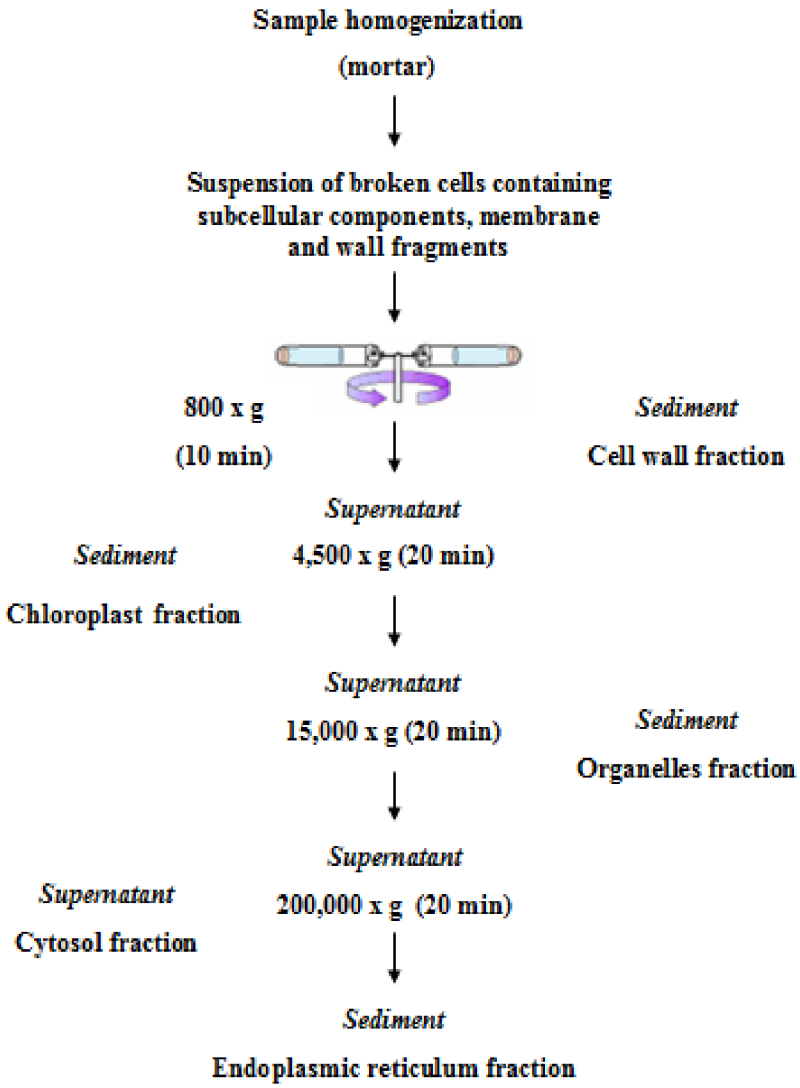

Fig. 20: Differential ultracentrifugation – fractionation of parsley cells

3.4 Separation methods

Isolated polymers (polysaccharides and proteins, respectively) were separated in analytical scale for identification as well as using preparative methods for their purification and further study. Extracted material required first the removal of low molecular substances by dialysis. Dialysis processes were performed using dialysis tubing cellulose 10 kDa cut off membrane (Sigma Aldrich) against distilled water. Desalted samples were concentrated by lyophilization and further separated on the basis of molecular weights, charges, isoelectric points or affinity to suitable matrixes.

The Pharmacia (Sweden) Fast Protein Liquid Chromatography (FPLC) system was used for methods development as well as for the purification of pre-purified proteins. The system includes:

- Liquid Chromatography Controller LCC-500
- 2 x High precision Pump P-500 capable of continuous flow of buffer to the purification column
- pre-filter for additional buffer purification
- mixer to produce elution gradients
- channel switch Motor Valve MV-7
- sample loop 200 µl
- suitable column
- Single Path Monitor UV-1, a dual beam flow-through UV-absorptiometer for monitoring the UV-absorption of a flowing liquid at 280 nm
- Two-channel Recorder REC-482
- fraction collector FRAC-100

3.4.1 Gel permeation chromatography

GPC (gel permeation chromatography) separates on the basis of molecular weight and can be successfully used preparatively to remove low molecular compounds from a polymer. For gel filtration two different types of medium (gel) were used; Biogel (Biorad) and Sephadex (Pharmacia), according to the types of polymer (www.biorad.com, www.amersham.com).

Biogel P (Biorad) gels are porous polyacrylamide beads prepared by copolymerization of acrylamide and N,N'-methylene-bis-acrylamide. The gels are extremely hydrophilic and essentially free of charge and provide efficient, gentle gel filtration.

Sephadex (Pharmacia) is a cross-linked dextran which swells considerably in water. The degree of cross-linkage determines the porosity and hence the fraction range.

Hemicelluloses in polysaccharide samples could directly adsorb to cellulose. Therefore the non-cellulosic matrix of Biogel P was chosen for their separation. Proteins were separated using the Biogel as well as Sephadex columns.

Separation of polysaccharide

- **Biogel P-2** column (1.0 x 90 cm): the sample was dissolved and eluted with water at a flow rate of 0.1 ml/min, 3 ml/fraction. Fractions were evaluated for polysaccharide content by phenol-sulphuric acid method and fractions with polysaccharide content were collected.

Separation of proteins

- **Biogel P-30** column (3.0 x 130 cm): protein sample was dissolved in 0.05 M McIlvane buffer pH 5.4 with 0.15 M NaCl and eluted in the same solution at flow rate of 0.2ml/min, 4 ml/fraction. The polygalacturonase (PG) activity was measured and fractions with PG activity were collected.
- **Sephadex G-25** Medium column (1.5 x 90 cm): protein mixture was dissolved and eluted with water at flow rate of 0.3 ml/min, 10 ml/fraction. In eluted fraction, the protein content, PG activity and salts content were determined. Fractions with enzyme activity were collected.

- **Sephadex G-25 Fine** (DP 10, Pharmacia) column (1.5 x 6.5 cm): this column was used for desalting of small amount of sample. Proteins (2.5 ml) were eluted through the column by water (3.5 ml).

Size exclusion chromatography coupled with FPLC was used for further purification of partial refined proteins.

In this work two different types of columns for analytical gel filtration were chosen: Superose 12 (agarose matrix) and Superdex 75 (dextran matrix).

- **Superose 12^{TM} HR** column (1.0 x 30 cm) (Pharmacia, Sweden) is a cross-linked, agarose based medium optimized for high performance gel filtration of biomolecules. The narrow particle size distribution of Superose enables high flow rates at low back-pressure. Proteins in the Mr range of 10-10, 000 Da are separated.
- **Superdex 75** column (1.0 x 30 cm) (Pharmacia, Sweden) is produced by covalent binding of dextran to highly cross-linked porous agarose beads. The separation properties of the composite medium are predominantly determined by the dextran component. The steep selectivity curve gives excellent resolution of proteins and peptides in the molecular weight range 3 000 – 70, 000 Da.

Separating process

Separating process was identical for both chosen columns; Superose 12 and Superdex 75. The column was equilibrated for 1 hour with the eluent buffer at flow rate 0.5 ml/min. The protein sample was then dissolved in eluent and applied to the column and eluted by 0.05 M phosphate buffer, pH 7.0 containing 0.15 M NaCl. Fractions were evaluated for the protein content and enzyme activity was identified on the basis of its pH optimum and substrate preference. Fractions of interest were collected.

Analytical connecting of FPLC and column for gel filtration is suitable for monitoring of protein purification process as well as for molecular weight determination. This characterisation requires the calibration of the column with the well-characterised standards.

Calibration curve of protein standards

Calibration curve and determination of molecular weight of proteins was determined using MW-GF-1000 Molecular Weight Marker kit for gel filtration (Sigma). For Superose 12 and Superdex 75 column the same marker kit was used:

Standard	Molecular weight
• Cytochrome	12 400
• Carbonic anhydrase	29 000
• Albumin, bovine	66 000
• Alcohol dehydrogenase	150 000
• β-amylase	200 000
• Apoferritin	443 000
• Thyroglobulin	669 000

3.4.2 Affinity chromatography

Affinity chromatography is based on a specific interaction of the sample with suitable effectors possessing reactive group. This interaction results in forming of stable complexes

and their disintegration (destruction under changes of microenvironmental conditions). This method can be used for proteins as well as for polysaccharides separation.

In this work, two different types of "specific adsorption" were used: polysaccharides separation process was based on a strong hemicellulose-cellulose affinity and for protein purification the specific interaction between lectin (concanavalin A) and glycosyl part of protein (potentially mannnosylated protein) was used.

3.4.2.1 Separation of polysaccharides

- **Adsorption of xyloglucan to Whatman 3MM paper**:

Xyloglucan-cellulose binding assay:

Polysaccharide sample was dissolved in 5 ml of 25 mM acetate buffer, pH 5.6 to concentration of 1mg/ml, applied on the Whatman paper 3M (2.5 x 2.5 cm) and incubated at 37° C for 6 hours under gentle rotation.

Desorption of bound xyloglucan:

The Whatman paper was washed with water to remove unbound polysaccharides. Bound xyloglucan was released after incubation overnight with 4 M NaOH at room temperature under constant stirring. Alkali solution was neutralized with concentrated acetic acid until neutral pH. After lyophilization the saccharide content was determined by sulphuric acid-phenol method and the sample was desalted on Biogel P-2 column.

- **Adsorption of xyloglucan to Avicel PH -101**:

Preparation of avicel cellulose:

Avicel cellulose PH – 101 (Sigma, Germany) was first washed with 4 M NaOH with constant stirring overnight at room temperature. The cellulose suspension was centrifuged (15,000 x *g*, 10 min), sediment was filtrated through the nylon cloth and washed with water until neutral pH. The prepared avicel was lyophilized.

Xyloglucan-avicel binding assay:

Pre-washed cellulose and polysaccharide sample were dissolved in 25 mM acetate buffer, pH 5.6, mixed (100:1 in weight) and incubated at 37° C under rotation for 6 hours. The mixture was centrifuged (15, 000 x g, 10 min). In supernatant the unbound polysaccharides were determined using the sulphuric acid-phenol method.[81] Bound fraction was collected and lyophilized.

Release of bound xyloglucan:

The bound xyloglucan was released after incubation in 6 M NaOH at room temperature for 6 hours under rotation. Suspension was centrifuged (15, 000 x *g*, 10 min), the alkali supernatant was neutralized with concentrated acetic acid and lyophilized. The saccharide content in sample was determined (by sulphuric acid-phenol method). Sample was desalted on Biogel P-2 column.

3.4.2.2 Separation of proteins

Purification of glycoproteins on concanavalin A – matrix (ConA) is generally shown in Fig.21:

- **Con A-HEMA 1000 E** column (1.5 cm x 2.5 cm): ConA A-HEMA 1000 E was prepared by the reaction of concanavalin A (Fluka, Germany) with the epoxy groups of HEMA 1000 (Tessek, Czech Republic) in 0.1 M acetate buffer, pH 3.8 for 11 days at 4°C. Mixture of parsley root proteins was applied on column in 0.1 M acetate buffer, pH 4.7, with addition of 0.1 M NaCl, 0.001 M $MnCl_2$ and 0.001 M $CaCl_2$ at flow rate 1ml/fraction. As the eluting agent was used 0.1 M methyl-α-D-mannopyranoside (Sigma, Germany) in 0.1 M acetate buffer, pH 4.7 with 0.1 M NaCl. Protein content and enzyme activity were measured and fractions with PG activity were collected.
- **Con A-Sepharose** column (1.5 cm x 2.5 cm) (Pharmacia, Sweden): Con A-Sepharose is concanavalin A covalently bound to Sepharose. Purification was performed in 0.1 M acetate buffer pH 6.0 with 1 M NaCl, 0.001 M $CaCl_2$, $MgCl_2$ and $MnCl_2$ at flow rate of 1ml/fraction. For elution of bound proteins 0.1 M methyl-α-D-mannopyranoside (Sigma, Germany) in 0.1 M acetate buffer, pH 6.0 with 0.1 M NaCl was used. In all fractions the protein content and polygalacturonase activity were determined. Fractions with PG activity were collected.

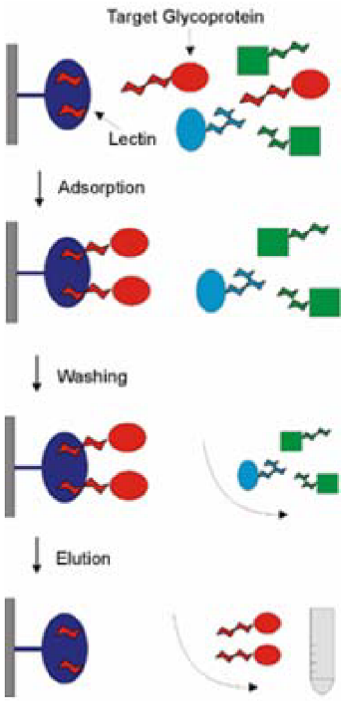

Fig. 21: Mixture of proteins is applied to the lectin (Concanavalin A) matrix. The target glycoprotein specifically binds to the lectin. Unbound compounds and contaminants are washed out and the target glycoprotein is desorbed with a special elution buffer.

3.4.3 Anion-exchange chromatography

Neutral and acidic polysaccharides were separated by the anion-exchange chromatography using Spheron DEAE (Lachema, Czech Republic) column. DEAE Spheron is a medium-basic anion-exchanger (ethyleneglycol methoxylate with diethylaminoethyl groups in side chains) and represents the non-cellulosic material suitable for separation of xyloglucan-like molecules.

Separating process

- **DEAE Spheron** column (1.0 cm x 3.0 cm): a polysaccharide sample dissolved in 0.02 M Tris/HCl buffer, pH 7 was applied to the column equilibrated in the same buffer and eluted at the flow rate of 1 ml/min (fraction) until no carbohydrate was detected in the elute by the anthrone assay. Material bound to the column (acidic polysaccharides) was then eluted by the 0.02 M Tris/HCl buffer with a linear gradient of NaCl (0-1 M) at the same flow rate. Fractions were assayed for total polysaccharide content.[81, 82, 83] Individual fractions were collected, desalted and freeze-dried.

3.4.4 Chromatofocusing

The chromatofocusing method coupled with FPLC device was used as the final step of protein purification, when the separation of individual enzyme forms was required.

Chromatofocusing offers the high resolution obtained by separations based on differences in isoelectric points, together with high capacity of ion exchange techniques. The pH is chosen so that the isoelectric points of the proteins of interest fall roughly in the middle of the pH gradient. The appropriate Polybuffer exchanger is then equilibrated with start buffer. The pH of start buffer is set slightly above the upper limit of the pH gradient, while the pH of eluent, Polybuffer, is adjusted to the value chosen for the lower limit of the pH gradient. The sample is equilibrated with eluent and applied to the column. The column is then eluted with Polybuffer and pH gradient is forming automatically. Proteins in the sample are eluted in order of their isoelectric points (from basic to acidic).

- **Mono P HR 5/20** column (0.5 x 20 cm) (Pharmacia, Sweden) is Polybuffer exchanger 94 used as a weak anion exchange medium for separating of proteins with pI 4-9.

Separating process

After equilibration of column with start buffer (for 2 hours), the sample was dissolved in the same solution to the saturated concentration and centrifuged. Then the sample (200 µl) was applied to the column. Proteins were eluted with Polybuffer at flow rate of 0.5 ml/min, 0.5 ml/fraction. Fractions were evaluated for the protein content and enzyme activity and accordingly collected.

Buffer system for pH 6-4 interval

- Starting buffer 0.025 M bis-Tris, pH 6.3, HCl
- Eluent Polybuffer 74, pH 4.0, HCl

The approximate value of protein pI can be determined from pH of elute which released it from medium.

3.4.5 Preparative isoelectric focusing

The process was performed at the same conditions as that used in analytical isoelectric focusing (described in chapter 3.5.1.). After IEF, the zones corresponding to the protein forms were cut out from the gel (segments with a diameter smaller than 2 mm) and washed out from these segments by water. The individual forms of pectate hydrolases were then detected on the basis of activities utilizing their pH optima and the substrate of preference.

3.5 Analytical methods

3.5.1 Isoelectric focusing

Electrofocusing technique was used for the separation of proteins according to their isoelectric points. For zone electrophoresis two different gels were prepared; separating polyacrylamide gel and detecting agarose gel.

Separating polyacrylamide gel

Flat-bed isoelectric focusing of proteins was performed in 1 mm thick polyacrylamide gels (13.5 x 17 cm) of the following composition:

• 3.6 ml	30 % acrylamide AA
• 3.0 ml	Bis-acrylamide Bis-AA (2 %)
• 1.0 ml	glycerol
• 1.5 ml	ampholytes pH 3-10 or pH 4.5 – 7 (SERVA)
• 7.0 ml	distilled water
	Degassing
• 5.0 µl	TEMED
• 0.8 ml	ammonium persulphate (APS)

Detecting agarose gel

Detecting gel was prepared from two solutions:

- 250 mg of decagalacturonic acid (DP 10) was dissolved in 5 ml of water
- 0.3 g of agarose was dissolved by boiling in 7.5 ml of water

Both solutions were mixed, briefly boiled and 2.5 ml of acetate buffer pH 5.0 was added (to avoid changes of pH). The casting was performed in gel casting cassette (18.0 x 16.0 cm) using 0.75 mm spacers. The solution mixture was quickly poured into a prewarmed (75 °C) casting assembly. The assembly was then cooled and the solidified gel was stored at 4 °C until use for detection.

The isoelectric focusing was performed under the following conditions:

•	Prefocusation	Start	120 V
		30 min	220 V
•	Focusation	30 min	400 V
		30 min	700 V
		30 min	900 V
		45 min	1100 V
		45 min	1250 V

Detection by zymogram technique [84]

The detection of pectate hydrolases was performed by zymogram ("sandwich") technique. The separating polyacrylamide gel after isoelectric focusing (signed as S in Fig. 22) was incubated with detecting agarose gel (signed as D in Fig. 22) and processed according the following scheme (Fig. 22):

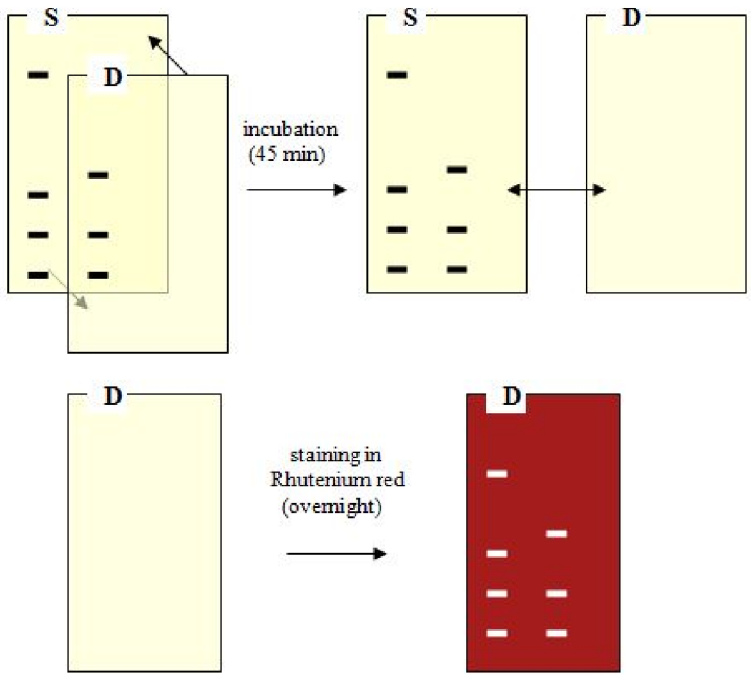

Fig. 22: Zymogram technique for detection of pectate hydrolases [84]

3.5.2 SDS-PAGE

The control of purification processes and molecular mass determination (Mr) of proteins were performed by SDS-polyacrylamide gel electrophoresis in 12% gel under reducing conditions (with β-mercaptoethanol) according to Laemli (1970). [85] A molecular weight calibration kit protein standard (Serva) was used for Mr determination of proteins. Proteins were detected by silver staining method [86] or by Coomassie blue staining.[87]

3.5.3 Thin Layer Chromatography (TLC)

Thin layer chromatography method was used for fast and easy evaluation of substrates purity, determination of degradation products of enzyme reactions (products of the hydrolysis of natural and unsaturated oligogalacturonates) as well as for the monitoring of polysaccharide content during the column separations.

Polysaccharides - TLC

Fractions eluted from Biogel P2 column were assayed for polysaccharide content by TLC using the commercial Silicagel 60 plates (Merck). Samples were spotted (1µl) onto plate and dried. Detection was performed according to the colorimetric orcinol method [81] with 5 % solution of orcinol.

Substrates purity - TLC

Substrates used for enzyme reactions were tested for purity using TLC. Oligogalacturonide samples (1µl) of different degree of polymerization (DP 2-10) were spotted onto the silica gel plate (Merck) and dried. Plate was developed in butanol-formic acid-water (2:3:1, v/v/v) mixture and detected by spraying with 20 % solution of ammonium sulphate. [88] Detected plates were visualized by gently heating. As a reference standard, D-galacturonic acid (1µmol/ml) was used.

3.5.4 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography was used for the purity control of prepared substrates used for enzyme reactions, too.

Oligogalacturonate samples (DP 2-10) after purification pathways were dissolved in 0.3 M sodium phosphate buffer, pH 4.4 to the concentration of 10 mM/ml and applied to the HPLC column.

Separating conditions

- HPLC — Shimadzu (Japan)
- Column — Shodex Asahipak NH_2P-50 4E (0.46 cm x 25.0 cm)
- Eluent — 0.3 M sodium phosphate buffer, pH 4.4
- Flow rate — 1.0 ml/min
- Detector — Shodex UV (210 nm)/ RI
- Column temperature — 40 °C

3.5.5 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transformed infrared (FTIR) spectroscopy

Spectra were measured by NICOLET Magna - IR 750 with DTGS detector and OMNIC 3.2 software. The isolated hemicellulose-pectin complex from the freeze dried and rehydrated polysaccharide sample was pressed into KBr pellets with sample/KBr ration 2/200 mg. The

single beam traversing each sample was ratioed with the single beam of the corresponding background. 128 scans at a resolution of 4 cm^{-1} were averaged.

Attenuated total reflectance (ATR) spectroscopy

Spectra were measured in solid state by ATR accessory MIRacle. This is single reflection Horizontal Attenuated Reflectance Accessory (HATR) with ZnSe crystal.

3.5.6 Nuclear Magnetic Resonance (NMR)

Spectra were measured in D_2O at 25 °C and 40 °C on Bruker 300 MHz Avance DPX and Varian 600 MHz UNITY INOVA 600 NB spectrometers, equipped with 5 mm multinuclear probe with inverse detection and 5 mm 1H{13C, 15N}PFG Triple Res IDTG600-5, respectively, (both with z-gradients). ^1H NMR spectra chemical shifts are referenced to internal acetone (2.217 ppm).

3.5.7 Edman degradation

N-terminal sequencing of apparently SDS-homogeneous protein was provided in Procise – Protein Sequencing System (Applied Biosystems, USA) by Ing. Zdeněk Voburka at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of Czech Republic, Prague. The principle of Edman degradation is shown in Fig. 23.

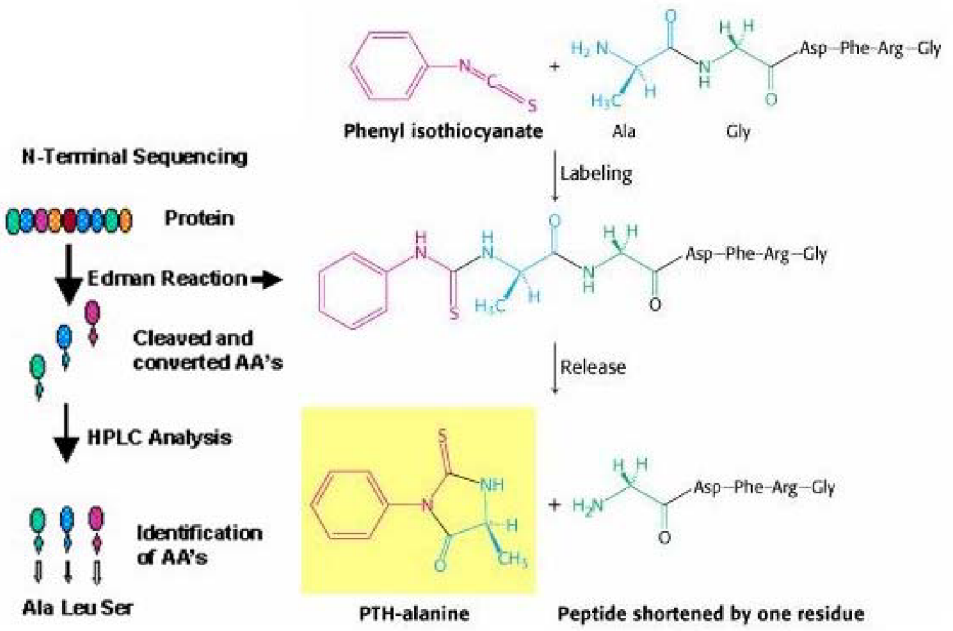

Fig. 23: Principle of Edman degradation and N-terminal sequencing [89]

3.5.8 MALDI – TOF/TOF mass spectrometric sequencing of proteins

Coomassie Blue stained proteins from isoelectric focusing were enzymatically digested and the resulting peptides were analyzed and sequenced by matrix assisted laser desorption mass spectrometry according Jensen method.[90, 91]

3.5.8.1 In-gel digestion of proteins

Coomassie Blue visualized protein bands of interest were excised from the gel whereas the bands were cut as close to the protein as possible to reduce the amount of background gel. Excised gel pieces (1x1 mm) were transferred into a 1.5 ml microcentrifuge tubes.

Washing of gel pieces

The gel particles were washed with water and water/acetonitrile (1:1), with two changes, for 15 minutes per change. All remaining liquid was removed and acetonitrile (ACN) was added to cover the gel particles. After the gel pieces have shrunk and discoloured, the acetonitrile was removed and the gel pieces were rehydrated in 0.1 M NH_4HCO_3. After approximately 5 min, an equal volume of acetonitrile was added (to get 0.1 M NH_4HCO_3/ACN, 1:1). After 15 min of incubation, all liquid was removed and gel particles were dried in a vacuum centrifuge.

Reduction and alkylation

The gel particles were swollen in solution containing 10 mM dithiotheitol in 0.1 M NH_4HCO_3 and incubated for 45 min at 56 °C to reduce the proteins. After incubation the excess liquid was removed and quickly replaced with the same volume of 55 mM iodoacetamide in 0.1 M NH_4HCO_3. The mixture was incubated for 30 min at room temperature in the dark. The iodoacetamide solution was removed and the gel particles were washed with 0.1 M NH_4HCO_3 for 5 min and then with acetonitrile for next 15 min. The washing cycle was repeated until all the Coomassie has removed.

In-gel digestion with trypsin/chymotrypsin

After removing of all the Coomassie Blue, the gel particles were completely dried in a vacuum centrifuge and then rehydrated with the digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$ and 12.5 ng/µl of trypsin (Promega, modified sequencing grade). After 45 min of incubation, the remaining supernatant was removed and replaced with 20 µl of the same buffer (without enzyme) to keep the gel pieces in the solution during enzymatic cleavage. Enzyme reaction was running at 37 °C overnight.

Extraction of peptides

The peptides were extracted from the gel pieces by addition of a sufficient volume of 25 mM NH_4HCO_3 to cover the gel and the sample was incubated for 15 min. Then the same volume of ACN was added and incubated for next 15 min. Extraction was repeated two times with 5% formic acid and ACN (1:1), extracts were pooled and dried in a vacuum centrifuge.

3.5.8.2 Purification and pre-concentration of peptide samples

For desalting and pre-concentration of peptide extracts, the small chromatography column, "ZipTip pipette tips" with C18 reversed phase were used (Millipore, USA).[92] The dried peptide samples were dissolved in 20 µl of 0.5% TFA and purified according to the ZipTip protocol (www.millipore.com).

ZipTip C_{18} purification protocol

- Wetting: 2 x 10 µl of 100% ACN
- Equilibrating: 2 x 10 µl of ACN: 0,1% TFA =5:95
- Sample binding: 10 x 10 µl of sample dissolved in 0,5% TFA
- Washing: 2 x 10 µl of 0.1% TFA
- Elution: 5 x 10 µl of ACN: 0.1% TFA = 60:40
- Recycling of the tip: 2 x 10 µl of ACN: 0.1% TFA = 60:40

3.5.8.3 MALDI-TOF/TOF mass spectrometry analysis

MALDI-TOF/TOF measurements in the positive reflectron mode were performed with Applied Biosystems 4700 Proteomics Analyzer (Fig. 24) (Applied Biosystems, Framingham, MA). This TOF/TOF instrument is equipped with a Nd:YAG laser (355 nm) of 3–7 ns pulse and 200 Hz firing rate. Both MS and MS/MS spectra were acquired using dual-stage reflectron mirror. Accelerating voltages applied for MS and MS/MS measurements were 20 kV and 8 kV, respectively. In MS/MS mode, collision energy of 1 kV was applied and nitrogen was used as a collision gas. Raw spectral data were further processed using Data Explorer 4.5 software (Applied Biosystems, Framingham, MA, USA). For measurement of protein digests, a solution of α-cyano-4-hydroxycinnamic acid (5 mg/ml) in ACN/0.1% TFA (3:2, v/v) was used. Digests desalted previously by ZipTip C_{18} were deposited on the previously deposited matrix layer and dried.

Data processing

MS data were further processed using GPS Explorer 3.6 software (Applied Biosystems, Framingham, MA, USA) connected to the Mascot program package (local installation, ver. 1.9.05) where the database searching was performed. The following parameters were used for the database searching: database – NCBInr (ver. 24.10.2006); taxonomy – all entries; enzyme – trypsin; allowed missed cleavages – 1; fixed modifications – carbamidomethyl (C); variable modifications – none; peptide tolerance – 30 ppm; MS/MS tolerance – 100 mmu; peptide charge – (+1); monoisotopic masses; instrument – MALDI-TOF/TOF.

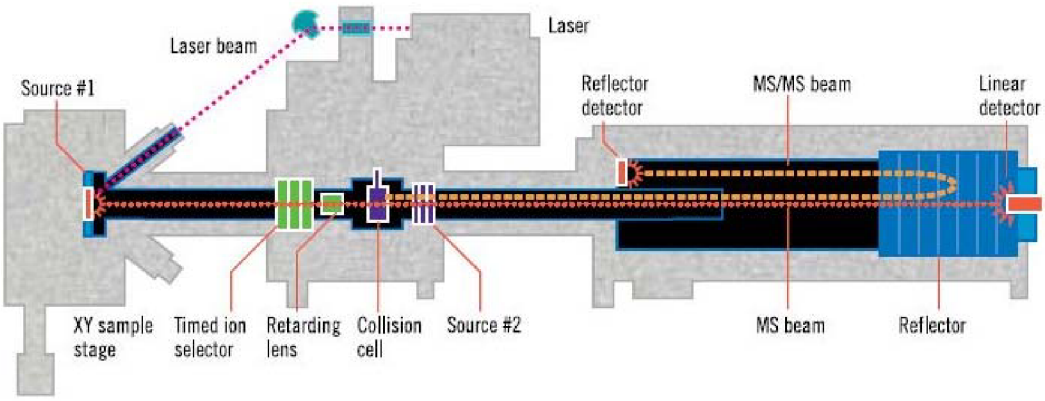

Fig. 24: The TOT/TOF optics acquires data in linear and reflector MS and true precursor-selected MS/MS modes (www.appliedbiosystems.com)

Spectra interpretation

De-*novo* interpretation was done manually as well as by using DeNovo Explorer (ver. 3.6, Applied Biosystems, Framingham, MA, USA). Similarity based searches were done using Pro BLAST program (ver. 3.6, Applied Biosystems, Framingham, MA, USA).

3.6 Biochemical assays

3.6.1 Determination of polysaccharide content

3.6.1.1 Determination of total neutral sugars

Total neutral sugars were assayed by phenol-sulphuric acid method.[81,82] To the polysaccharide sample (200 µl) 0.5 % solution of phenol (200 µl) and concentrated sulphuric acid (1 ml) were added, and within 20 min the absorbance measurements at 490 nm was provided.

3.6.1.2 Determination of uronic acids

Uronic acids content was determined according to the Blumenkratz method with 0.15% solution of meta-hydroxydiphenyl reagent in 0.5% NaOH. [83] Sample mixture was incubated with 0.0125 M solution of tetraborate in concentrated sulphuric acid and after heating in a water bath at 100 °C for 5 min the meta-hydroxydiphenyl reagent was added and absorbance at 520 nm was measured. For quantitative determination the calibration curve of D-galacturonic acid (0.01 – 0.12 µmol/ml) was made.

3.6.2 Determination of protein content

The occurrence of proteins in the sample was evaluated either upon monitoring the UV-absorption at 280 nm (Jennway 6305 Spectrophotometer) of effluent during chromatography or using the quantitative method according to Bradford. [93] For quantitative proteins determination the calibration curve of albumin was done.

3.6.3 Measurement of enzyme activities

3.6.3.1 Substrates

Activity assay was performed using a different substrates: pectate (polymer), oligogalacturonic acids of different degree of polymerization (DP 2-15), unsaturated oligogalacturonic acids (DP 2,3) as well as xyloglucan (tamarind seeds, Glyloid), xyloglucan oligosaccharides ([^{3}H]XXXG) [94,95] and arabinoxylan (Megazyme).

Preparation of pectate

Commercial citrus pectin (Genu Pectin, Copenhagen, Denmark) was purified by washing with acidified 60% ethanol (5 ml conc. HCl/100 ml of 60% ethanol), followed by 60% and 96% neutral ethanol according to Kohn and Furda. [96] Pectate (DP 152) was prepared from this pectin by repeated deesterification with 0.1 M NaOH at pH 10, 22 °C. Deesterified product was precipitated by HCl added until the pH of solutions was adjusted to 2.5 and then neutralized by means of KOH.

Preparation of oligogalacturonic acids

1% sodium pectate solution was carried out in 0.5 M acetate buffer at pH 4.6. 0.26 mg of polygalacturonase (Leozým, Liko Leopoldov, Slovakia) was added to 100 ml substrate and incubated at 30 °C for 10 min. The enzyme was deactivated after 10 minutes by boiling. The reaction mixture was filtrated and freeze-dried. Products of hydrolysis were separated by chromatography on two subsequently connected Sephadex G-25 Fine (Pharmacia) columns (5.6 x 120 cm) equilibrated and eluted with 0.05 M phosphate buffer at pH 7.0. Purified oligo-(D-galacturonic) acids (OGAs) with DP 2-8 were desalted using a Sephadex G-10 (2.0 x 100 cm) column (Pharmacia, Sweden) and lyophilized. [97]

Unsaturated oligogalacturonic acids preparation

Unsaturated oligogalacturonic acids (DP 2,3) were obtained after enzymatic degradation of potassium pectate by bacterial pectate lyase (1% pectate treated by lyase of *Erwinia* sp., pH 8.0, sustained by addition of 0.1 M KOH, 30 °C, 20 h) and freeze-dried. Products of hydrolysis were separated by gel permeation chromatography as described for preparation of OGAs. [97]

Preparation of fluorescently labelled oligogalacturonic acids

The preparation of fluorescently labelled oligosaccharides was done according to Kosík and Farkaš (2008). [95] The starting oligosaccharides (mixture of OGAs of DP 2-8) were in the first step converted to corresponding 1-amino-1-deoxy-alditols (glycamines) by incubation with ammonium acetate and $NaCNBH_3$ at 80 ºC for 2-4 h and in the second step, the glycamines were reacted with Lissamine™ rhodamine B sulfonylchloride to obtain fluorescent sulforhodamine conjugates of the glycamines. All operations were carried out in a single centrifuge test-tube and the products from the individual reaction steps were isolated on the basis of their differential solubility in organic solvents. The purity of the product was checked by TLC on silicagel plates using the solvent system n-buthanol-ethanol-water (5:3:2).

3.6.3.2 Activity assay

Pectate hydrolase activity

Pectate hydrolase activity was assayed in 0.1 M acetate buffer at a pH value corresponding to the pH optimum of the individual form (pH optimum was evaluated in the range of pH 3.6-5.6) at 30 °C by measuring the increase of colour intensity at 530 nm in the reaction mixture containing solutions of substrates (1 mM solution of oligogalacturonates or 0.5% solution of sodium pectate). [98, 99]

The enzyme activity was then expressed in μmol reducing groups liberated within 1 min by 1 mg protein and determined by means of standard graph for D-galacturonic acid.

Xyloglucan endotransglycosylase activity

The transglycosylating activity of XET (enzyme separated from pectate hydrolases during purification process) was determined as described by Fry, 1992. [100] Reaction mixtures consisted of 10 μl of an assay mix containing 0.3% xyloglucan (donor substrate), 2.10^4 cpm of [^{3}H]XXXG (acceptor substrate) in 0.1 M succinate buffer, pH 5.5 and 10 μl of enzyme preparation. Time course assays were done at 25 °C over 1 hour with reactions stopped by the addition of 40 μl of 40% formic acid. Reaction mixture was spotted onto a 3 x 4 cm of Whatman 3MM paper, which was dried and then washed for 1 hour in running tap water to remove the unreacted [^{3}H]XXXGol. The paper was dried and polymeric material was assayed for ^{3}H by scintillation counting. [101]

Hetero-transglycosylation activity of crude extract from parsley roots

The mixture of fluorescently labelled oligogalacturonic acids (OGA-SR) as an acceptor substrate (10 μl) and 0.3% arabinoxylan as a donor substrate (50 μl) were incubated in succinate buffer pH 5.5 with 10 μl of centrifuged concentrated crude extract of proteins from parsley roots or nasturtium seeds [59] Operations were carried out in a single centrifuge test-tube at laboratory temperature for 24 hours. After incubation the samples were repeatedly washed with water and precipitated with ethanol (to get 60% ethanol) and centrifuged (10,000 x g, 5 min). Supernatants after each washing and precipitation step were spotted onto the Whatman 3MM paper and air dried. The content of unreacted OGA-SR in supernatant after individual washing steps and the effect of hetero-transglycosylating reaction (washed precipitate dissolved in 50 μl of distilled water) were detected under UV light.

3.6.4 Determination of action pattern

The action pattern of enzymes was demonstrated by the correlation of viscosity decrease of polymeric substrate with the decrease of its degradation. [102]

The reaction mixture of enzyme (1 mg/ml) and pectate substrate (0.5%) was incubated in Ubbelohde viscometer at 30 °C for 0 – 360 min and after each 3 min the decrease of viscosity of solution was monitored. At the same time intervals the measurements of liberated reducing groups was performed. Together with the molecular mass of pectate it was used for counting of the degree of pectate degradation.

Calculations

The decrease of viscosity *P (%)* was calculated according to equation (1): [102]

$$P = \frac{t - t_{H_2O}}{t_0 - t_{H_20}} \cdot 100 \qquad (1)$$

where t is the flow time of reaction mixture in given time, t_0 is the flow time of reaction in the initial point and t_{H_20} is the flow time of distilled water. The decrease of viscosity was then correlated by the degradation degree of polymeric substrate. Degradation degree represents the percentual amount of splitted bounds within the polymer molecule.

3.6.5 Determination of K_M values

Michaelis parameters of purified enzymes from parsley juice and pulp were determined utilizing the initial velocities at five concentrations of substrates, ranging from 0.1 – 0.5 mM for substrates with DP 2 – 10 or 0.05 – 0.25% for pectate. Reactions were performed at 30 °C, in acetate buffer of appropriate pH value. Michaelis parameters were calculated by nonlinear regression.

3.6.6 Determination of activation energy

Two parameters are needed to characterize the thermal stability of a given enzyme. One is the rate of inactivation at a specified temperature, expressed either as a rate constant or as a D value (equation 4), and the other is a measure of how the rate of inactivation varies with temperature, given either by an activation energy (Ea) or as z value. [103] With these two parameters the rate of enzyme inactivation at any temperature, and accordingly the expected level of residual activity remaining after a given heat treatment, can be calculated.

The determination of the rate of inactivation (thermal stability) was evaluated after 2 hour incubation of the enzyme solution at temperature from 20 °C – 70 °C and the following activity assay at 30 °C. The energy of activation (Ea) was then determined as the temperature profile of enzyme activity depending on time. [103]

Calculations

The rate constants k for first-order inactivation was determined from the slopes of the inactivation time courses according to equation (1): [103]

$$\log(A / A_0) = -(k / 2.303)t \qquad (1)$$

where A_0 is the initial enzyme activity and A is the activity after heating for time t. Slopes of these lines were determined by linear regression and the calculated rate constants replotted in Arrhenius plots.

Activation energies (Ea) were calculated from the slopes of these Arrhenius plots according to equation (2):

$$\ln(k) = -E_a / RT + c \qquad (2)$$

where R is the gas constant (8.314 J.mol^{-1}.K^{-1}) and T is the temperature in K. Slopes were calculated by linear regression.

For each enzyme a reference temperature was chosen near the middle of the temperature range used for inactivation determinations. The rate constant for inactivation at this reference

temperature (k_{ref}) was determined from the value of ln(k_{ref}) at this temperature given by the regression line in the Arrhenius plot.

With these two parameters, Ea and k_{ref}, the inactivation rate constant (k) at any temperature (T) can be calculated from equation (3):

$$\ln(k) = \ln(k_{ref}) - (E_a / R)(1/T - 1/T_{ref}) \qquad (3)$$

In some cases inactivation is given as a D value, the time required to reduce the enzyme activity to 10 % of its original value. The D value is directly related to the inactivation rate constant k by equation (4):

$$D = 2.303 / k \qquad (4)$$

3.6.7 Deglycosylation with N-glycosidase F

Proteins obtained from single SDS-PAGE band were tested for potential glycosylation using the N-glycosidase F deglycosylation kit (Table 2) for asparagine–linked glycan chains of glycoproteins (Roche, Germany). The principle of deglycosylation with N-glycosidase F is shown in Fig. 25.

Table 2: Deglycosylation kit composition

Bottle	*Contents*
1	N-glycosidase F, recombinant from *E.coli*
2	Denaturation buffer, pH 8.6 (Contains sodium phosphate and ionic detergent)
3	Control glycoproteins: • human transferring • ribonuclease B • human α1-acid glycoprotein
4	Reaction buffer, pH 7.2 (Contains sodium phosphate and non-ionic detergent)
5	Premixed protein molecular weight markers: • Phosphorylase B, 97.4 kDa • Bovine serum albumin, 66.2 kDa • Aldolase, 39.2 kDa • Triose phosphate isomerase, 26.6 kDa • Trypsin inhibitor, 21.5 kDa • Lyozyme, 14.4 kDa

For deglycosylation four microtubes were prepared. Two vials for control glycoproteins (standards) and two for the analyzed glycoproteins. To each sample (5 µl) reduced denaturation buffer was added (5 µl) and reactions were incubated for 3 min at 95 °C. After the heating step the contents were centrifuged. To each vial 10 µl of reaction buffer was added and mixed. One control sample (standard) and one analyzed glycoprotein were digested with 10 µl of reconstituted *N*-glycosidase F for 1 hour at 37 °C. Second standard and analyzed glycoprotein (blanks) were prepared by incubation with 10 µl of reaction buffer under the same conditions. After reaction time, the appropriate aliquot of the sample with an equal

amount of SDS-sample buffer were mixed and heated for 3 min to 95 °C. Then the analysis on SDS-PAGE was performed.

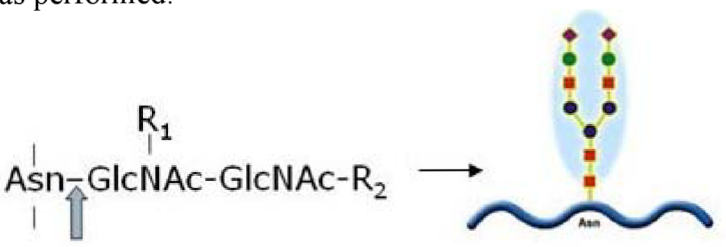

Fig. 25: Principle of deglycosylation
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4. RESULTS AND DISCUSSION

4.1 Hemicellulose-pectin cross-linking

4.1.1 Purification of polysaccharides

Purification of polysaccharides from cell suspension cultures obtained by cultivation of parsley roots was performed according to Fry (2001) [47] (Fig. 26). Cells were incubated in the solution of 6 M NaOH with 1% $NaBH_4$ at 37 °C for 24 hours under gently shaking. Cell suspension was then filtrated through the very fine nylon cloth and alkali extract was neutralized using the ice acetic acid. Neutral sample solution was then dialyzed and freeze-dried. Obtained crude polysaccharide extract was used for further purification procedure (Fig. 26).

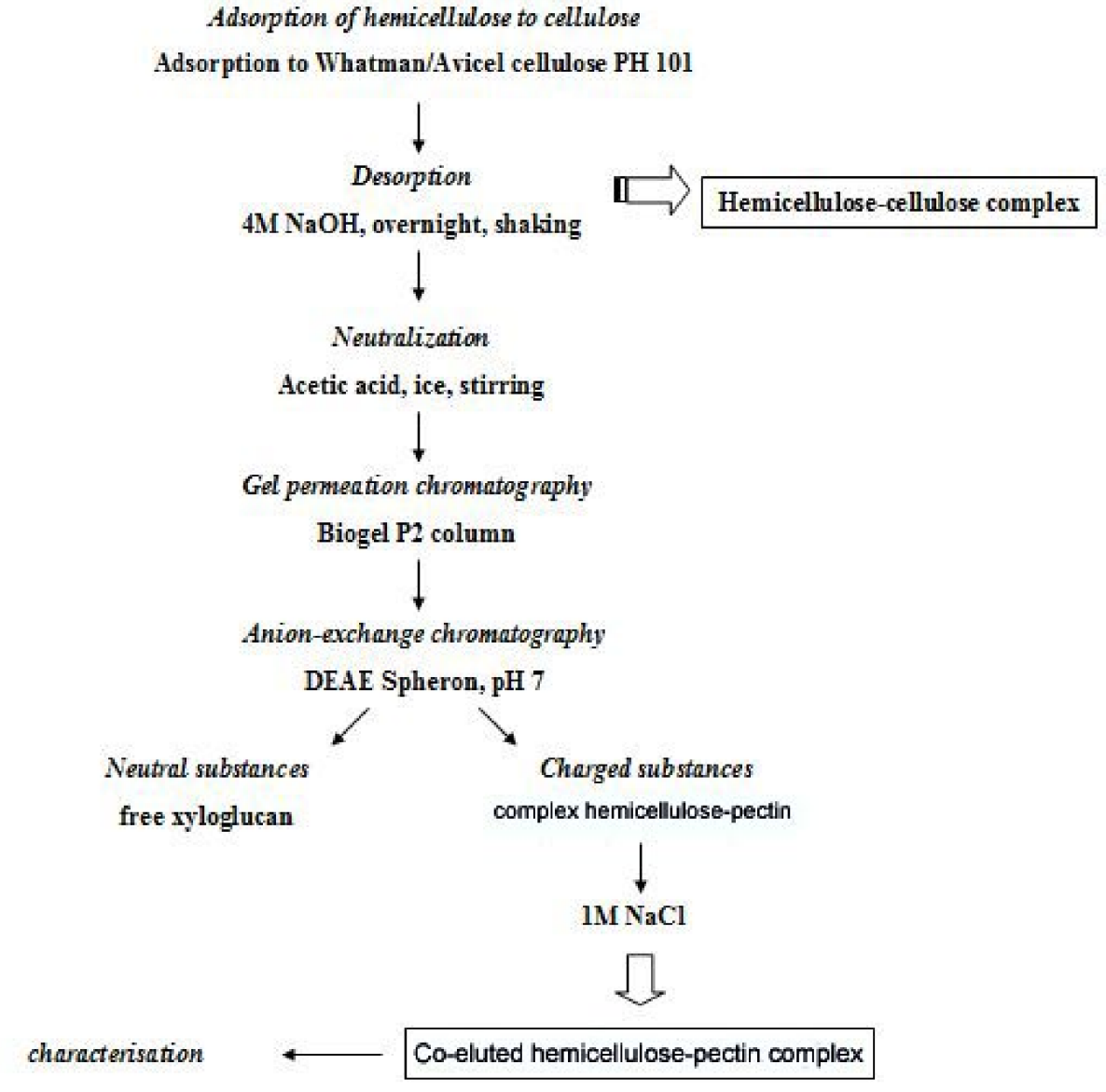


Fig. 26: Purification process developed for charged polysaccharide complexes

Adsorption of hemicelluloses to cellulose

For this type of affinity chromatography two types of cellulose were used pre-washed Avicel cellulose and Whatman 3MM paper. Polysaccharide sample was incubated with both types of cellulose under the same condition and the amount of adsorbed hemicelluloses was determined by phenol-sulphuric acid method (Fig. 27).

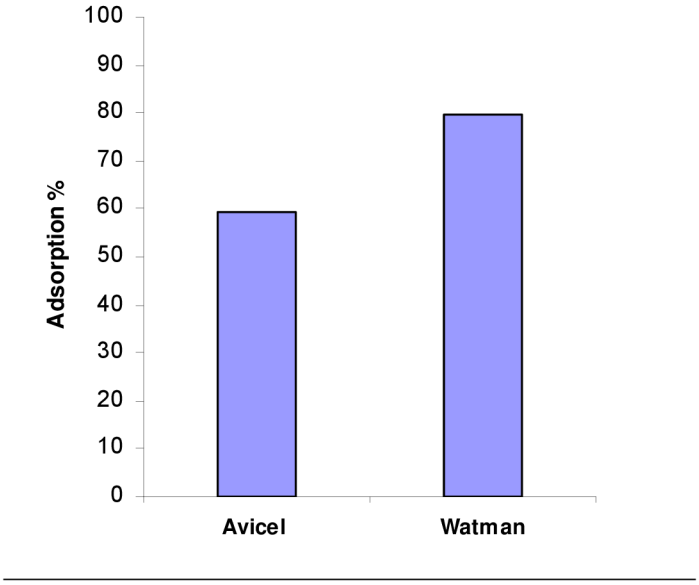


Fig. 27: Affinity chromatography: Adsorption of hemicelluloses to cellulose

As can be seen the amount of hemicellulose bound to Avicel cellulose was about 60 % and to Whatman paper was more than 80 % (Fig. 27). As a result the Whatman paper was used for the next adsorption process.

Hemicellulose sample was desorbed in 4 M NaOH, overnight at room temperature. After neutralisation, released polysaccharides were washed with water and dialyzed using the Amicon device through the polyethersulfone ultrafiltration membrane (10 kDa, Millipore) at nitrogen atmosphere. After first washing the amount of desorbed hemicellulose was monitored according phenol-sulphuric acid method. More than 90 % of polysaccharides were obtained.

Gel permeation chromatography on Biogel P2 column

The concentrated sample was freeze-dried and then applied to Biogel P2 column. Gel permeation chromatography was used for removing of the salt residues and separation of hemicellulose complex from other residual sugars. All fractions eluted from column were evaluated for the sugar content according the phenol-sulphuric acid (Fig. 28).

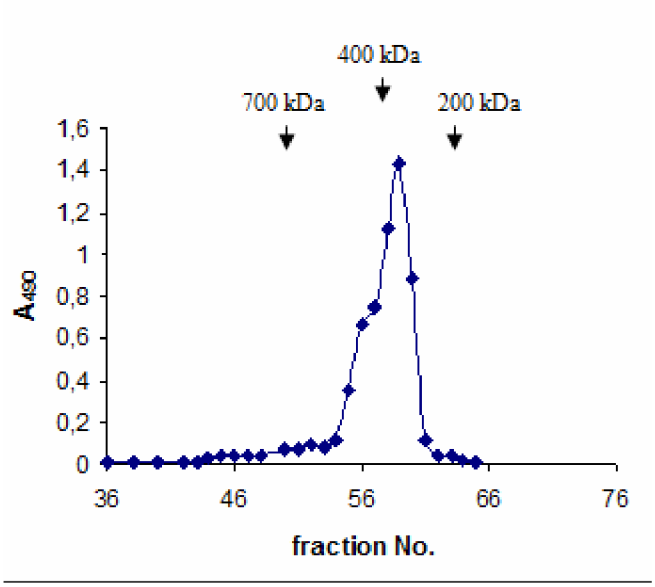


Fig.28: Gel permeation chromatography on Biogel P2 column: separation of released polysaccharides from Whatman paper

From the separation process results that the obtained polysaccharides fraction is composed mainly from the hemicellulose complex. For the determination of approximate molecular mass of this complex, dextran fractions were used as molecular weight standards (Fig. 28). Supposed molecular weight of hemicellulose complex is in the range of 300-500 kDa.

The main peak of polysaccharides was collected, freeze-dried and applied to DEAE Spheron column.

Anion-exchange chromatography on DEAE Spheron column

Partially refined polysaccharides sample was obtained using anion-exchange chromatography on DEAE-Spheron, which separated neutral and acidic polysaccharides. Xyloglucan, as a main hemicellulose occurring in the sample, represented the neutral component and passed through the column without retention (free xyloglucan). On the other hand, all acidic components interacted with charged column medium.

Providing that sample adsorbed/desorbed from Whatman paper and separated from gel permeation chromatography contain hemicellulose (neutral) as well as pectin part (acidic), it can be supposed that the obtained acidic fraction includes a co-eluted hemicellulose-pectin complex.

Neutral fractions as well as acidic fractions after elution with 1 M NaCl were monitored for the neutral sugars (hexose, pentose) [81] and uronic acids [82] content (Fig. 29). Acidic fractions of interest were collected, desalted and freeze-dried.

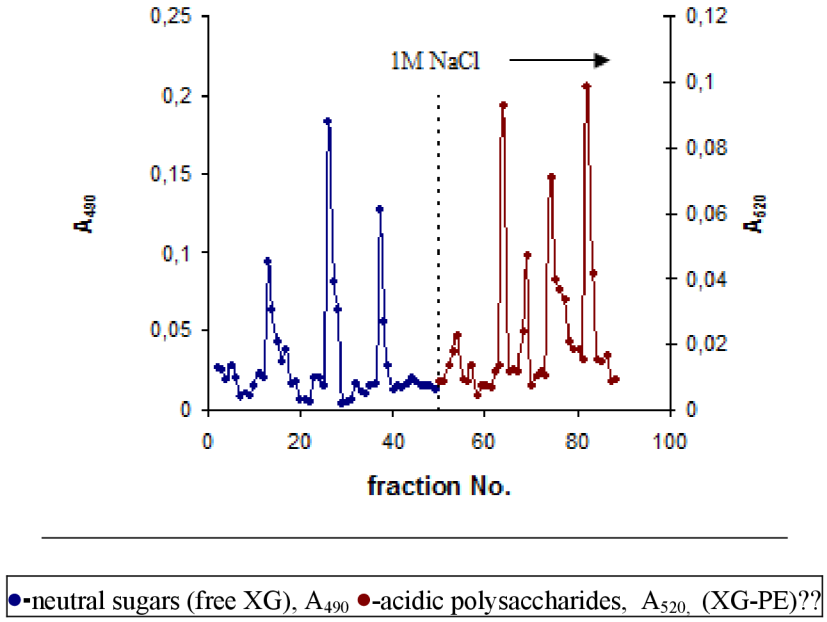


Fig. 29: Anion-exchange chromatography: DEAE Spheron column: separation of neutral and acidic polysaccharides

For the confirmation of the existence of hemicellulose-pectin complex further analysis of acidic fractions obtained from DEAE-Spheron column was required. Sample was used for FTIR (Fourier transformed infrared) and NMR (Nuclear magnetic resonance) analysis.

4.1.2 Analysis of isolated polysaccharides

Fourier transformed infrared (FTIR) spectroscopy

Infrared spectroscopy can be extensively applied in plant cell wall polysaccharide analysis whereas cellulose and pectic components are the most widely studied polysaccharides by this technique. According to Kačuráková (2000) report, the IR data of model components can be used for identification of polysaccharides.[104] These model data were used also for the interpretation in this work.

Polysaccharide sample was analyzed using Fourier transformed infrared (FTIR) and Attenuated total reflectance (ATR) spectroscopy. Measured spectra were interpreted on the bases of model data for individual polysaccharides and their characteristic band maxima in the near 1200-800 cm^{-1} FTIR region (Table 3). These specific band maxima are due to the influence of the constituent monosaccharides of the studied pectic and hemicellulosic polysaccharides.

Excepting model data taken from literature, [104-107] also data of standard polysaccharides measured in this work were used for interpretation of obtained FTIR spectra (Table 3).

Table 3: FTIR frequencies of the studied plant cell wall polysaccharides: Model data used for spectra interpretation: No.1-18 data taken from literature, No. I-IV– measured data of polysaccharides used as a standard compounds (vs- very strong, s-strong IR band intensity)

No.	*Compound*	*(C-OH), (C-O-C), (C-C), ring*	*(C1-H), ring*
1	Pectin	1144s, 1100vs, 1047, 1017vs	953,896,857,835
2	Rhamnogalacturonan	1150, 1122, 1070vs, 1043vs, 989s	951,916,902, 846, 823
3	Galactan	1155, 1134,1072vs, 1038vs	893, 883
4	Arabinan	1141, 1097, 1070, 1039vs	918, 895, 807
5	Arabinogalactan	1074vs, 1045vs	897, 868, 808
6	Arabinogalactan	1139, 1078vs, 1043, 985	880, 842
7	Arabinogalactan(type II)	1156, 1078vs, 1040	916, 892, 879
8	Arabinogalactan(type II) and Glucomannan (mix.)	1146, 1066vs, 1034	896, 872, 809
9	Arabinogalactorhamnoglycan	1049vs	914, 837, 810
10	Xyloglucan	1153, 1118, 1078vs, 1041vs	945, 897
11	Glucan	1151, 1104, 1076, 1041vs, 1026vs	916, 840
12	Glucomannan	1150, 1092vs, 1064vs, 1034vs	941, 898, 872, 814
13	Galactoglucomannan	1149, 1064, 1034vs, 960	934, 897, 872, 813
14	Arabinoglucuronoxylan and Galactoglucomannan (mix.)	1161, 1151, 1109, 1070, 1038vs	898, 881, 809
15	Pectin	1152, 1004vs, 1082, 1051,1022vs, 972	891, 834
16	GX	1147, 1084, 1047vs, 985	897
17	Starch	1155, 1110, 1082, 1026vs	931, 850
18	Cellulose		
I	Rhamnogalacturonan I	1146,1077s,1040s, 1020vs	959, 896
II	Arabinoxylan	1087s, 1044vs	898, 861, 809
III	Galactomannan	1153, 1071, 1029,	871, 812
IV	Xyloglucan	1153, 1079vs, 1041vs	943, 897

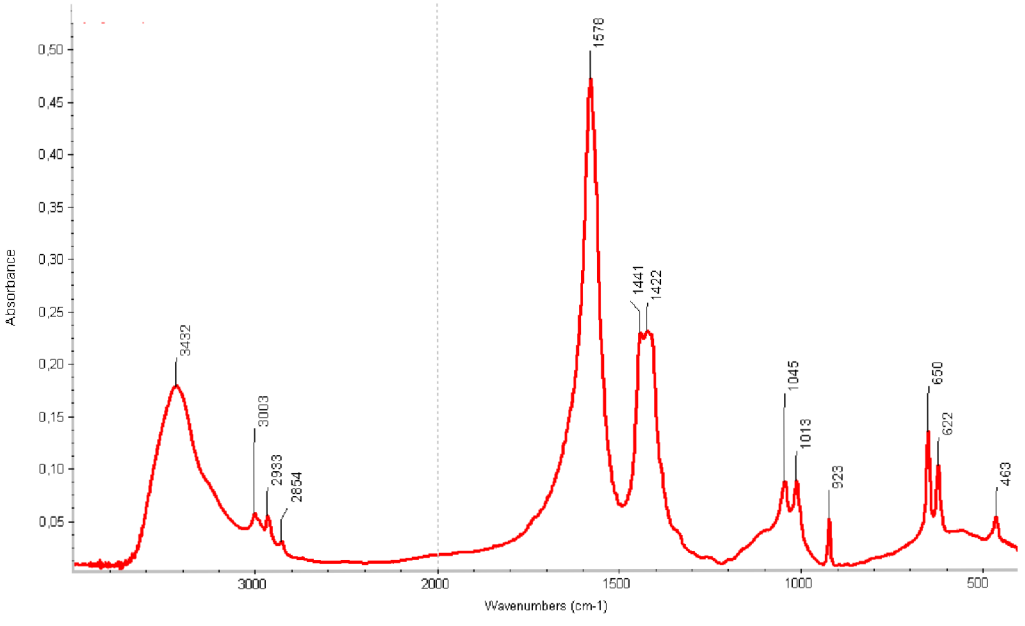


Fig. 30: FTIR spectrum of hemicellulose-pectin complex

Attenuated total reflectance (ATR) spectroscopy

ATR spectra measured in solid state were interpreted according to data obtained from polysaccharide standards (Table 4) and compared with results form FTIR spectroscopy.

Table 4: ATR frequencies of polysaccharides used as a standard compounds (vs- very strong, s-strong IR band intensity)

No.	*Compound*	*(C-OH), (C-O-C), (C-C), ring*	*(C1-H), ring*
I	Rhamnogalacturonan I	1020	831
II	Arabinoxylan	1039	896
III	Galactomannan	1057, 1018	812
IV	Xyloglucan	1028	897

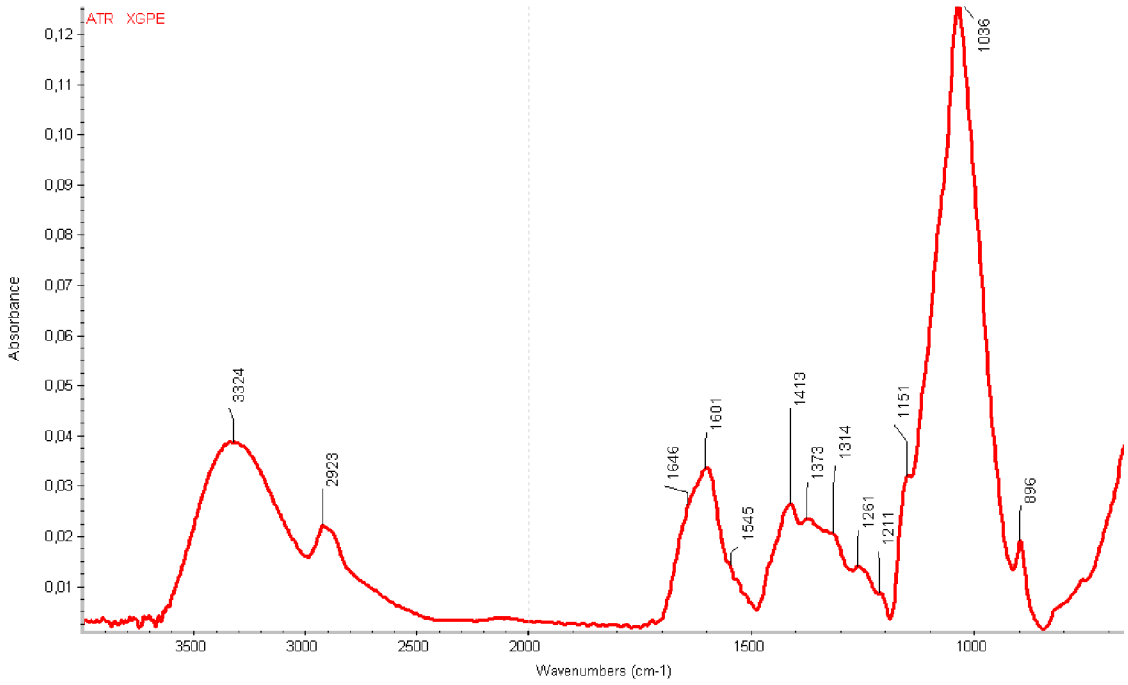


Fig. 31: ATR spectrum of hemicellulose-pectin complex

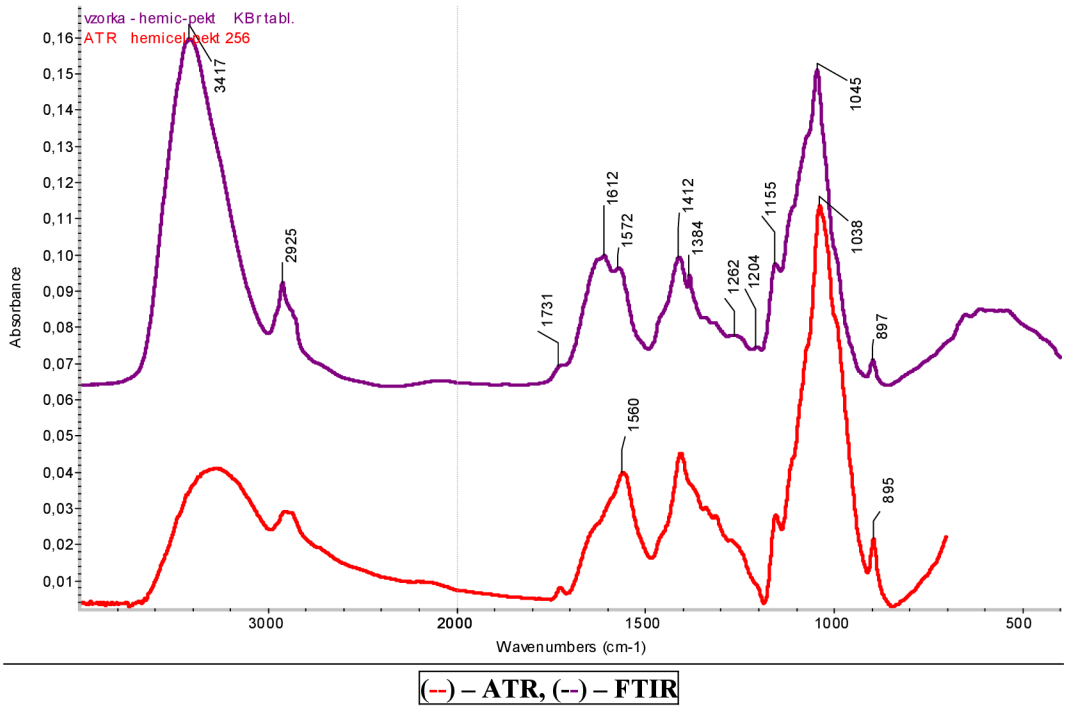


Fig. 32: FTIR/ATR spectra of hemicellulose-pectin complex

Table 5: Summary of FTIR/ATR frequencies of hemicellulose-pectin sample

Compound	*(C-OH), (C-O-C), (C-C), ring*	*(C1-H), ring*
Hemicellulose-pectin sample	1155, 1151, 1045vs, 1036vs, 1013, 923	896,897

The FTIR data showed that each polysaccharide has a specific band maximum in the 1200-1000 cm^{-1} region (Table 3,4). This region is influenced by ring vibrations overlapped with stretching vibrations of C-OH side groups and the C-O-C glycosidic bond vibrations.[104]

Hemicelluloses

Hemicellulose standards used in this work included xyloglucan (Tamarind seeds, Dainippon Pharmaceutical), galactomannan (Sigma), and arabinoxylan (Wheat arabinoxylan, Megazyme).

It was found the band maximum at 1041 cm^{-1} for xyloglucan, a band position which corresponds to β-glucan (Table 3). Also further obtained bands at 1153 cm^{-1}, 1079 cm^{-1} and bands from anomeric region with maximum at 897 cm^{-1} confirmed previous published results. [104] In addition, data from ATR analysis showed band maxima at 1028 cm^{-1} and 897 cm^{-1} (Table 5), which correspond to α-glucan.

According to Kačuráková (2000), [104] the IR bands of β-(1→6) or β-(1→3)- linked galactan occur at about 1078-1072 cm^{-1} and the β-(1→4)-mannan can be found at 1066-1064 cm^{-1}. In addition, the main chain forming arabinans was found at 1039 cm^{-1} while the side chain arabinans occur at about 1044 cm^{-1}. In this work, band maximum for galactomannan at 1071 cm^{-1} and for arabinoxylan at 1044 cm^{-1} was found (Table 3), what corresponded to previous results. [104,105]

Pectins

For interpretation of measured spectra standards of homogalacturonan (in the form of pectate) and rhamnogalacturonan I (Megazyme) were used.

In the pectin and rhamnogalacturonan the bands at 1100 cm^{-1} and about 1017 cm^{-1} are usually strongest.[105,106] The unique spectral shape of pectin is due to the high homogalacturonan content. In rhamnogalacturonan the band shape is different with the main maxima at about 1070 and 1043 cm^{-1}. In the case of α-linked arabinogalactan, the side chain of rhamnogalacturonan I, the IR maximum occurs at 1039 cm^{-1}. The β-arabinogalactans have two bands at about 1078 cm^{-1} and 1045 cm^{-1} (Table 3). These two bands may belong to their particular components; the former to galactopyranose in the backbone and the latter to arabinofuranose units in side chain.

FTIR data of this work corresponds to previous results.[104-107] Spectra of rhamnogalacturonan I confirmed the main band about 1020 cm^{-1}. In addition results indicate the presence of arabinogalactans with band maximum at 1078 cm^{-1}.

Glycosidic linkage

The IR regions of bands at about 1160-30 cm^{-1} are influenced by the glycosidic linkages (C-O-C). Galactose units with any link type and position were found at about 1155 cm^{-1}, xyloglucan at 1153 cm^{-1} and at lower frequencies 1051-1039 cm^{-1} were polysaccharides with mannose, arabinose and rhamnose constituents.[104]

Anomeric region

The anomeric region is complicated by band overlap. On the other hand, the bands can be unique for each sugar constituent and can give additional information to region at 1200-1000 cm^{-1}. This characteristic absorption bands in anomeric region for α-linkage (834 cm^{-1}) and β-linkage (898 cm^{-1}) can distinguish well aldopyranoses and the furanoid compounds at 879 and 858 cm^{-1} in carbohydrates. [108] The most important bands are at about 898 cm^{-1} for β-anomer and about 845 cm^{-1} for α-anomer form of the pyranoid ring. Galactose and mannose show bands at 875 and 810 cm^{-1}. Our results for galactomannan with band in anomeric region at 812 cm^{-1} confirmed these previous results. [104,108] This band maximum arises from mannose monomer units of galactomannan.

NMR- ^{1}H analysis

The ^{1}H NMR spectrum of a polysaccharide can generally be divided into three major regions: the anomeric region (4.5 - 5.5 ppm), the ring proton region (3.1 - 4.5 ppm) and the alkyl region (1.2 - 2.3 ppm). For identification of compounds in sample the anomeric region is the most suitable.[109, 110]

Spectra of measured polysaccharide sample were compared with ^{1}H chemical shifts reported in the literature [109, 110] and accordingly interpreted (Fig. 33). On the basis of previous results from FTIR analysis, the main compounds of interest were arabinoxylan (Megazyme) and homogalacturonan (pectate) that were used as well as reference substances. Unfortunately, high heterogeneity and deficient amount of polysaccharide sample caused the spectra interpretation more difficult. Therefore, obtained preliminary results require further analysis.

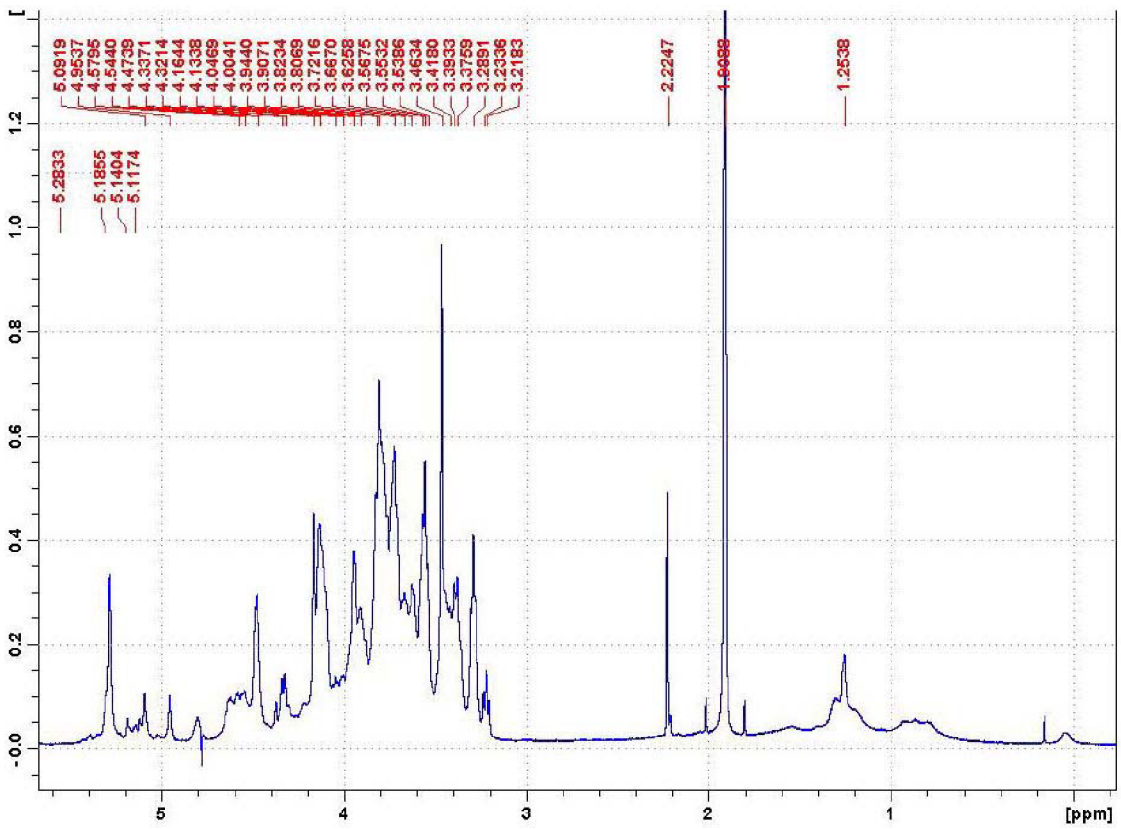


Fig. 33: NMR – ^{1}H spectrum of polysaccharide sample isolated from parsley suspension cells

4.1.3 Identification of analysed polysaccharides

FTIR

On the basis of FTIR model data and results obtained from analysis of polysaccharide sample and reference compounds (see chapter 4.1.2) it can be supposed that isolated complex includes hemicellulose as well as pectin part.

The very strong band with maximum at 1045 cm^{-1} and band found in anomeric region at 896 cm^{-1} corresponded to arabinoxylan. This band at 1045 cm^{-1} (from region influenced by glycosidic bonds) represented the side chains of arabinans. In addition, bands at frequencies of about 1151-1155 cm^{-1} corresponded to polysaccharides with arabinose constituents. From these results yields that hemicellulose compound found in the complex sample is an arabinoxylan.

In the case of pectin and pectic components there are specific band maxima at around 1605-1630 cm^{-1} and 1420 cm^{-1} influenced by carbonyl groups of pectin. Accordingly, these carboxylate regions were found also in complex sample. There were found band maxima at 1612 and 1412 cm^{-1} (symmetric stretch of carboxylate) what corresponded to model and standard data of pectic compounds.[104, 106, 107] In addition, the band maximum at 1262 cm^{-1} responds to COC ester region. In anomeric region the band at 896 cm^{-1} typical for pectin was found. From these results it can be supposed that measured sample contains pectic

compounds. The signal at 1731 cm^{-1} can be attributed to the carbonyl group stretching of uronic acid carboxyls.

Unfortunately, due to the heterogeneous and complex macromolecule structure of pectin, it is impossible to determine accurately the structure of pectin compound included in measured polysaccharide sample. On the other hand, there was not found band maximum at about 1070 cm^{-1} typical for rhamnogalacturonan.[104] The absence of RG-I band can indicate that the linkage between hemicellulose (arabinoxylan) and pectin is formed by other type of pectic compound, probably via its side chains, *e.g.* homogalacturonan or xylogalacturonan.

NMR

On the basis of previous results from FTIR there was supposed that polysaccharide sample includes hemicellulose as well as pectin components. This presumption indicated also results obtained from NMR analysis (Fig. 33).

NMR analysis indicated signals at 5.34 (not shown), 5.28 and 5.26 ppm (Fig. 33) corresponding to anomeric protons of α-L-arabinofuranoses substituted at O-3 (mono substituted) and at both O-3 and C-2 (disubstituted) of xylose residues, respectively. Signals obtained at 4.58, 4.54 and 4.47 ppm were due to the anomeric protons of β-D-xyloses residues which can be substituted at C-2 and C-3 (disubstituted), C-3 (monosubstituted) or unsubstituted. A signal obtained at 5.14 ppm could be influenced by the α-anomer of unsubstituted xylopyranosyl residues at the reducing end (Fig. 33). The signals for other protons of arabinose and xylose were observed in the region of 3.20–4.30 ppm. These obtained results corresponded with structure described for arabinoxylan (Fig. 34).[109] They are in agreement with previously published results. [110,111]

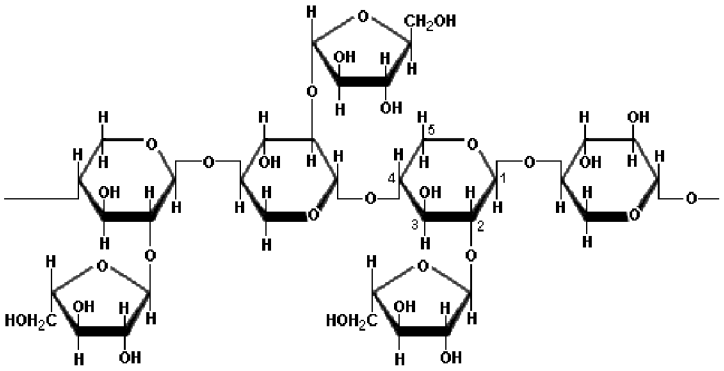


Fig. 34: Structure of arabinoxylans [109]

A ^{1}H NMR spectrum of the sample indicated signals of chemical shifts at 5.09, 3.91, 3.76 and 4.37 ppm (Fig. 33) what can correspond to 1,4-linked-α-D-galacturonic acid residues (GalA), the main backbone of homogalacturonan. In addition, the low signals at about 1.80 and 2.0 ppm indicated presence of methyl ($-CH_3$) groups (Fig. 33).

Results obtained from ^{1}H NMR confirmed previous FTIR results. On that account it can be supposed that polysaccharide sample isolated from parsley root suspension cells consists of arabinoxylan bound with homogalacturonan.

On the other hand, due to the high heterogeneity of plant sample, the spectrum interpretation was too difficult. Therefore, to prevent misinterpretation of results obtained form NMR analysis, further analyses as well as structure studies are required.

4.1.4 Hetero-transglycosylating reaction between OGA-SR and AX

The first reference of hetero-transglycosylating activity was described in crude protein extract from nasturtium seeds (*Tropaeolum majus*) by Ait-Mohand and Farkaš in 2006. [59] They used different combinations of donor: acceptor pairs, where as the glycosyl acceptors served the oligosaccharides fluorescently labelled with sulforhodamine while the different, unstained, high M_r-polysaccharides served as glycosyl donors. They detected hetero-transglycosylating activities with the following donor: acceptor pairs: XG:XGOs-SR, XG:CEOs-SR, XG:LAOs-SR, HEC:XGOs-SR and CMC:XGOs-SR. [59]

On the basis of results obtained from FTIR and NMR where the linkage between homogalacturonan and arabinoxylan were indicated, the crude extracts of proteins from parsley roots and nasturtium seeds were used for evaluation of potential enzymes capable to catalyze hetero-transglycosylating reaction of appropriate type. OGAs-SR were used as an acceptor substrate and arabinoxylan as a donor substrate.

Obtained results show that both protein precipitates contain unknown type of enzymes (probably transglycosylases, because no nucleotide sugars were added) enabling the hetero-transfer between OGAs and AX (Fig. 35).

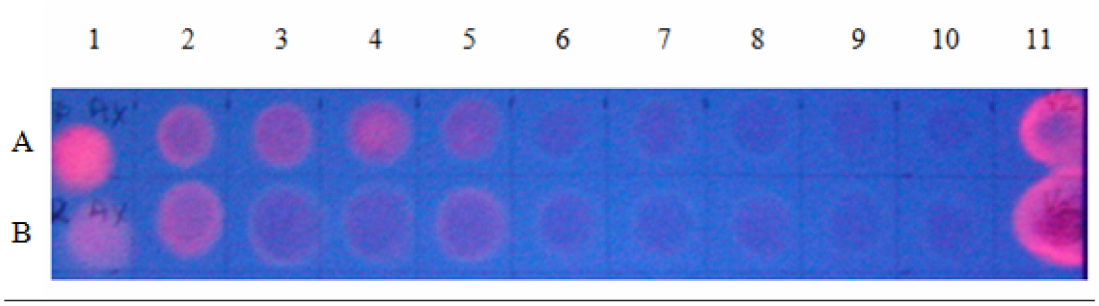


Fig.35: Hetero-transglycosylating reaction between OGA-SR and AX:
A –crude extract of proteins from parsley roots, B – crude extract of proteins from nasturtium seeds,
1-10 – individual washing steps, unreacted OGA-SR,
11 – resulting precipitated sample dissolved in 50 µl of water, incorporated OGA-SR into AX

4.2 Exopectate hydrolases from *Petroselinum crispum*

Plant pectate hydrolases are in general supposed to be bound on primary cell wall where they cause the homogalacturonan degradation. Polygalacturonases can be classified into two groups depending on action pattern; enzymes randomly cleaving substrate (polygalacturonases, EC 3.2.1.15) and enzymes terminally cleaving substrate (exopolygalacturonases, EC 3.2.1.67). [112] The biological function, structure as well as gene-expression of polygalacturonases have been studied in detail, while the research of

exopolygalacturonases (exoPGs) is still on its beginning. ExoPGs have not been fully characterized yet in terms of developmental roles but could clearly have significant involvement in cell expansion processes. These enzymes are supposed to play a key role in the turnover of biologically active oligogalacturonates as signalling molecules affecting plant growth and development. [10,11]

The aim of this work was to purify and characterize the pectate hydrolases from parsley roots as a new plant source. Enzymes obtained from roots were compared with proteins found in callus and cell suspension cultures.

4.2.1 Purification of pectate hydrolases

Pectate hydrolases were at first isolated from precipitate of parsley juice. Purification process included different separation methods involving gel-permeation and affinity chromatographies as well as preparative isolelectric focusing (Fig. 36).

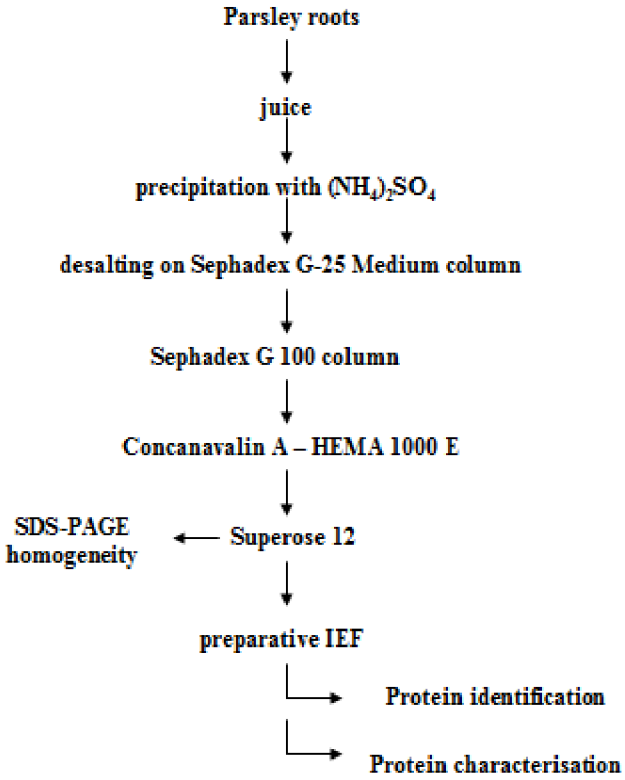


Fig. 36: Scheme of purification pathway used for parsley juice proteins with polygalacturonase activity

The process of proteins isolation after their precipitation was started by removing of low molecular substances on a Sephadex G-25 Medium (Pharmacia, Sweden) column (Fig. 37). The desalted protein mixture was applied on a Sephadex G-100 (Pharmacia, Sweden) column (Fig. 38). Fractions with polygalacturonase activity were collected, desalted by dialysis and freeze dried. The next step was provided on a concanavalin A-HEMA 1000 E column (Fig. 39). This chromatography is based on interaction between Concanavalin A and glycosyl

group of enzyme, and so it can be used only for proteins with appropriate glycosylation. Enzyme fractions released from Concanavalin A (after desalting on DP 10 column) were further separated on FPLC with Superose 12 column (Fig. 40 a,b).

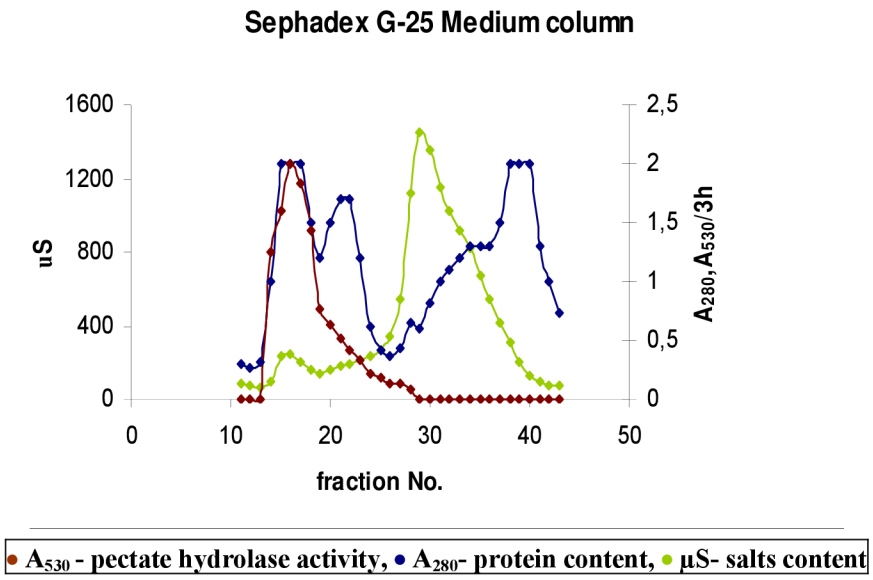


Fig. 37: Sephadex G-25 Medium column: Desalting of crude protein extract and removing of low molecular substances after precipitation with ammonium sulphate, dialysis and lyophilization

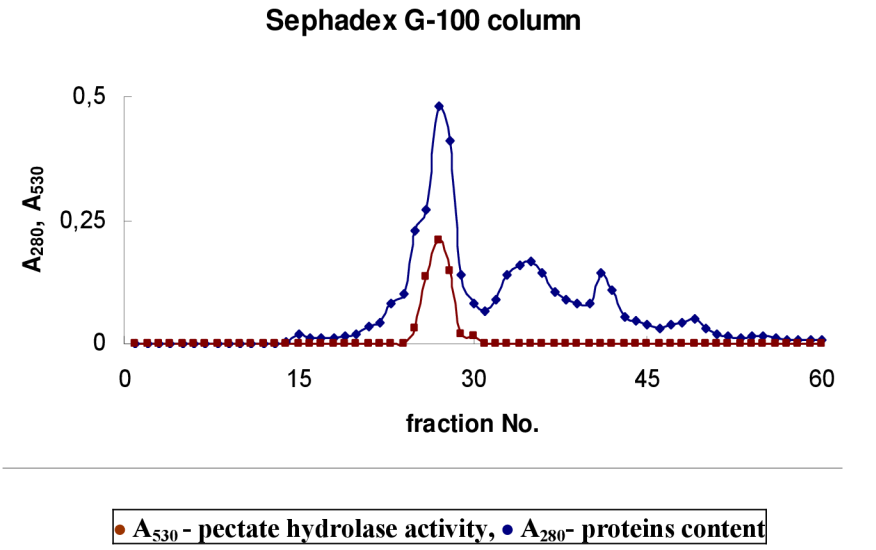


Fig. 38: Sephadex G-100 column: further separation of desalted proteins after Sephadex G -25 Medium step

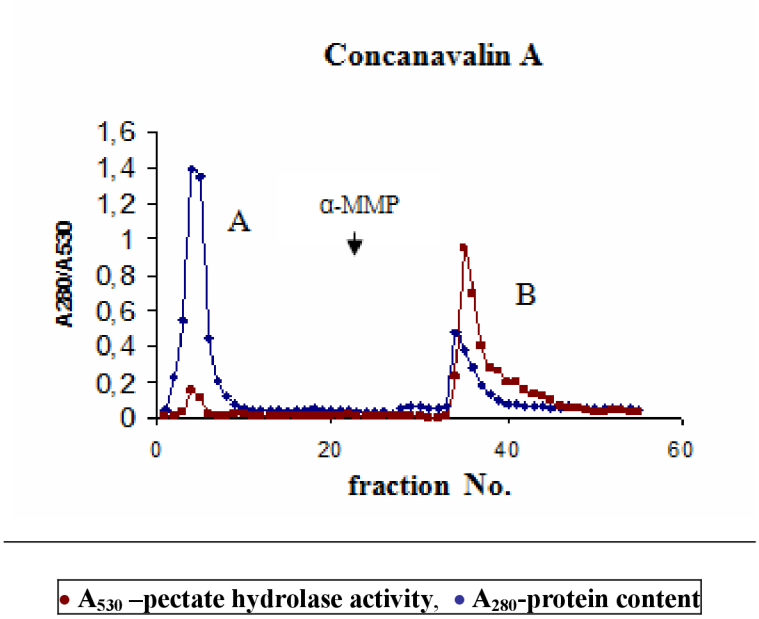


Fig.39: Concanavalin A HEMA 1000 E: A- the protein fraction unbound to concanavalin A, B- the bound enzyme fraction eluted with α- MMP

Both fractions unbound to ConA (Fig. 39, peak A) as well as eluted by α-MMP (Fig. 39, peak B) were collected and used for next purification step on Superose 12 column connected to FPLC device (Fig. 40a,b). Fractions eluted by α-MMP were used for the confirmation of potential glycosylation in reaction with *N*-glycosidase F.

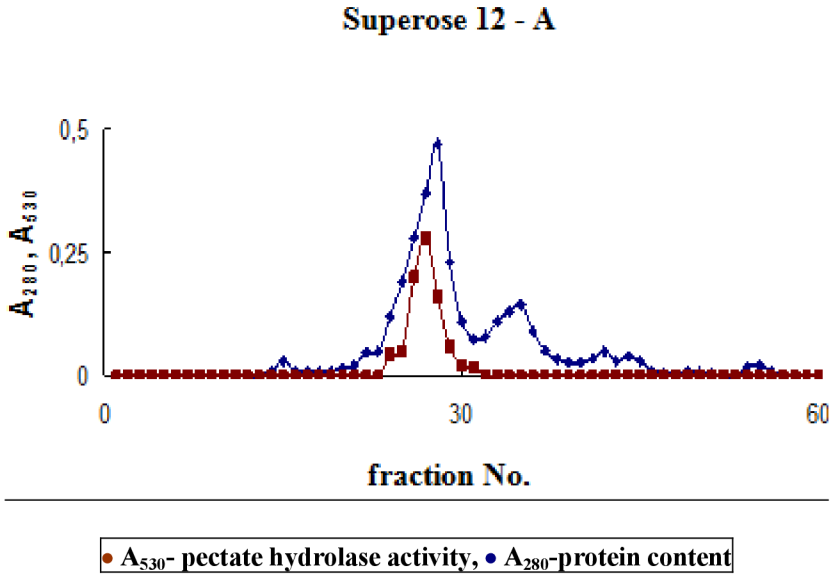


Fig. 40 a: Superose 12: Separation profile of proteins no interacting with Con A (Fig. 39, peak A)

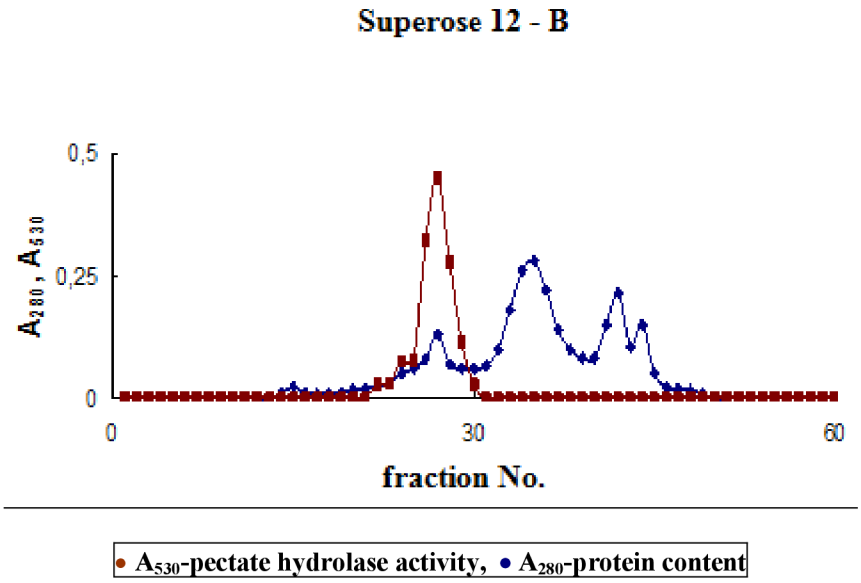


Fig. 40 b: Superose 12: Separation profile of proteins eluted with α-MMP (Fig 39, peak B)

Protein sample after last purification step on Superose 12 column (fraction after interaction with α-MMP) was used for further characterization.

Control of purification process and mass determination

The purification procedure of pectate hydrolase from the protein precipitate of juice from parsley roots was monitored after each chromatography step by SDS-PAGE. As can be seen in Fig. 41, single protein band was obtained after Superose 12 step.

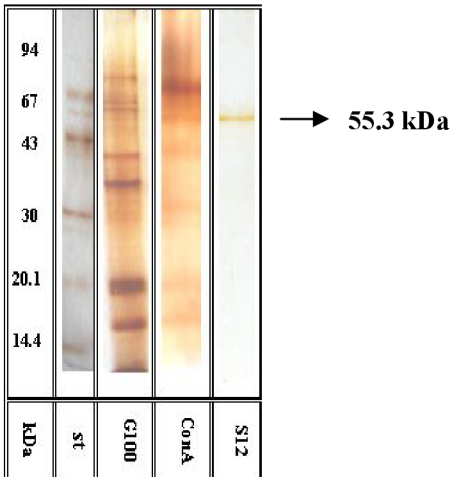


Fig. 41: SDS-PAGE of purified pectate hydrolase from parsley roots juice after individual purification step: G-100, Sephadex G-100, ConA, concanavalin A-HEMA, S12, Superose 12.

4.2.2 Characterization of pectate hydrolase

The molecular weight of protein was determined. Standard calibration proteins in the range 14.4-94 kDa (Serva) were used for molecular weight evaluation. Results indicate that the SDS homogeneous band responds to molecular weight of 55.3 kDa (Fig. 41).

The affinity of parsley pectate hydrolase to concanavalin A indicated that this enzyme is glycosylated. This presumption was confirmed by *N*-glycosidase F cleavage what resulted in slight molecular weight decrease (1-2 kDa) of protein (Fig. 42).

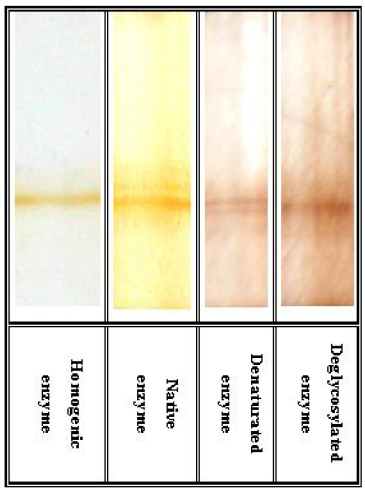
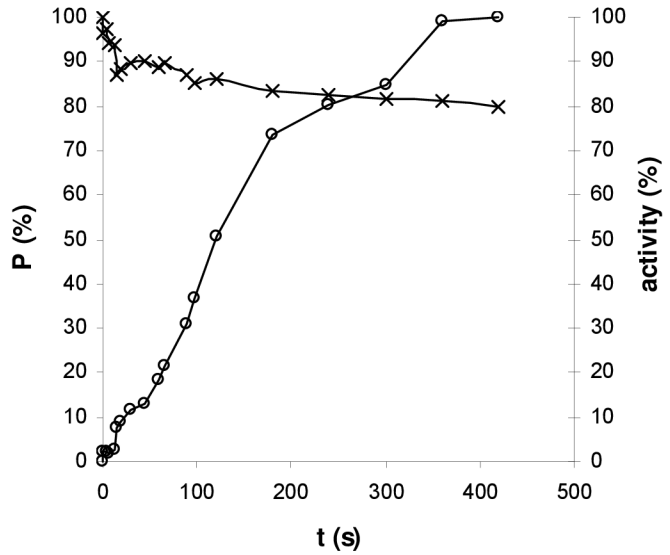


Fig. 42: SDS-PAGE after N-glycosidase F cleavage: decrease of Mr, confirmation of protein glycosylation

Determination of action pattern

The action pattern of isolated enzyme was determined using the correlation of viscosity of pectate decrease with its degree of degradation measured during enzyme reaction. [102] A very slow decrease of viscosity *P* (%) with simultaneous fast increase of reducing groups (A_{530}) representing the ratio of substrate cleavage was monitored (Fig. 43). Accordingly, the degradation degree of substrate was determined and the typical exo mechanism of enzyme action was evaluated (Fig. 44).



A_{530}, (-○-)-reducing groups, P, (-×-)- viscosity

Fig. 43: Viscosity and reducing groups of pectate during its reaction with pectate hydrolase from parsley roots

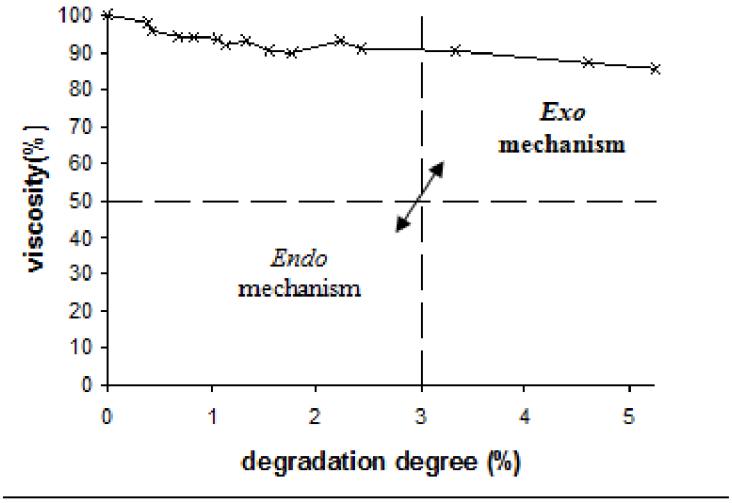
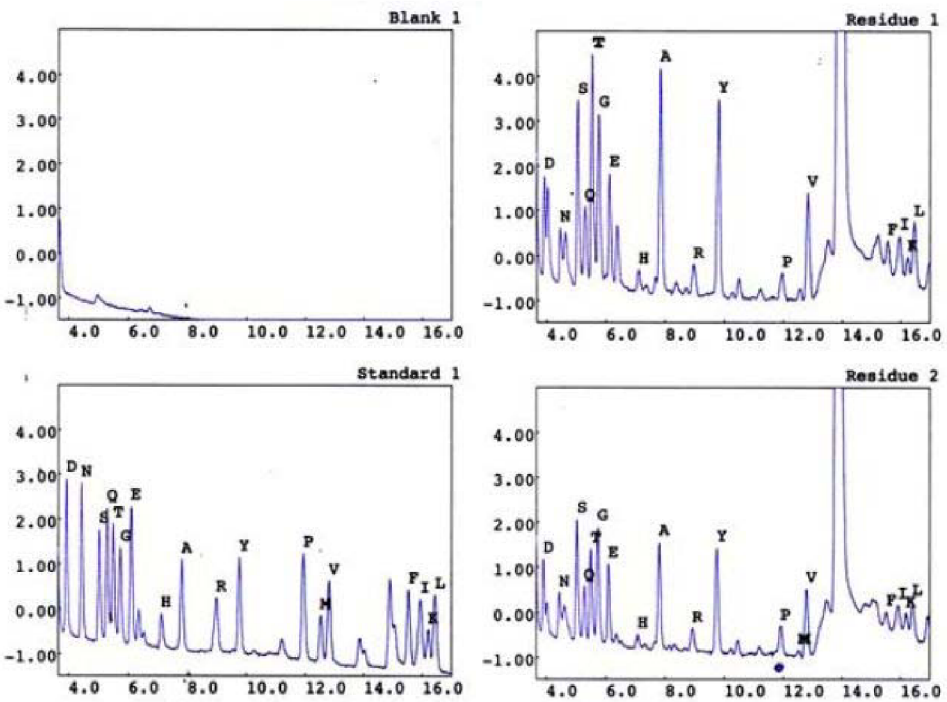


Fig. 44: Action pattern determination of pectate hydrolase from parsley roots

As a typical exopolygalacturonase, the enzyme from parsley roots was able to cleave di-D-galacturonic acid as a specific substrate of difference between endo- and exo-PGs and the only product detectable by TLC was D-galacturonic acid (MGA).[112] The orientation of substrate splitting was evaluated using substrates modified by endopectate lyase. Reaction of these unsaturated oligogalacturonates (DP 2,3 marked on the nonreducing end by the double bond) with pectate hydrolase from parsley did not lead to any splitting of these modified substrates, *e.g.* these enzymes utilized the cleavage of substrates from nonreducing end as was described for enzymes from carrot roots.[113-116]

Edman degradation

N-terminal sequencing of SDS-homogeneous protein sample was provided in Procise-protein sequencing system (Applied Biosystems, USA).



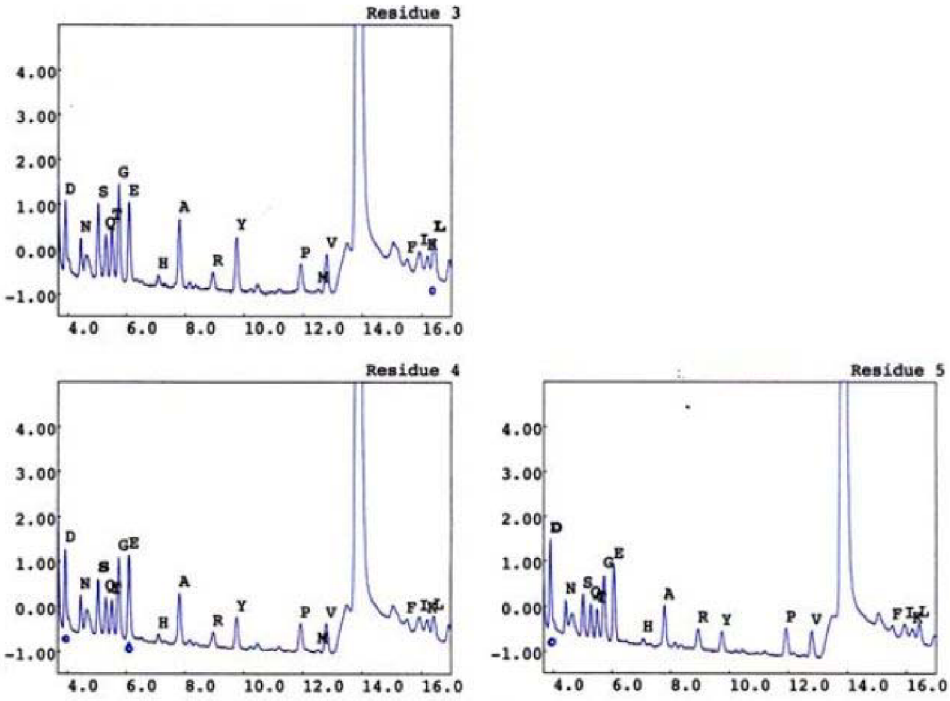


Fig. 45: Edman degradation: amino acids N-terminal analysis

Unfortunately, the protein with the molecular weight of 55.3 kDa and apparently homogeneous by SDS-PAGE included still a mixture of proteins as was confirmed by N-terminal analysis. Alanin (A), threonin (T) and serin (S) seemed to be the N-terminal amino acids of the major forms of present proteins (Fig. 45).

Determination of pH optima

The protein mixture from parsley juice was examined for the presence of pectate hydrolases on basis of activity on pectate and pentagalacturonate at various pH values (Fig. 46) as described for enzymes from carrot roots. [114,115]

One form of typical exopolygalacturonase preferring polymeric substrate and having pH optimum at 5.2 and three forms of oligogalacturonate hydrolases (with preference for OGAs with pH optima 3.6, 4.2 and 4.6) were found in parsley juice showing a wide variety of exo-enzymes able to completely degrade parts of pectate in wall pectin.

Pectate hydrolases with terminal action pattern preferring oligomeric substrates were described as typical enzymes produced by various microorganisms.[112] To prevent misinterpretation of results obtained with roots (both juice and pulp) cropped from the field (possibility of contamination) sterile cell cultures from these roots were prepared and used for pH optima determination. The comparison of the occurrence of pectate hydrolases in roots, callus and cell suspension showed the presence of the same enzyme forms (Fig. 46). Ratio of individual forms varied considerably, probably due to various stages of plant growth and different conditions.

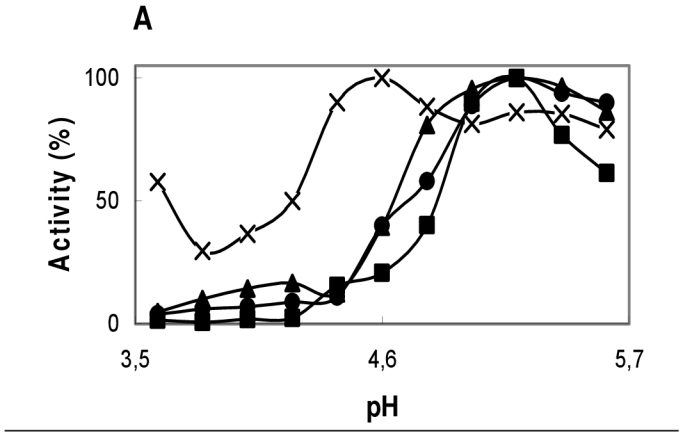


Fig. 46: pH optima of pectate hydrolases in parsley; pectate hydrolase activity dependence on substrate:
(A) – 0.5% sodium pectate (PGA), (B) -1 mM pentagalacturonate (GA5)

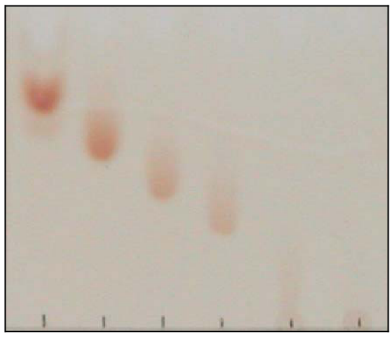
Substrates

Oligogalacturonic acids (DP 2-10) were prepared by enzymatic hydrolysis of pectate, gel filtration on Sephadex G-25 Fine column in 0.05 M phosphate buffer, pH 7.0 and desalting on a Sephadex G-15 column.

These oligogalacturonates were used for characterization of substrate specificity of enzyme forms as well as for identification of individual enzyme in their mixture. Accordingly, the control of substrates purity was required. This was performed by TLC (Thin layer chromatography) and HPLC (High performance liquid chromatography).

Thin Layer Chromatography

Oligogalacturonic acids of DP 2-10 were analyzed on Silica gel sheets (Merck) using butanol/formic acid/ water (2:3:1) as the eluent (Fig. 47). The spots were detected by 20% solution of ammonium sulphate in water, D-galacturonic acid (MGA) was used as a reference substance.

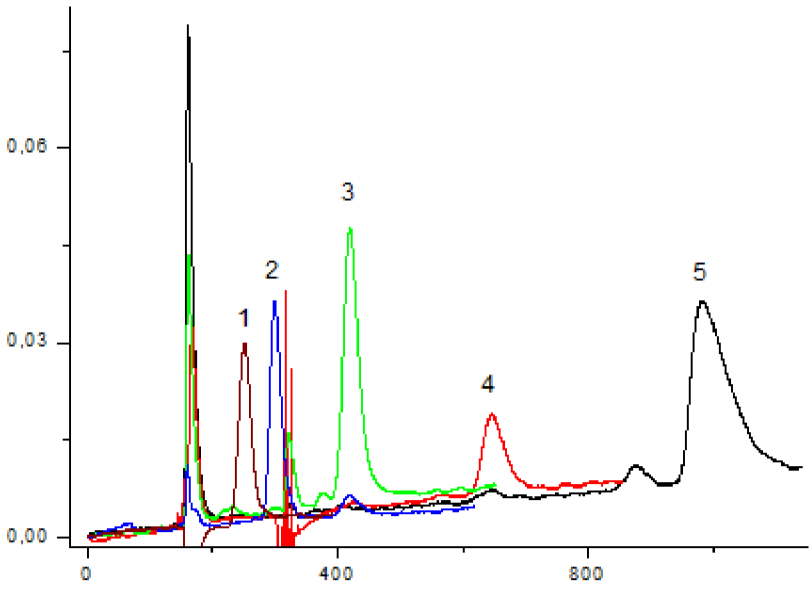


GA2 GA3 GA4 GA5 GA8 GA10

Fig. 47: TLC chromatogram of oligogalacturonic acid standards: Control of purity

HPLC

The purity of oligogalacturonic acids of DP 2-5 was confirmed by HPLC using the Shodex Asahipak NH_2P-50 4E column. The solution of 0.3 M phosphate buffer, pH 4.4 was used. Separated OGAs were detected using Shodex UV detector (Fig. 48).



1 (--)-MGA, 2 (--) GA2, 3 (--) GA3, 4 (--) GA4, 5 (--) GA5

Fig. 48: HPLC chromatogram: Separation and purity control of oligogalacturonate standards used for enzymes characterization

Determination of initial rates

At least four pectate hydrolases with pH optima 3.6, 4.2, 4.6 and 5.2 were detected in parsley juice. The comparison of these forms according to their substrate preference is shown in Fig. 49. Enzymes with more acidic pH optima 3.6, 4.2 and 4.6 can be supposed to be

oligogalacturonate hydrolases (OGHs). The enzyme with pH optimum 5.2 is a typical exopolygalacturonase. The enzymes detected in the parsley juice were further characterized.

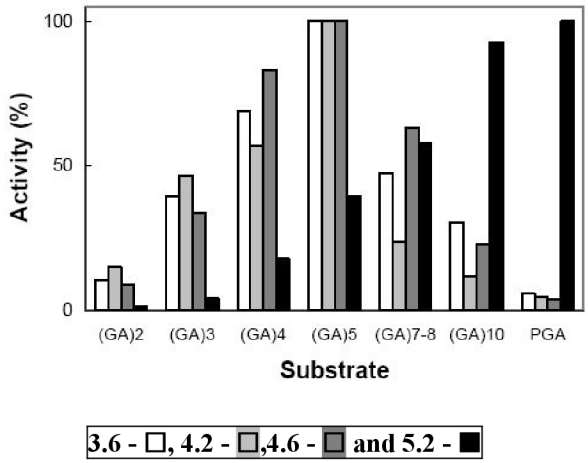


Fig.49: The initial rates of pectate hydrolases from parsley roots juice on substrates with various DP

First the partial separation of individual forms was performed using preparative isoelectric focusing (IEF) in the region of pH 3-10. After IEF the gel was divided into segments with a diameter smaller than 2 mm. Proteins were washed out from these segments by water and forms of pectate hydrolase were detected on the basis of pH optima and preferred substrates (Fig. 50, part A). The isoelectric points of enzymes were then evaluated by IEF utilizing IEF standards and the zymogram technique for the localization of enzymes in the gel. The pI values were very close together, in the range of 5.3-5.6 (Fig. 50, part B).

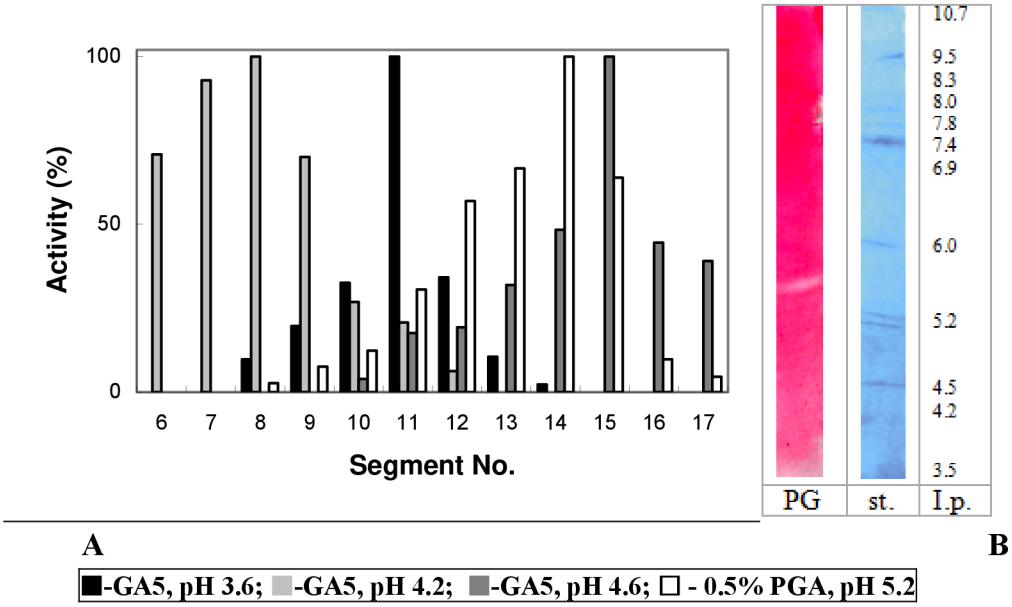
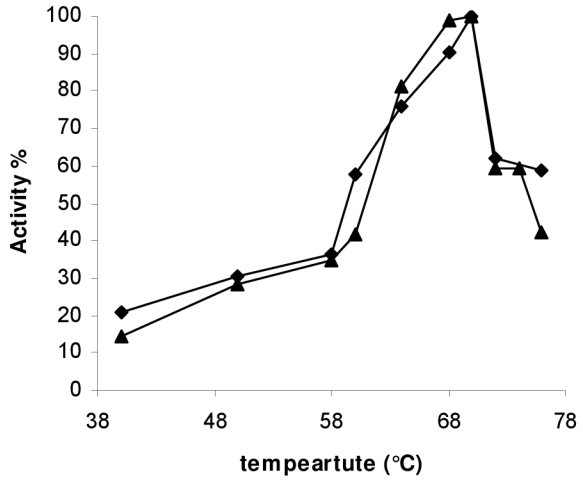


Fig.50: IEF of pectate hydrolases from parsley juice: A- The representation of individual enzyme forms in segments after preparative IEF detected on the basis of differences in pH optima and preferred substrates, B-Determination of pI for individual pectate hydrolases using the zymogram technique and IEF standards

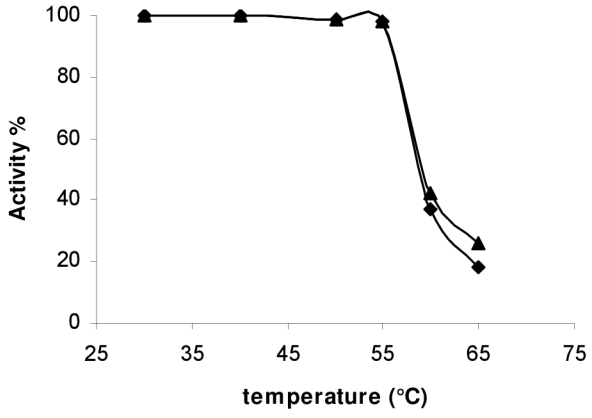
Determination of temperature optima and thermal stabilities

The temperature optimum of all forms was between 60 and 70 °C. Enzymes were still stable by 55 °C (100 % recovery of activity after 2 hours.).For instance the temperature optimum of the exopolygalacturonase and OGH with pH optimum at 4.2 was very similar (Fig. 51). Both enzymes showed very high temperature stability, too (Fig. 52).



(-▲-) – OGH, enzyme with pH optimum at 4.2, (-♦-) –exoPG, enzyme with pH optimum at 5.2

Fig. 51: Temperature optimum of chosen pectate hydrolases from parsley juice



(-▲-) – OGH, enzyme with pH optimum at 4.2, (-♦-) –exoPG, enzyme with pH optimum at 5.2

Fig. 52: Thermal stability of chosen pectate hydrolases from parsley juice

In comparison to enzymes (exoPGs as well as OGHs) found in carrot roots, [113-116] the temperature optima and thermal stabilities of enzymes from parsley juice were in the same region.

Kinetic parameters determination

Michaelis parameters of enzymes purified from parsley juice were determined utilizing the initial velocities at five concentrations of individual substrates, ranging from 0.05 to 0.5 mM (substrates with DP 2 and 5) or 0.05 to 0.25% pectate. Reactions were incubated at 30 °C and K_M values were calculated by nonlinear regression (Table 6).

Table 6: Summary of Michaelis parameters obtained for individual enzyme forms

Substrate pH	(GA) 2		(GA) 5		PGA	
	K_M ($mol.l^{-1}$)	V_{max} (μmol/min)	K_M ($mol.l^{-1}$)	V_{max} (μmol/min)	K_M ($mol.l^{-1}$)	V_{max} (μmol/min)
3.6	$1.02x10^{-4}$	0.044	$6.36x10^{-5}$	0.184	Nd	Nd
4.2	$1.13x10^{-4}$	0.033	$7.01x10^{-5}$	0.065	Nd	Nd
4.6	$1.25x10^{-4}$	0.025	$6.26x10^{-5}$	0.201	Nd	Nd
5.2	Nd	Nd	$6.78x10^{-5}$	0.110	$5.08x10^{-5}$	0.187

As expected from results for carrot enzymes, [116] the affinity of both types of enzyme, OGH and exopolygalacturonase, increased with increasing DP of substrate (decrease of K_M value with DP increase). On the other hand, the increase of the maximal rate was stopped by OGH when polymeric substrate was used (Fig. 53a,b). The initial rates on substrates with various DP (Fig. 53a,b) indicated that these maximal rates were reached for DP 5 or 6 as it was determined for carrot OGH.[116]

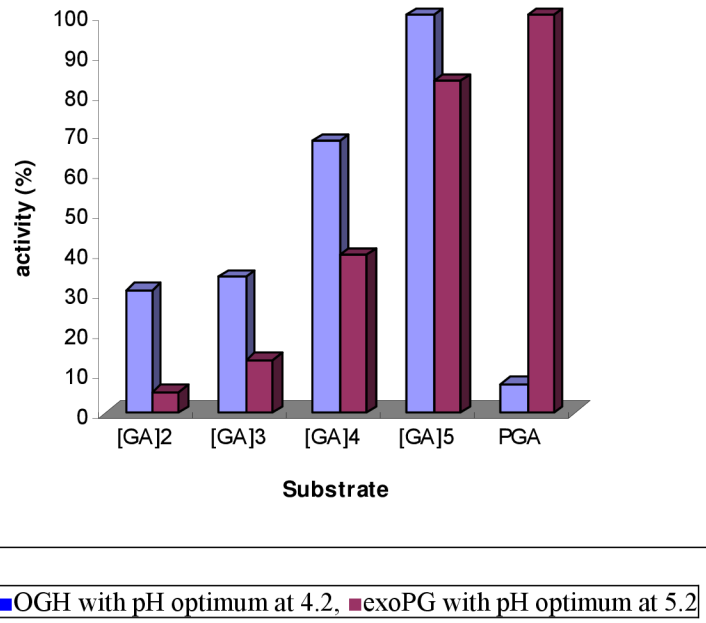


Fig. 53a: Initial rates comparison of OGH with pH optimum at 4.2 and exoPG, activities for substrates with various DP

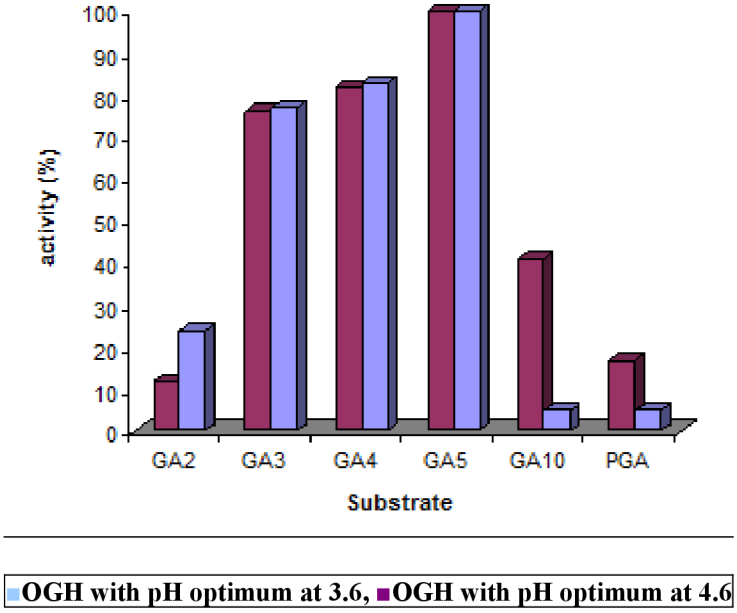


Fig. 53 b: Initial rates comparison of chosen OGHs, activities for substrates with various DP

Conclusion

Two types of pectate hydrolases with terminal action pattern on substrate were found in parsley juice. Exopolygalacturonase (exoPG) and three forms of oligogalacturonate hydrolases (OGHs) were isolated, purified, further characterized and in-between compared. The comparison of their molecular masses, isoelectric points, temperature optima, thermal stability and also action pattern showed very similar results. On the other hand there were observed differences in their pH optima and substrate specificity in respect to degree of substrate polymerization.

ExoPG, which prefers the polymeric substrate, has pH optimum at 5.2. By contrast, the other types of pectate hydrolases, OGHs, favour oligomeric substrate, hexagalacturonic acid. OGH with preference for hexamer includes three isoforms, with pH optima 3.6, 4.2 and 4.7.

In general, exopolygalacturonase from carrot roots with pH optimum 5.0 [113] is very similar to exopolygalacturonase found in parsley with pH optimum 5.2, and OGH from carrot with pH optimum 3.8 [116] corresponds to three OGH isoforms from parsley (pH optima 3.6, 4.2 and 4.6). [117] This similarity indicating the same function in roots of both plant sources was supported by results obtained from kinetic analyses provided on di-D-galacturonic acid, penta-D-galacturonic acid and pectate. [116]

The production of individual enzyme forms in roots was compared to enzymes produced by root cells cultivated on solid and liquid medium.

These results will serve in future as a basis for structural evaluation of individual pectate hydrolases as well as for structure-function studies.

4.3 Oligogalacturonate hydrolase from the pulp of parsley roots

Plant exoPGs were supposed to prefer only polymeric substrate and the ability to cleave substrate with lower degree of polymerization (DP) was strictly attributed to enzymes produced by microorganisms.[112, 113] First description of plant enzyme preferring oligogalacturonates (oligogalacturonate hydrolase, OGH) appeared in 2005 when an enzyme from carrot roots was described.[116] In addition, more forms of OGH were found in parsley roots juice (Chapter 4.2). All three forms of this enzyme were similar to protein from carrot roots with preference for hexagalacturonate (OGH6). The pH optima determination of proteins extracted from parsley roots pulp (Fig. 46) indicated the presence of enzyme with preference for substrates with higher DP and occurring exclusively in pulp. In contrast to carrot, the enzyme system in parsley seemed to be even more enriched in connection to degradation of substrates with different DP.

The further aim of this study was to purify and characterize OGH from parsley roots pulp indicating a unique substrate preference.

4.3.1 Purification of oligogalacturonate hydrolase

The pulp protein mixture presented a very heterogeneous material what required more complicated purification procedure (Fig. 54) as that for juice mixture (Fig. 36).

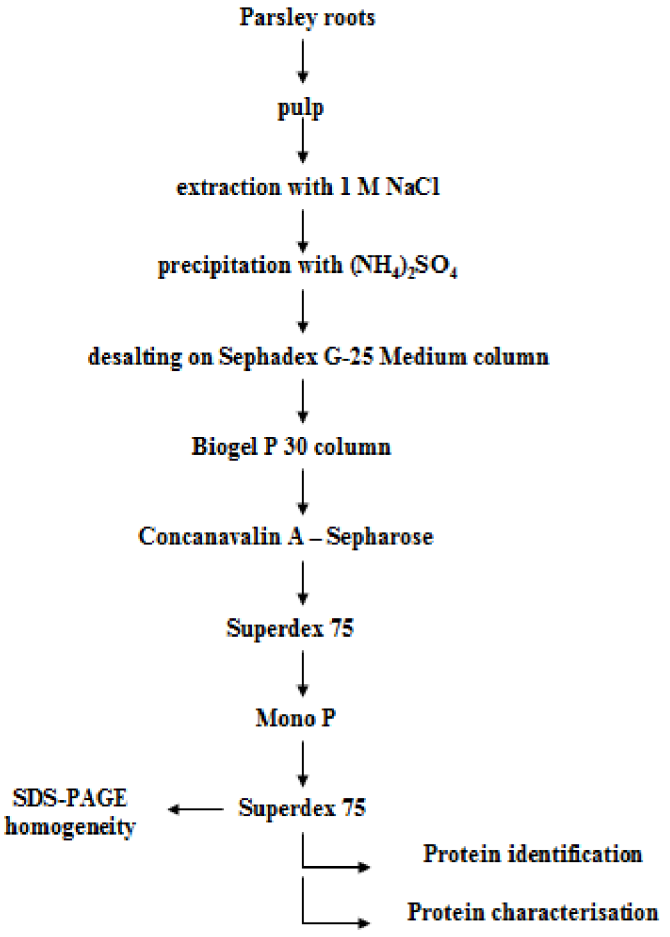


Fig. 54: Scheme of purification pathway used for parsley pulp protein mixture

The first step of purification was provided on Sephadex G-25 Medium column (Fig. 55). The desalted sample was then applied on Biogel P 30 column (Biorad) where exoPGs (approx. Mr about 55 kDa) and XET enzymes (approx. Mr about 32 kDa) were separated (Fig. 56). Fractions with exopolygalacturonase activity were collected and separated on a Concanavalin A-Sepharose (Pharmacia, Sweden) column (Fig. 57). The next purification step was provided on Superdex 75 column connected to FPLC device (Fig. 58).

The improvement of purification was achieved with chromatofocusing on Mono P column (Fig. 59) followed by rechromatography on Superdex 75, where a huge amount of contaminants was removed.

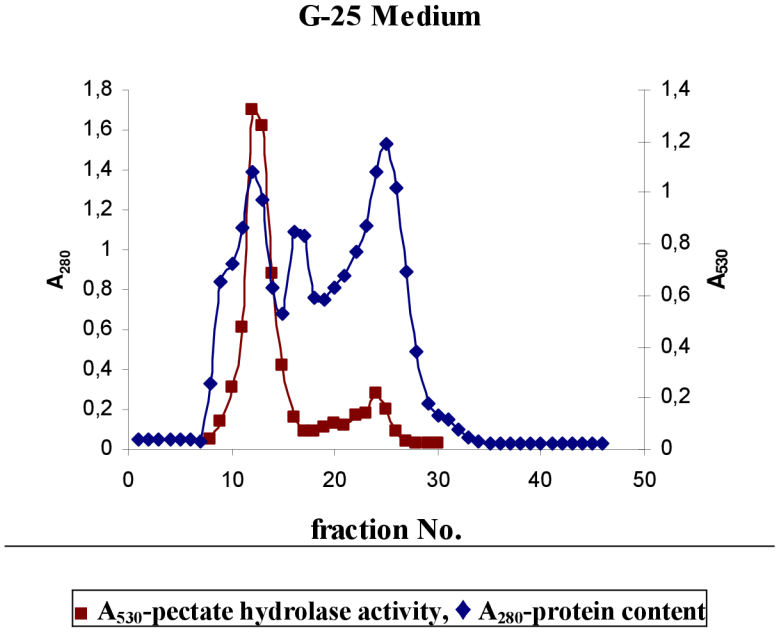


Fig. 55: Sephadex G-25 Medium: Separation of protein mixture from parsley pulp after previous extraction with 1 M NaCl and precipitation with ammonium sulphate

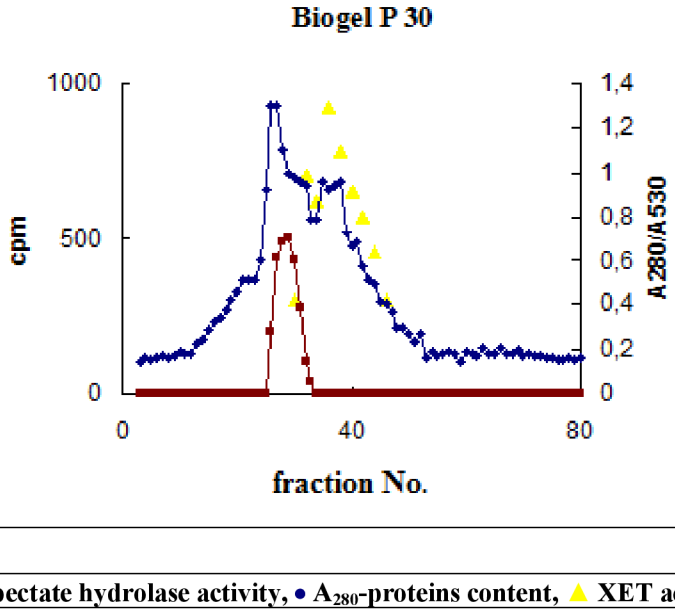


Fig. 56: Biogel P 30 column: Separation of exoPGs (approx. 55 kDa) and XETs (approx. 32 kDa)

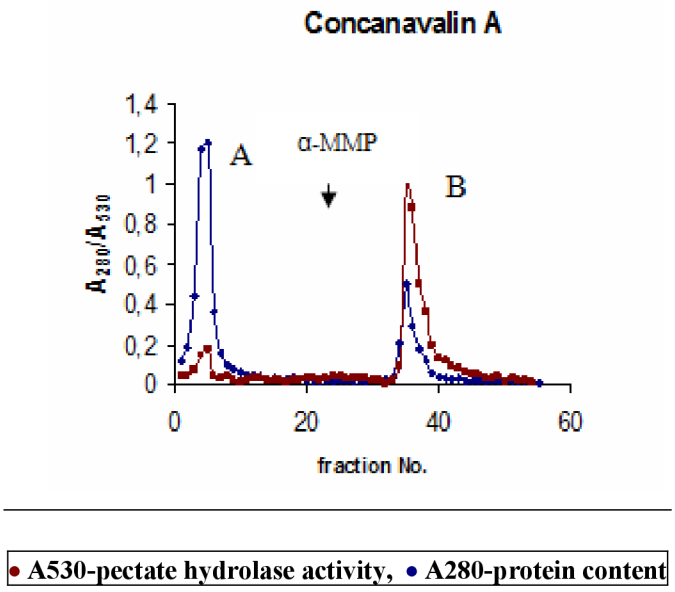


Fig. 57: Concanavalin A – Sepharose: A- the proteins unbound on concanavalin A, B enzyme fraction eluted from column with α- MMP

The affinity of enzyme to Concanavalin A indicated an glycosylation of the same type as glycosylation confirmed in enzymes (exoPGs) from parsley juice.

Fractions eluted with α-MMP were collected, desalted on DP 10 column and separated by gel permeation chromatography on Superdex 75 column (Fig. 58).

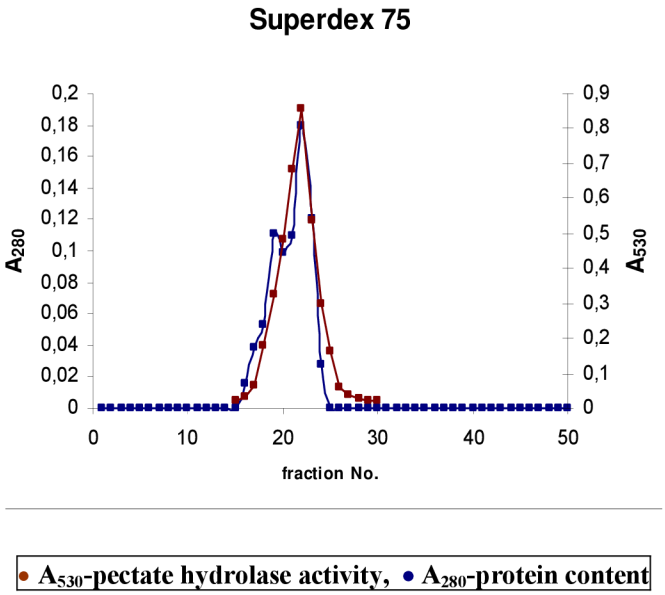
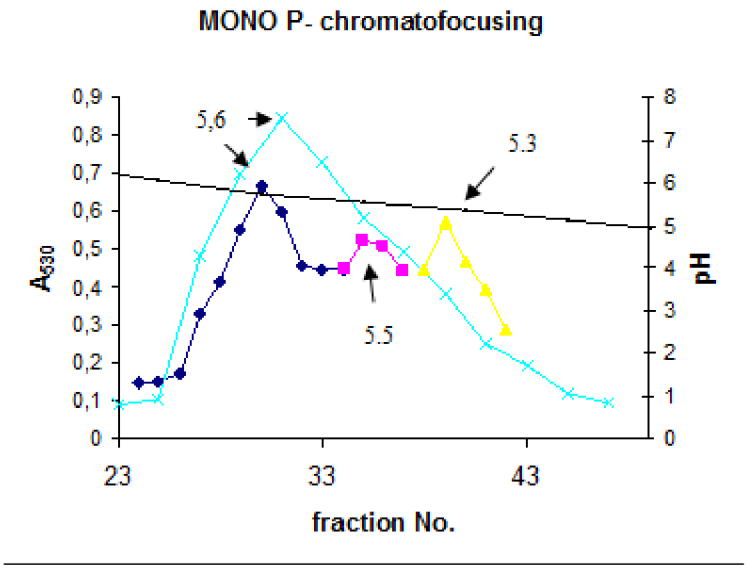


Fig. 58: Superdex 75: Separation profile of enzyme eluted from ConA by α-MMP

The attempt to separate individual enzyme forms was provided on Mono P column connected to FPLC (Fig. 59), where desalted fractions 22-23 from Superdex 75 column were applied.

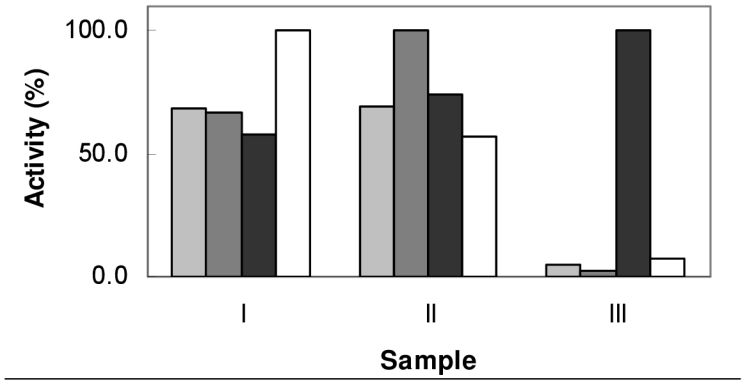


Proteins (-×-), activity peak I (-♦-), activity peak II, (-■-), activity peak III (-▲-)

Fig. 59: Chromatofocusing on Mono P column: Determination of isoelectric point of OGH found in parsley pulp

Rechromatography on Superdex 75 (not shown) was used for removal of PB 74 from proteins collected in fractions of peaks I-III (Fig. 59).

The activity curve on PGA indicated the possible separation of individual forms (three separated peaks I, II and III) on Mono P column. Enzymes were identified according to their pH optima and the preference for individual substrate. Decamer and pH value of 4.6 were used for identification of wanted OGH (Fig. 60).



GA5, pH 3.6 - ■, GA5, pH 4.2 - ■, GA10, pH 4.6 - ■, PGA, pH 5.2 - □

Fig. 60: Identification of individual pectate hydrolases separated on Mono P column (Peaks I-III). Identification provided on different substrates.

The first peak (I) corresponded to the mixture of exopolygalacturonase and OGHs with pH optima 3.6 and 4.2. The second peak (II) contained mainly OGH with pH optimum 4.2. The third peak (III) corresponded clearly to OGH which was the subject of this part of study.

Control of protein purification process

The control of purification process of OGH was evaluated after each chromatography step, including Biogel P 30 column, Concanavalin A-Sepharose as well as Mono P and Superdex 75 steps (Fig. 61). As can be seen, protein of OGH was still not completely homogeneous (Fig. 61).

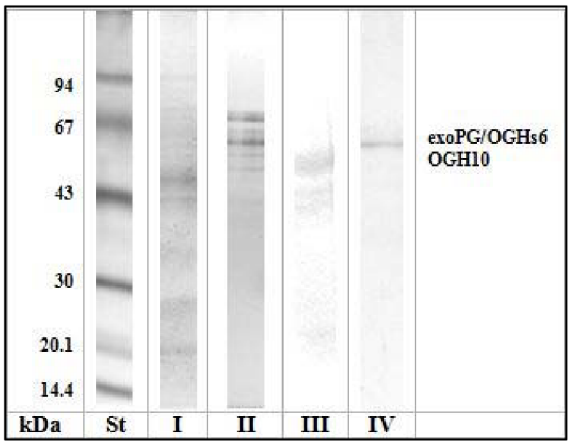


Fig: 61: SDS-PAGE of purification path of OGH from parsley roots pulp: I - the protein extract after Biogel P-30 column, II - enzymes from the pulp of roots purified with the same purification process as the apparently SDS-PAGE homogeneous proteins from juice, III - OGH after Mono P and Superdex 75 step and IV – purified pectate hydrolases from juice.

4.3.2 Characterization of oligogalacturonate hydrolase

The preferred substrate of purified OGH (OGH10) produced in parsley roots pulp was decagalacturonate (Fig. 62), what means the main difference between this enzyme and the others. In addition, this enzyme does not correspond to any enzyme form described generally in plants.

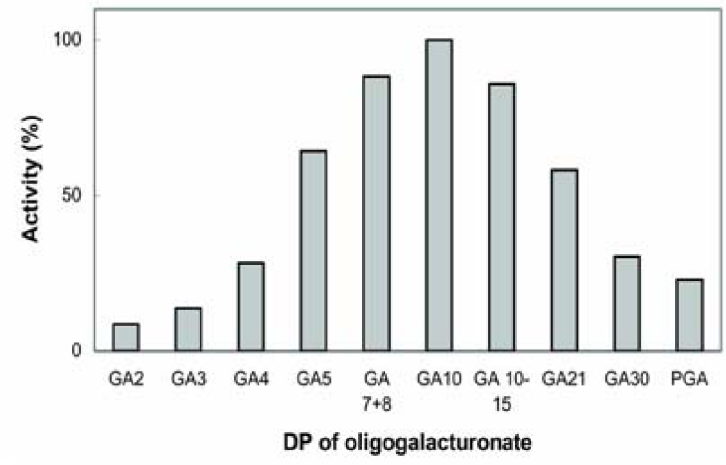


Fig. 62: The initial rates of OGH with unique substrate preference from parsley pulp for substrates with various DP.

Found OGH with unique preference for GA10 substrate was released from the Mono P chromatofocusing column at pH about 5.3 (Fig.59) what means that this enzyme has the most acidic isoelectric point from all pectate hydrolases obtained from parsley roots.

Mass determination of OGH was performed using molecular weight standards (Serva). Molecular weight was determined to be about 53.5 kDa what was in good agreement with the result obtained with gel filtration on Superdex 75 column. Gel permeation chromatography on Superdex 75, calibrated with the reference proteins, indicated the molecular weight of this enzyme about 53 kDa.

Determination of action pattern

The orientation of substrate splitting was evaluated using substrate modified by endopectate lyase (mixture of dimers and trimers marked on nonreducing end by double bond). Degradation of an unsaturated oligogalacturonates with OGH10 did not lead to any splitting of this modified substrate. Therefore, it was concluded that OGH10 prefers the cleavage of substrate from nonreducing end as an exopolygalacturonase or OGH from carrot roots as well as other OGHs found in parsley juice. [116,117]

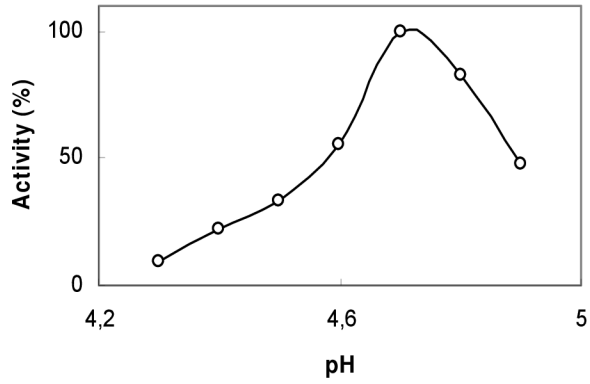
Determination of pH optimum of OGH10

For further characterization of this enzyme the decagalacturonate (1μmol/ml) was used as a substrate. Sharp pH optimum at 4.7 was found with half activity at pH 4.6 and 4.9, respectively (Fig. 63 A).

Determination of temperature optimum and thermal stability

The temperature optimum of OGH10 (Fig. 63 B) was determined near by 60 °C, what is comparable with other OGHs from this source (juice). This value responds to the energy of activation of 37 kJ/mol.

In comparison to other OGHs the thermal stability (Fig. 63 C) was slightly lower, near to 50 °C. In case of thermal stability determination, the activities were measured after 2h of incubation.



A

A - pH optimum determination

Fig. 63: Characterization of OGH from parsley roots

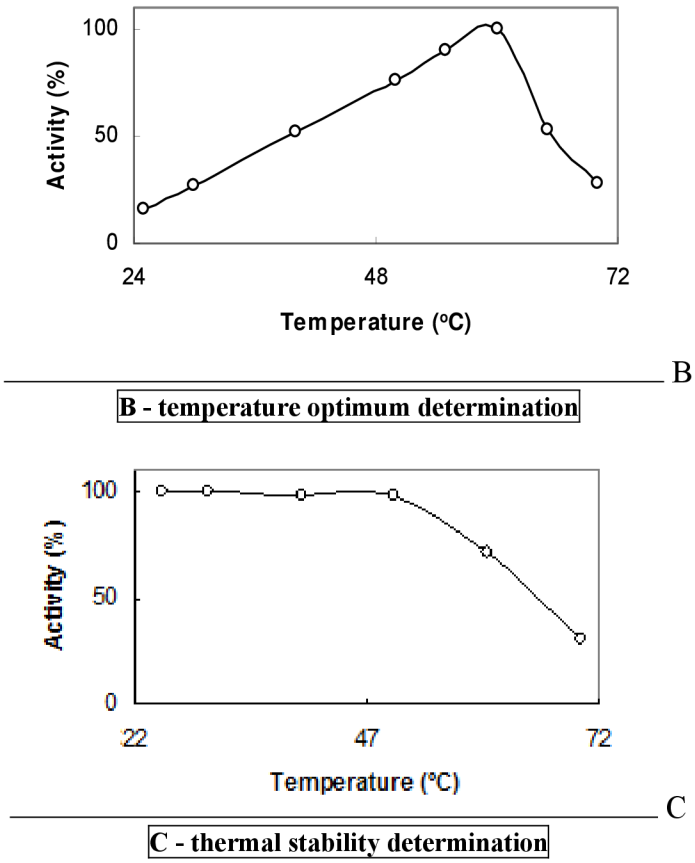


Fig. 63: Characterization of OGH from parsley roots

Kinetic parameters determination

Michaelis parameters were determined utilizing the initial velocities at five concentrations of substrate, ranging from 0.1 – 0.5 mM decagalacturonate in 0.1 M acetate buffer, pH 4.7, at 30 °C and the K_M value was calculated by nonlinear as well as by linear regression (Fig. 64).

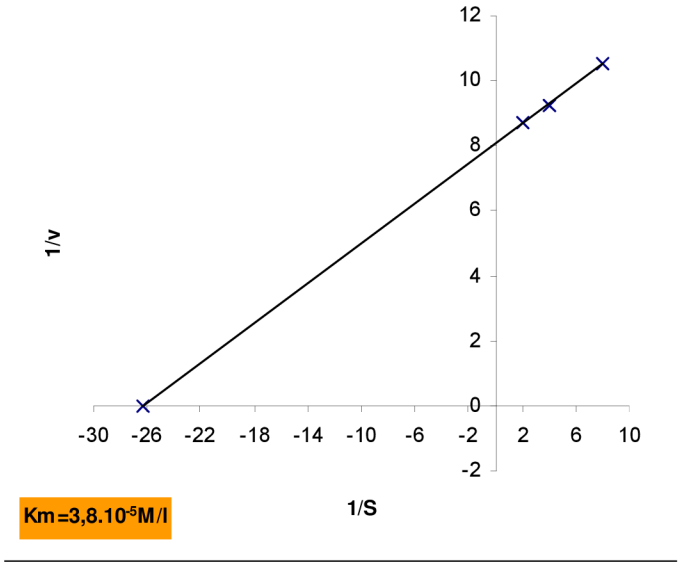


Fig. 64: Determination of Michaelis parameters for OGH10

The kinetic analysis of degradation of the preferential substrate showed that the affinity of this enzyme to decagalacturonate was slightly higher (K_M equal 3.8 x 10^{-5} mol/l) than the affinities of OGHs 6 or exoPG on pentagalacturonate (Table 6). On the other hand value of maximal initial rates described for this enzyme was comparable (0.124 µmol/min.mg) with the others (Table 6). The expected inhibition by the sole product of the enzymatic reaction, D-galacturonic acid, was not observed in this case.

Conclusion

The main form of pectate hydrolase, OGH 10, found in parsley pulp showed an unique substrate preference within all plant exopolygalacturonases. This enzyme clearly preferred substrates with degree of polymerization about 10 in contrast to other plant oligogalacturonate hydrolases preferring hexagalacturonic acid or typical exopolygalacturonases with preference for pectate. *112-116*

Enzyme had a sharp pH optimum corresponding to pH 4.7, but the temperature optimum, the presence of glycosylation as well as the cleavage of pectate from nonreducing end were similar to other pectate hydrolases found in this source.

This form of pectate hydrolase with preference for decagalacturonate as a substrate was not found yet in plants and results will serve as a basis for its structural evaluation as well as for structure-function studies.

4.4 Localization of exopectate hydrolases in the cell structures of parsley

As generally supposed, exoPGs should be bound on the plant cell wall. *112, 113* The occurrence of enzyme forms in the juice of parsley roots *117* or carrot *114-116* is in the contradiction with this assumption. From this reason the localization of these enzymes in cell structures was provided.

The localization of individual enzymes within the cell structures was provided using the method of differential ultracentrifugation. Individual fractions (cell walls, plasts, organelles, endoplasmic reticulum and cytosol) were analyzed for pectate hydrolase activity making use of their different pH optima and substrate of preference.

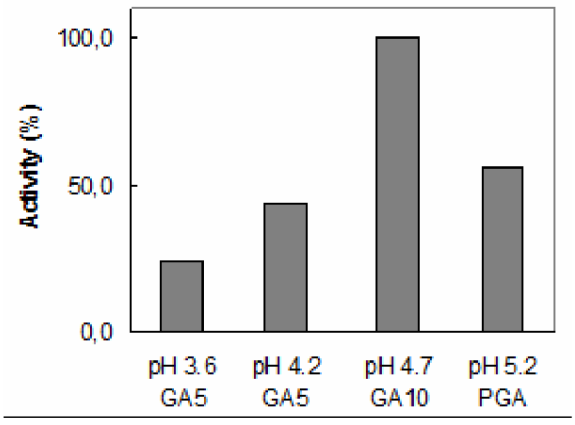


Fig. 65: The abundance of individual exopectate hydrolases activities in cell wall fraction

The main pectate hydrolase activity was connected with the plant cell wall as was reported previously. [112, 113] but this activity is especially represented by the oligogalacturonate hydrolase with unique substrate preference for decagalacturonate (GA10) while the activity of typical exoPG was just half time lower (Fig. 65). The main form detected in cytosol was OGH6 with pH optimum at 4.6 (Fig. 66).

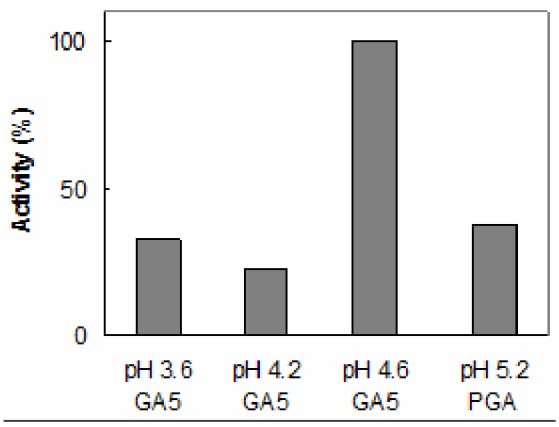


Fig. 66: The abundance of individual exopectate hydrolase activities in cytosol

4.5. Comparison of pectate hydrolases from parsley root cells

Three types of pectate hydrolases with terminal action pattern on substrate were found in parsley roots. Exopolygalacturonase (exoPG) and two types of oligogalacturonate hydrolases (OGHs) were isolated, partially purified, further characterized and in-between compared. The comparison of their molecular masses, isoelectric points, temperature optima, thermal stability and also action pattern showed very similar results. On the other hand there were observed differences in their pH optima and substrate specificity in respect to degree of substrate polymerization.

ExoPG, which prefers the polymeric substrate, has its pH optimum at 5.2. By contrast, the other types of pectate hydrolases, OGHs, favour oligomeric substrates, hexagalacturonate and decagalacturonate, respectively. First type of OGH with preference for decamer has its pH optimum at 4.7. The second type of OGH favours hexamer as a substrate and includes three isoforms, with pH optima 3.6, 4.2 and 4.6. (Table 7):

Table 7: Characterization of individual exopectate hydrolases isolated form parsley roots

pH optimum	Occurrence			DP of preference substrate	Enzyme	pI
	Parsley roots	Plant tissue	Cell components			
3.6	juice, pulp	callus,cells	organelles	5-7	OGH6	5.45
4.2	juice, pulp	---	plasts, wall	5-7	OGH6	5.35
4.6	juice	callus,cells	cytosol	5-7	OGH6	5.60
4.7	pulp	---	wall	10	OGH10	5.30
5.2	juice, pulp	cells	wall, ER	PGA	exoPG	5.55

The main difference between pectate hydrolases isolated from parsley roots is mainly the substrate preference accompanied by the sharp pH optimum decreasing with the DP decrease of substrate.

4.6 Primary structure studies of exopolygalacturonase from parsley roots

Partially purified proteins (without chromatofocusing step) from the pulp of parsley roots were separated by IEF (pH 4.5-7.0) utilizing Coomasie blue detection (Fig.67 B) Bands in gel were handled as described in chapter 3.5.8 and studied with MALDI-TOF/TOF mass spectrometric analysis.

The best results were achieved with the sample obtained from IEF band No.2 (Fig. 67, part B). Enzyme was identified as exoPG (Fig. 67, part A).

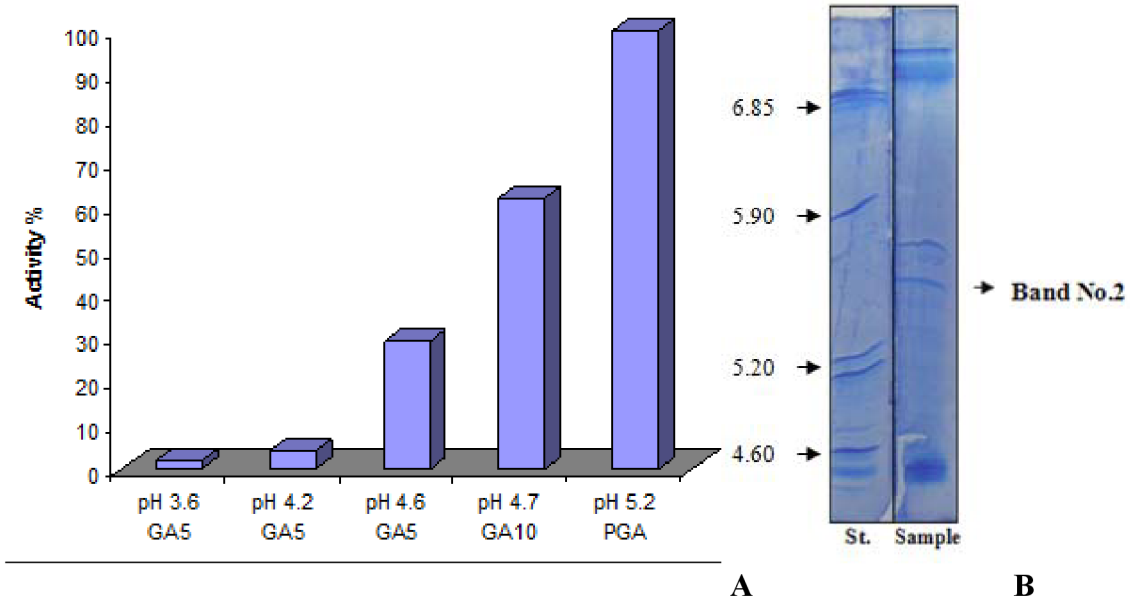


Fig. 67: IEF of partially purified pectate hydrolases from parsley roots pulp: A - identification of enzyme of IEF band No.2 (on the basis of pH optima and substrate preferences), B - Coomassie blue detection of proteins separated by IEF (St. – standard)

MALDI-TOF/TOF mass spectrometric sequencing of exoPG

The amino acids sequencing of exoPG was evaluated using MALDI-TOF/TOF mass spectrometric analysis.

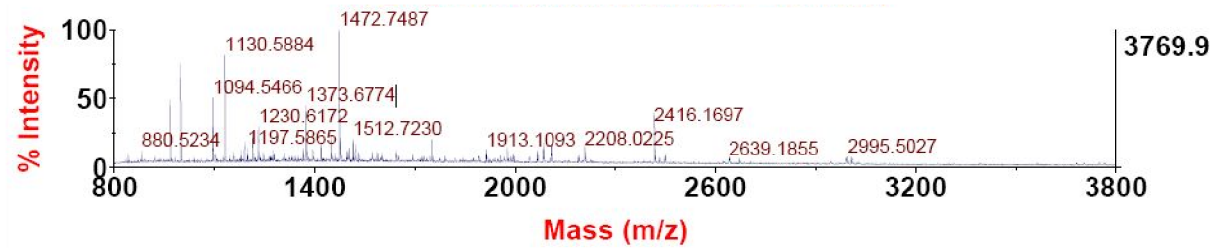


Fig. 68: MALDI-TOF/TOF MS spectrum from tryptic digest of exoPG

Peptide 1094.5466

a) MS/MS spectrum

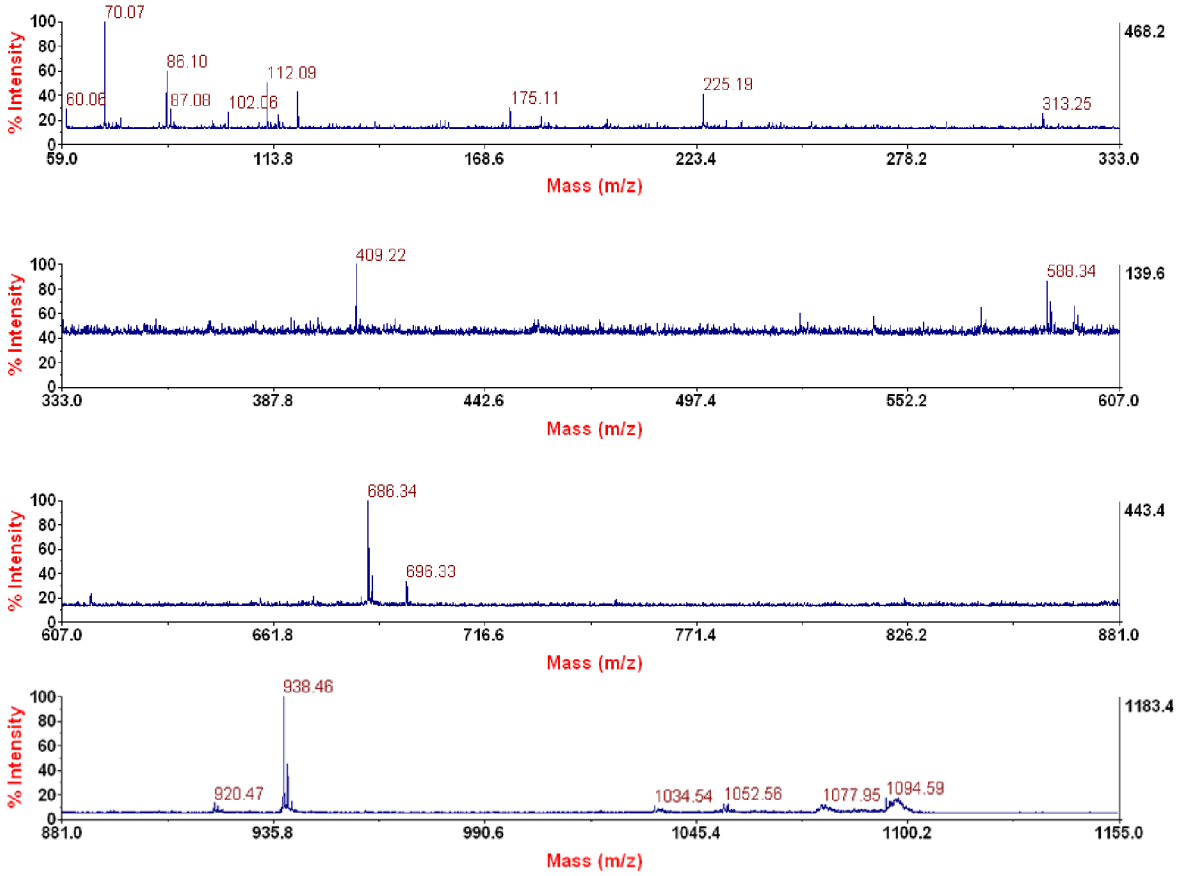


Fig. 69: MALDI-TOF/TOF MS/MS spectrum of peptide at 1094.5466

b) Sequences proposed by DeNovo program

Sequence	**Positive hit (%)**
-RIETADPHR-	79.0
-RITEADPHR-	77.8
-RIETGEPHR-	77.3
-RIETADVPHG-	67.3
-RNKTADPHR-	67.0
-RIETADRPH-	66.6
-RIETADSSDT-	64.2
-DHRDPWLR-	64.0
-HDRDPWLR-	64.0
-RLPNNLVLR-	63.0

c) Manual interpretation of MS/MS spectrum

-R[IL]KTADPHR-
-R[IL]KTADVPHG-

Peptide 1130.5884

a) MS/MS spectrum

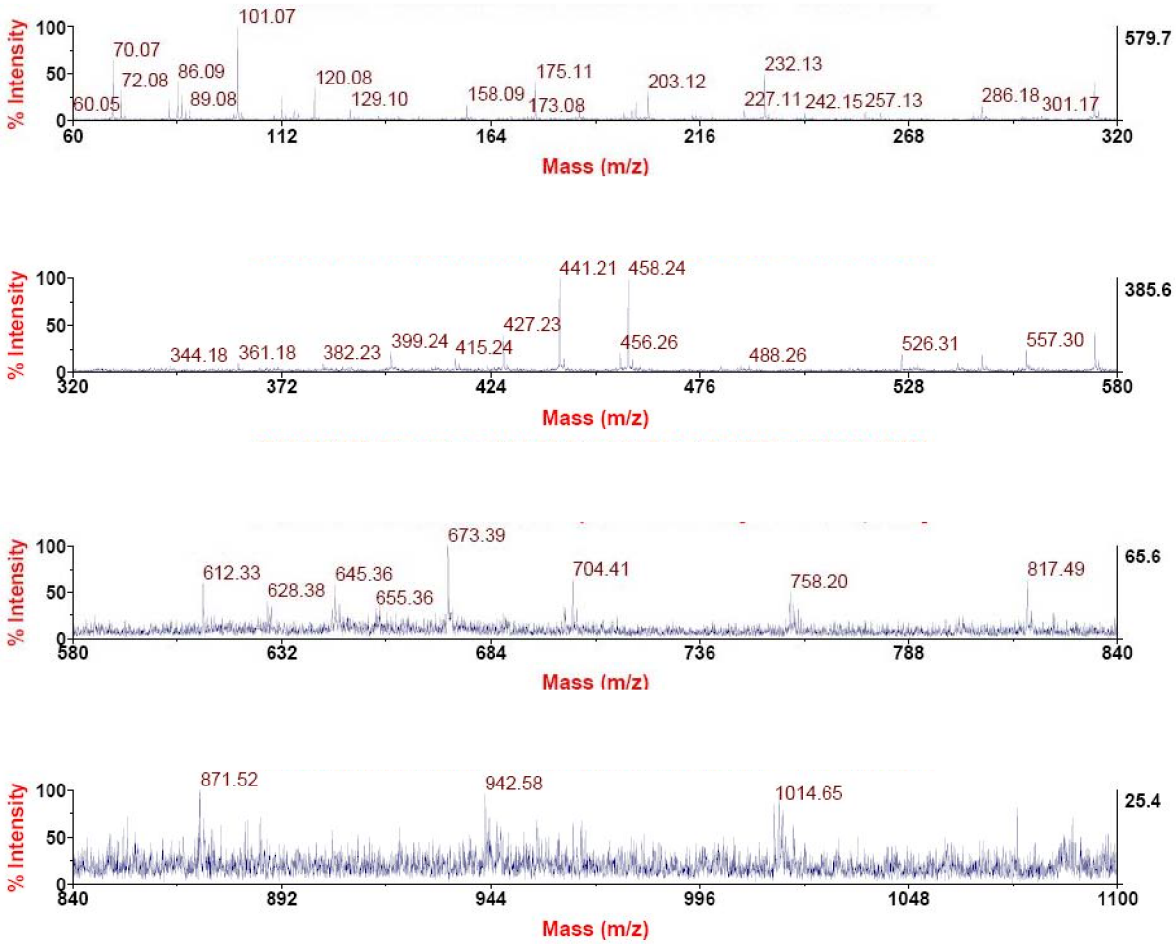


Fig. 70: MALDI-TOF/TOF MS/MS spectrum of peptide at 1130.5884

b) Sequences proposed by DeNovo program

Sequence	Positive hit (%)
-KAGGLFVPEGR-	88.9
-KANLFVPEGR-	86.6
-AKGGLFVPEGR-	84.9
-NAKLFVPEGR-	84.5
-NAAGLFVPEGR-	84.3
-KGAGLFVPEGR-	83.9
-AGAGGLFVPEGR-	83.8
-KAGGLVFPEGR-	82.9

c) Manual interpretation of MS/MS spectrum

(NA)[KQ][LI]FVPEGR
(G[KQ])Q[LI]FVPEGR
GGAQ[LI]FVPEGR

Peptide 2160.1050

a) MS/MS spectrum

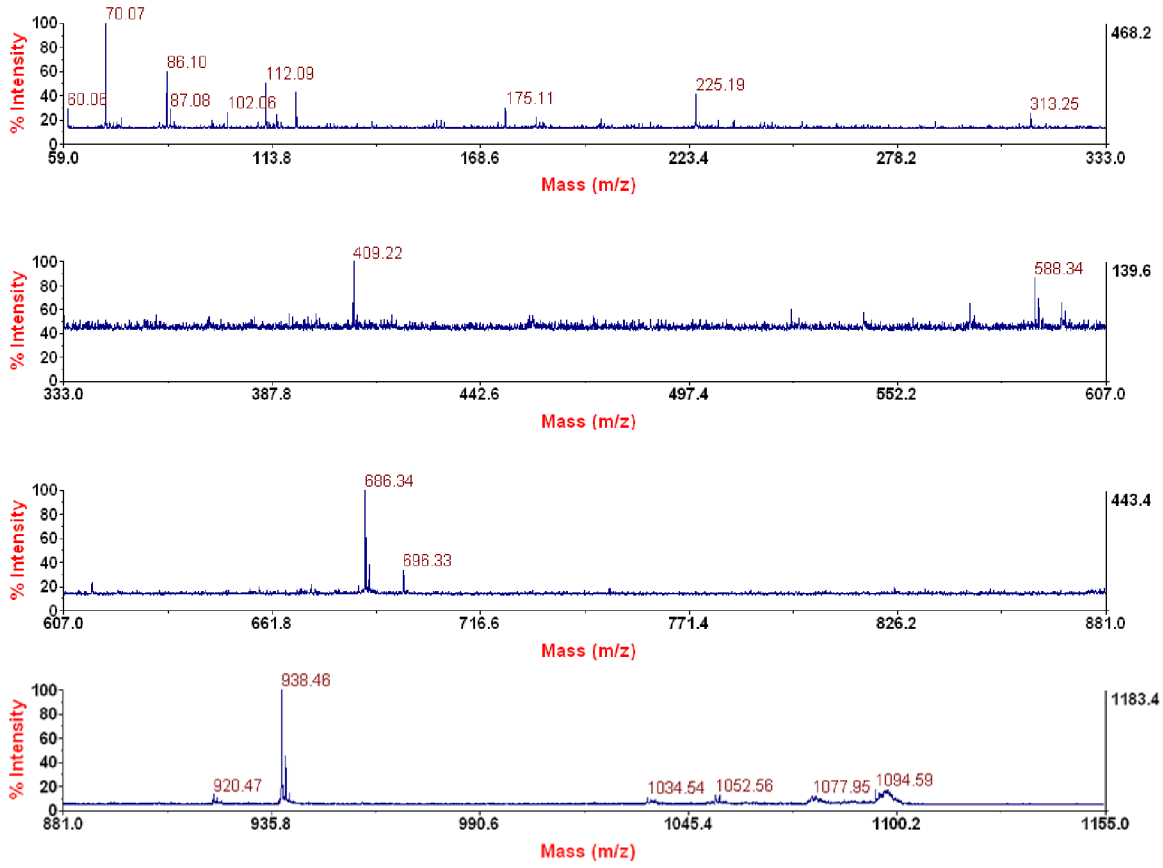


Fig. 71: MALDI-TOF/TOF MS/MS spectrum of peptide at 2160.1050

b) Sequences proposed by DeNovo program

Sequence	**Positive hit (%)**
-(1061.515)WLHYKVPR-	66.0
-(1061.515)WLYHKVPR-	66.0
-(1061.515)LWYHKVPR-	66.0
-(1061.515)LWHYKVPR-	66.0
-(1061.515)WLWNKVPR-	64.8
-(1061.515)LWWNKVPR-	64.8
-(1061.515)WLNWKVPR-	64.8
-(1061.515)LWNWKVPR-	64.8
-SSPVKYHLW(1043.504)-	59.5
-SSPVKHYWL(1043.504)-	59.5

c) Manual interpretation of MS/MS spectrum

-[IL]WWNKVPR-

Peptide 2416.1697

a) MS/MS spectrum

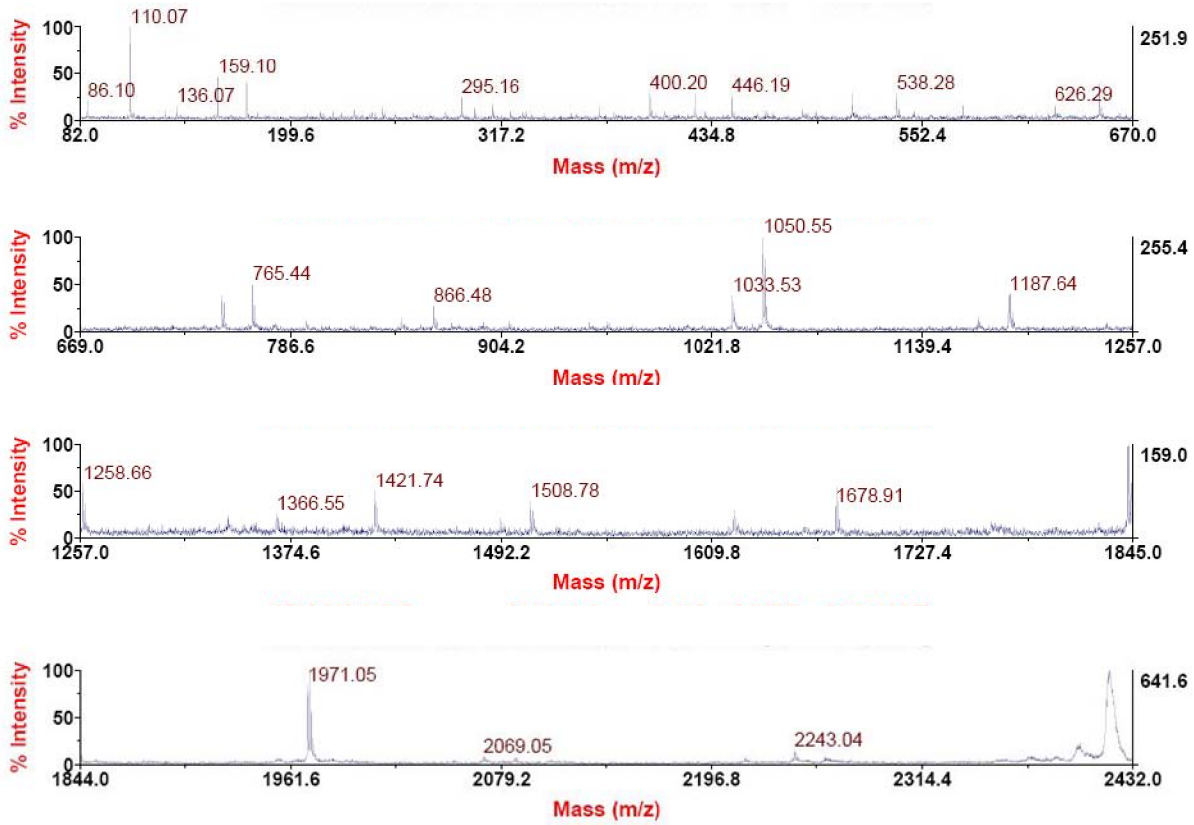


Fig. 72: MALDI-TOF/TOF MS/MS spectrum of peptide at 2416.1204

b) Sequences proposed by DeNovo program

Sequence	Positive hit (%)
-RSSDEYGISYAHSPTNIIIHR-	83.4
-RSSDEYGEAYAHSPTNIIIHR-	82.9
-RSSDEYLGSYAHSPTNIIIHR-	82.5
-RSSDEYGISYAHSPTNIPEHR-	82.3
-RSSDEYGISYAHSTPNIIIHR-	82.1
-RIINTPSHAYSIGYEDRR-	80.9
-RSSDEYGISPHHSPTNIIIHR-	79.7
-RSSDEYGISYAHSPTRAIIHR-	78.2
-RSSDEYGISYAHSSKPIIIHR-	75.7

c) Manual interpretation of MS/MS spectrum

(SNE)DEYG[LI]SYAHPSTN[LI][LI][LI]HR
(SGW)DEYG[LI]SYAHPSTN[LI][LI][LI]HR

Comparison of found sequences with sequences of PGs and exoPGs

The obtained sequence fragments of *Petroselinum crispum* exoPG (exoPG-*PC*) were compared with known sequences of plant PGs and exoPGs. The highest similarity was found with enzymes from *Vitis vinifera*, *Eucalyptus globulus*, *Orysa sativa* and *Arabidopsis thaliana* (Fig.73). (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

```
exoPG-PC        ------------------------------------------------------------
Vitis           MRRFFTLVDVLLVLLLFSEAPWAVWGSPHCDQTSSGVIRPHSVAITEFGAVGDGVTLNTK 60
Eucalyptus      ------------------------------------------------------------
Orysa           --------------------------------MGQWVAAPPVYDLREFGGVGDGRTLNTE 28
Arath           MKRSFLLLYVLLVQAFYGAWCSVGESLHCEYSNLASLHRPHSVSITEFGAVGDGVTLNTK 59

exoPG-PC        ---------------GGAQLFVPEGRWL-------------------------------- 10
Vitis           AFQNAIFYLNSFADKGGAQLFVPAGRWLTGSFDLISHLTLWLDKDAVILGSMNSNDWPVI 120
Eucalyptus      ------------------------------------------------------------
Orysa           AFVAAVA---SIAERGGGRLVVPAGRWLTAPFNLTSRMTLFLAAGAEILGVQDERYWPLM 85
Arath           AFQNALFYLNSFSDKGGAQLFVPAGQWLTGSFDLISHLTLWLDKGATILGSTAKN-WPVV 118

exoPG-PC        ------------------------------------------IWWNWKVPR------- 10
Vitis           DPLPSYGRGRELPGRRHRSLIYGCNLTDVIVT-GDNGTIDGQGSIWWNWFQKKTLNYTRP 179
Eucalyptus      ----------------------------------------GSMWWERFRNKTLDYTRP 18
Orysa           SPLPSYGYGREHRGPRYGSLIHGQDLKDVTITGGQNGTINGQGQSWWSKFRKKVLNHTRG 145
Arath           DPLPSYGRGRELPGRRHRSLIYGQNLTDVVIT-GENGTIDGQGTVWWDWFRNGELNYTRP 177

exoPG-PC        ------------------------------------------------------------
Vitis           HLVEFINSTGVVISNVTFLNSPFWTIHPVYCSQVIIQNVTILAPLD-SPNTDGIDPDSSN 238
EUCGG           HLVELMNSTGVVISNLTFLNSPFWTIHPIYCSHVIVQNVTIRAPLD-SPNTDGIDPDSSD 77
Orysa           PLVQLMRSSNITISNITLRDSPFWTLHIYDCKDVTISDTTILAPIVGAPNTDGIDPDSCE 205
Arath           HLVELMNSTGLIISNLTFLNSPFWNIHPVYCRDVVVKNLTILAPLE-SPNTDGVDPDSST 236

exoPG-PC        ------------------SGWDEYGISYAHPSTNIIIHR-------------------- 29
Vitis           DVCIEDCYISTGDDLIAIKSGWDEYGISYARPSTNIIIRRLVGKTNSSAGIAIGSEMSGG 298
Eucalyptus      DVCIEDCYISTGDDVIAIKSGWDEYGISYGRPSKNIIIRRLIGETHSSG-IAIGSEMSGG 136
Orysa           NVVIKNCYISVGDDGIAIKSGWDQYGIAYGRPSTNIIIHNVTIRSMVSAGVSIGSEMSGG 265
Arath           NVCIEDCYIVTGDDLVSIKSGWDEYGISYARPSSKIKINRLTGQTTSSSGIAIGSEMSGG 296

exoPG-PC        ------------------RIKTADVPHG-------------------------------- 5
Vitis           VSEVHAESLQFFNSKTGIRIKTSPGRGGYVRNIYISDMNLVDVKIAIRFTGQYGEHPDEF 358
Eucalyptus      VSDVHAEDIVFFNSTTGIRIKTSPGRGGYVRNIFISNVSLANVKVAIRFTGRYGEHPDES 196
ORYSJ           VSNVLVENVHIWDSRRGVRIKTAPGRGAYVSNITYRNITLEHIRVGIVIKTDYNEHPDEG 325
Arath           VSEIYIKDLHLFNSNTGIRIKTSAGRGGYVRNVHILNVKLDNVKKAIRFTGKYGEHPDEK 356

exoPG-PC        ------------------------------------------------------------
Vitis           YDPTALPIIENITVKDVMGENIKFAGLLEGIEGDNFVNICLSNITLNVTSES--PWNCSY 416
Eucalyptus      YDPKAMPKIERITFKDIHGENITVAGLMEGIEGDNFINICLYNITLSVNSIS--PWNCSN 254
Orysa           FDPKAVPIIENISYSSIHGHGVRVPVRIQGSAEIPVKNVTFHDMSVGLVDRKNHVFQCSF 385
Arath           YDPKALPAIEKITFENVNGDGIGVAGLLEGIEGDVFKNICFLNVTLRVKKNSKKPWECSN 416
```

Fig. 73: Amino acid sequence alignment of peptides at 1094.5466, 1130.5884, 2160.1050 and 2416.1204 m/z with sequences of enzymes with PG activity from NCBInr database (shown below):

Oryza (ORYSJ)

Oryza Sativa susbsp.japonica (Rice)

EAZ44872.1, putative uncharacterized protein

Glycosyl hydrolase 28, polygalacturonase activity, catalysis of the hydrolysis of 1,4-α-D-galacturonic linkages in pectate and other galacturonans.

Vitis (VITVI)
***Vitis Vinifera* (Grape)**
CAO40225.1, unnamed protein product with unclear function
Glycosyl hydrolase 28, polygalacturonase activity, catalysis of the hydrolysis of 1,4-α-D-galacturonic linkages in pectate and other galacturonans.

Eucalyptus (EUCGG)
Eucalyptus globulus subsp. globulus (Tasmanian blue gum)
ABG34278.1, polygalacturonase fragment
Glycosyl hydrolase 28, polygalacturonase activity, catalysis of the hydrolysis of 1,4-α-D-galacturonic linkages in pectate and other galacturonans.

Arath (ARATH)
Arabidopsis thaliana
CAB71871.1, putative protein
Glycosyl hydrolase 28, polygalacturonase activity, catalysis of the hydrolysis of 1,4-α-D-galacturonic linkages in pectate and other galacturonans.

Classification of plant PGs and exoPGs

In agreement with the proposed classification system, plant PGs and exoPGs were divided into five clades A-E. [118-120] This division was based on the evolutionary tree of all known PG structures. The similarity of found sequence fragments with some parts of known PG structures indicates that this enzyme belongs to the clade C (Fig. 74) covering all pollen and flower PGs as well as plant exoPGs. [118]

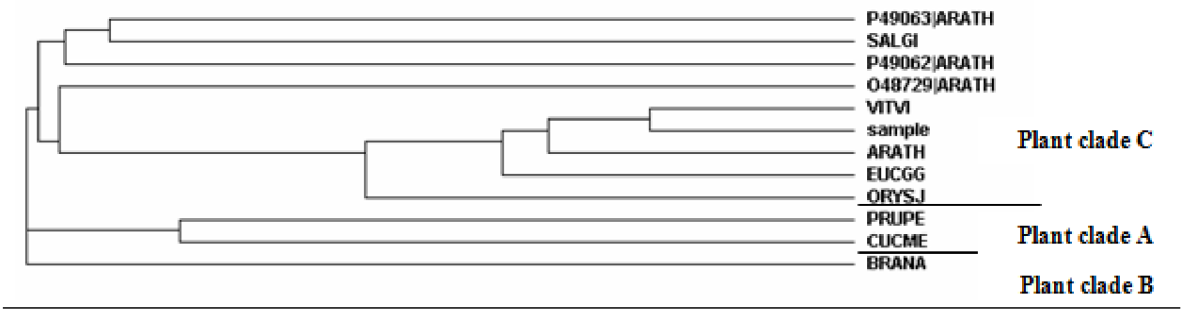


Fig.74: Cladogram of exoPG (sample) from parsley roots, enzymes with PG activity with the most similar primary structure and chosen PGs and exoPGs from previous report (shown below): [118]

Plant exopolygalacturonases

P49063.ARATH	Arath1.epg	EC 3.2.1.67	*Arabidopsis thaliana*
P49062.ARATH	Arath4.epg	EC 3.2.1.67	*Arabidopsis thaliana*
O48729.ARATH	Arath5.epg	EC 3.2.1.67	*Arabidopsis thaliana*

Plant pollen polygalacturonases

Q9MBCO. SALGI	Salgi1.pp	EC 3.2.1.15	*Salix gilgiana*

Plant endopolygalacturonases*

P48979.PRUPE	Prupe1.pg	EC 3.2.1.15	*Prunus persica*
Q42399.BRANA	Brana1.pg	EC 3.2.1.15	*Brassica napus*
Q81244.CUCME	Cucme1.pg	EC 3.2.1.15	*Cucumis melo*

Plant PGs* and exoPGs sequences were used to prove the correctness of obtained cladogram (Fig. 74) and phylogam (Fig. 75)

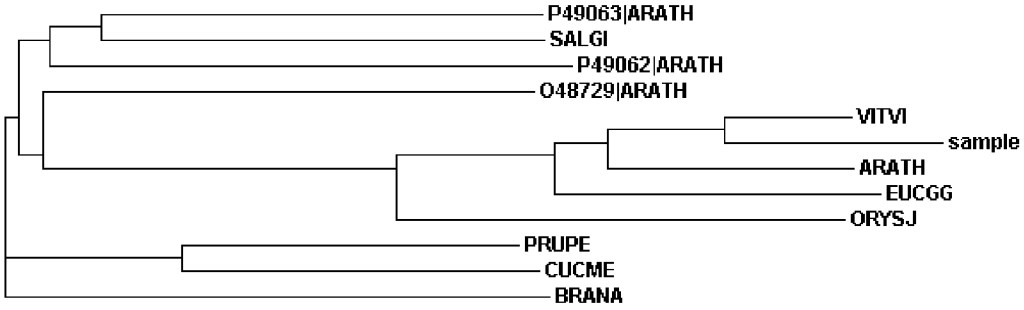


Fig. 75: Phylogam of exoPG (sample) from parsley roots, enzymes with PG activity with the most similar primary structure and chosen PGs and exoPGs from previous report [118]

5. CONCLUSION

This work can be divided into two parts and generally represents the contribution to the complex study about the plant cell wall. Thesis includes the problem of polysaccharide networks formed within the cell wall as well as enzymes involving in degradation or modification of individual polysaccharide components. Parsley roots (*Petroselinum crispum*) pulp, juice and suspension cultures were used as a source of plant material.

First part, named "Hemicellulose-pectin cross linking" was focused on the presence of hetero-polysaccharide complex in plant cell wall. Aim of this study was to confirm the existence of such complex and identify its individual hemicellulose and pectin components.

The second part, "Exopectate hydrolases", was target on the pectate hydrolases attacking the backbone of homogalacturonan, one of the main domains of pectic molecule. Chosen enzymes were isolated, purified and characterised.

Within the polysaccharide part of work, the hemicellulose-pectin conjugate was isolated from suspension cultures of parsley roots and partially purified. First, hemicelluloses were separated using the affinity and gel permeation chromatographies (adsorption to cellulose, LC on Biogel P2) and then hemicellulose-pectin complex was refined as co-elute by anion exchange chromatography (DEAE Spheron). From the gel permeation chromatography the molecular weight in the range of 300-500 kDa was determined.

Isolated heteropolysaccharide complex was then analysed using FTIR spectroscopy. From obtained results the hemicellulose compound was identified as arabinoxylan. On the other hand, homogalacturonan (1,4-linked-α-D-galacturonic acid) was supposed to represent the pectic component in the sample. On the basis of FTIR results, the polysaccharide sample was further analysed by NMR. Acquired spectra indicated that analysed sample is composed from hemicellulose (arabinoxylan) and pectin part (probably homogalacturonan). These results confirmed previous FTIR data. Unfortunately, polysaccharide sample represented the heterogeneous plant matrix what caused the spectra interpretation more difficult. Therefore, for determination of individual polysaccharide structures as well as for conclusive confirmation of obtained results, the further analyses are required.

The next step was to check out the potential hetero-transglycosylating reaction between arabinoxylan and homogalacturonan in plant tissues. Crude extracts of proteins isolated from parsley roots and nasturtium seeds were used for proof of this hetero-transglycosylating activity. Fluorescently labelled oligogalacturonic acids (OGA-SR) was used as an acceptor substrate and arabinoxylan (AX) as a donor substrate. Obtained positive results show that plant protein precipitates contain unknown type of enzymes (probably transglycosylases) enabling the catalysis of this hetero-transfer between OGAs and AX. This type of hetero-transfer was not described yet.

In the enzymology part of dissertation, five forms of exopolygalacturonase (EC 3.2.1.67) were identified in parsley roots as well as in their callus and suspension cells. Enzymes in the protein extracts of plant tissue were easily distinguished from each other according to different pH optima and substrate preferences. Presence of these enzyme forms in individual cell structures was determined using the differential centrifugation method. Although exoPGs were generally supposed to be bound to the cell wall, this assumption was absolutely valid

only for one enzyme form. This can indicate further functions of these enzymes in the plant tissue.

At first, exopolygalacturonases not bound to the cell structures were isolated and purified to apparent homogeneity according to SDS-PAGE using combination of affinity (ConA-Sepharose) and gel permeation (Sephadex G-100, Sephadex G-25 and Superose 12) chromatographies. Molecular weight obtained for all enzymes was about 55.3 kDa and deglycosylation with N-glycosidase F resulted in slight weight decrease (about 1 kDa). Edman degradation showed that N-terminal amino acids of two exopolygalacturonases were probably threonin and alanin. Unfortunately, the presence of other two enzyme forms made further research more complicated. The individual forms were separated by preparative IEF in close pH range (4.5 – 7.0), characterized and in between compared. The temperature optima and thermal stabilities were very similar. On the other hand, the most important difference between individual forms was observed in respect to their preference for substrate with concrete degree of polymerization. From this point of view, only one form (with pH optimum 5.2) was a typical exopolygalacturonase with preference for polymeric substrate. Other three forms (with pH optima 3.8, 4.2 and 4.6) showed preference for penta- or hexagalacturonate. Accordingly, the term "oligogalacturonate hydrolases" seemed to be more suitable.

Later, there was found the fifth exopolygalacturonase form bound to the wall of parsley roots cells. This form represented the major pectate hydrolase bound to this structure. It showed a unique substrate preference of a plant pectate hydrolases because it clearly preferred substrates with higher degree of polymerization (about DP 10) in contrast to other "oligogalacturonate hydrolases" with substrate preference for hexagalacturonate or typical exopolygalacturonase preferring pectate. The pH optimum 4.7 was found with half activities at pHs 4.6 and 4.9, respectively.

The isolation and purification of this enzyme required more steps as those described for other exopolygalacturonases from this plant source. The separation of individual form occurred utilizing chromatofocusing at pH of elution about 5.3.

The characterization of this form showed similarities with other pectate hydrolases from parsley roots. The thermal stability was slightly lower (stable until 50 °C), the temperature optimum was 60 °C (energy of activation being 37.0 kJ/mol), molecular weight 53.5 kDa, enzyme was glycosylated (interaction with ConA), K_M (decamer as a substrate) was equal $3.8.10^{-5}$ mol/l, V_{max} 0.124 μmol/min.mg and enzyme utilized cleavage of substrate from nonreducing end.

Pressey and Avants (1975) discussed the impossibility of natural complete hydrolysis of pectin in plants or plant tissues without endopolygalacturonase production due to the decrease of reaction rate of plant exopolygalacturonases acting on oligomeric substrates.[121] García-Romera and Fry (1995) presented hypothesis describing the oligogalacturonides with lower DP as a biologically inactive and therefore no more degradable. [122] Oligogalacturonate hydrolase (OGH6) preferring hexagalacturonate with still high reaction rate on dimer in cooperation with typical exoPG found in carrot roots showed that the complete hydrolysis of pectin from the point of view of present work is thinkable in this plant tissue. [116]

On the other hand, the present knowledge gives no explanation (except the microbial attack and following cleavage of pectin with endoPG) for the "preparation" of oligogalacturonates suitable as a substrates for referenced OGH. [116]

The pectate hydrolase system in parsley [117] was even more enriched in connection to substrates with different DP and pH optima in comparison to carrot. [114-116] OGH10 with

unique substrate preference for higher oligogalacturonates is described in this work and represents the main pectate hydrolase of the cell wall. In addition, this form of pectate hydrolase with preference for decagalacturonate as a substrate was not found yet in plants.

Generally, it seems that the DP decrease of preferred substrate of individual form is functionally bound with the decrease of extremely sharp pH optima of enzymes. The relationship between the decreasing pH of primary cell wall during auxin activation of proton pump bound on plasmatic membrane [123] and DP decrease of linear parts of pectin molecule side chains [9] regulated by pectate hydrolases with terminal action pattern may be indicated.

The OGH enzyme with unique substrate preference for decamer could also be important in determining the half-lives of OGAs that may be endogenous signals regulating aspects of programmed developmental events or responses to "invanders." [10] Therefore, this enzyme and other OGHs may be of importance to the plant from several perspectives.

6. REFERENCES

1) Minic, Z., Jouanin, L..: Plant glycoside hydrolases involved in cell wall polysaccharide degradation, *Plant Physiology and Biochemistry*, 2006, Vol.44, pp. 435-449, ISSN 0981-9428
2) Carpita, N.C., Gibeaut, D. M.: Structural models of primary cell walls in flowering plants; consistency of molecular structure with the physical properties of the walls during growth, *Major review: The Plant Journal*, 1993, Vol.3 (1), pp. 1-30
3) Willats, W.G.T, McCartney, L., Mackie, W., Knox, J.P.: Pectin: cell biology and prospects for functional analysis, *Plant Molecular Biology,* 2001, Vol.47, pp. 9-27
4) Cosgrove, D. J.: Growth of the plant cell wall, *Nature review*: *Molecular cell biology*, 2005, Vol.6, pp.850-861
5) Rose, J.K.C., Bennett A. B.: Cooperative disassembly of the cellulose-xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening, *Trends in plant science*, 1999, Vol.4, No.5, pp.176-183
6) Reiter, W-D.: *Arabidopsis thaliana* as a model system to study synthesis, structure, and function of the plant cell wall, *Plant Physiology and Biochemistry.* 1998, Vol.36 (1-2), pp. 167-176
7) Rizk, S.E., Abdel.Massih, R.M., Baydoun, E.A.-H, Brett, Ch.: Protein- and pH-dependent binding of nascent pectin and glucuronoarabinoxylan to xyloglucan in pea, *Planta* 2000, Vol. 211, pp. 423-429
8) Thompson, J.E., Fry, S.C.: Evidence for covalent linkage between xyloglucan and acidic pectins in suspension-cultured rose cells, *Planta* 2000, Vol. 211, pp. 275-286
9) Vincken, J.P., Schols, H.A., Oomen, R.J., McCann, M.C., Ulvskov, P., Voragen, G.J., Visser, R.G.F.: If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture, *Plant Physiology*, 2003, Vol.132, pp. 1781-1789,
10) Verhertbruggen, Y. , Knox, J.P.: Pectic Polysaccharides and Expanding Cell Walls, *The Expanding Cell*, 2006, DOI 10.1007/7089
11) Reymond, P., Gruenberger, S., Paul, K., Mueller, M., Farmer, E.E.: Oligogalacturonide defense signals in plants: Large fragments interact with the plasma membrane *in vitro*, *The proceedings of the National Academy of Sciences, USA*, 1995, Vol.92, pp. 4145-4149
12) Peréz, S., Rodríguez-Carvajal, M.A., Doco, T.: A complex plant cell wall polysaccharide: rhamnogalacturonan II.A structure in quest of a function, *Biochemie*, 2003, Vol. 85, pp. 109-121
13) Abdel-Masih, R.M., Baydoun E., Brett, Ch.T.: In vitro biosynthesis of 1,4-β-galactan attached to a pectin-xyloglucan complex in pea, *Planta* 2002, Vol. 216, pp. 502-511
14) Takeuchi, Y., Tohbaru, M., Sato, A.: Polysaccharides in primary cell walls of rice cells in suspension culture, *Phytochemistry*, 1994 , Vol. 35, No.2, pp. 361-363
15) Vidal, S., Doco, T., Williams, P., Pellerin, P., York, W.S., O´Neil, M.A.: Structural characterization of the pectic polysaccharide rhamnogalacturonan II: evidence for the backbone location of the aceric acid-containing oligoglycosyl side chain, *Carbohydrate Research,* 2000, Vol. 326, pp. 277-294

16) Fry, S.C.: Unzipped by expansins, *Current Biology*, 1994, Vol. 4, No. 9, pp. 815-817,
17) Fry, S.C.: Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells, *New Phytology,* 2004, Vol. 161, pp. 641-675
18) Doblin, M.S., Kurek, I., Jacob-Wilk, D., Delmer, D.P.: Cellulose biosynthesis in plants: from genes to rosettes, *Plant Cell Physiology*, 2002, Vol. 43, pp. 1407-1420
19) Marga, F., Grandbois, M., Cosgrove, D.J., Baskin, T.I.: Cell wall extension results in the coordinate separation of parallel microfibrils: evidence from scanning electron microscopy and atomic force microscopy, *Plant Journal,* 2005, Vol. 43, pp. 181-190
20) Deguchi, S., Tsujii, K., Horikoshi, K.: Cooking cellulose in hot and compressed water, *Chemical Communications*, 2006, Vol. 31, pp. 3293-3295
21) O´Neil, M., York, W.S.: The composition and structure of plant primary cell walls, In: *The Plant Cell Wall, Annual Plant Reviews,* 2003, Vol. 8, (J.K.C. Rose, ed.), CRC Press, Boca Raton, FL., pp. 1-54
22) Zhan, D., Janssen, P., Mort, A.J.: Scarity of complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin, *Carbohydrate Research*, 1993, Vol. 238, pp. 271-286
23) Schröder, R., Wegrzyn, T.F., Bolitho, K.M., Redgwell, R.J.: Mannan transglycosylase: a novel enzyme activity in cell walls of higher plants. *Planta*, 2004, Vol. 219, pp. 590-600
24) O´Neil, M., Albersheim, P., Darvill, A.: The pectic polysaccharides of primary cell walls, *Methods in Plant Biochemistry*, 1990, Vol.2, pp. 415-441
25) Albersheim, P., Darvill, A.G., O´Neil, M.A., Schols, H.A., Voragen, A.G.J.: An hypothesis: the same six polysaccharides are components of the primary cell walls of all higher plants, In: Visser and Voragen, *Pectins and pectinases*, 1996, Elsevier, Wageningen, The Netherlands, ISBN 0-444-82330, pp. 147-55
26) Mohnen, D.: Biosynthesis of pectins and galactomannans, *Comprehensive Natural Products Chemistry*, 1999, Vol.3, pp. 497-527
27) Ridley, B.L., O´Neil, M.A., Mohnen, D.: Pectins: structure, biosynthesis and oligogalacturonide-related signaling, *Phytochemistry* 2001, Vol. 57, pp. 929-967
28) Thibault, J.F., Tor, M., Barry, C.S., Vrebalov, J., Orfila, C., Jarvis, M.C.: Studies of the length of homogalacturonic regions in pectins by acid-hydrolysis, *Carbohydrate Research* 1993, Vol. 238, pp. 271-286
29) O´Neil, M.A., Warrenfeltz, D., Kates, K., Pellerin, P., Doco, T., Darvill, A.G., Albersheim, P: Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester – in vitro conditions for the formation and hydrolysis of the dimer, *Journal of Biological Chemistry*, 1996, Vol. 271, pp. 22923-22930
30) Matoh, T., Kawaguchi, S., Kobayashi, M.: Ubiquity of a borate-rhamnogalacturonan II complex in the cell walls of higher plants. *Plant Cell Physiology,* 1996, Vol. 37, pp. 636-640
31) McNeil, M., Darvill, A.G., Fry, S.C., Albersheim P.: Structure and function of the primary cell walls of plants, *Annual Review of Biochemistry,* 1984, Vol.53, pp. 625-663

32) Delmer, D.P.: Cellulose biosynthesis: exciting times for a. difficult field of study, *Annual Review of Plant Physiology and Plant Molecular Biology*, 1999, Vol.50, pp. 245-276
33) Geshi, N., Jørgensen, B., Ulvskov, P.: In vitro biosynthesis of 1,4-ß-galactan attached to rhamnogalacturonan, *Planta,* 2004, Vol.218, pp. 862-868
34) Sterling, D.J., Quigley, H.F., Orellana, A., Mohnen, D.: The catalytic site of the pectin biosynthetic enzyme α-1,4-galacturonosyltransferase (GalAT) is located in the lumen of the Golgi. *Plant Physiology*, 2001, Vol.127, pp. 360-371
35) Richmond, T.A., Somerville, C.R.: The cellulose synthase superfamily. *Plant Physiology,* 2000, Vol. 124, pp. 495-498
36) Dhugga, K.S., Barreiro, R., Whitten, B., Stecca, K., Hazebroek, J., Randhawa, G.S., Dolan, M., Kinney, A.J., Tomes, D., Nichols, S., Anderson, P.: Guar seed β-mannan synthase is a member of the cellulose synthase super gene family. *Science,* 2004, Vol. 303, pp. 363-366,
37) Liepman, A.H., Wilkerson, C.G., Keegstra, K.: Expression of cellulose synthase-like (*Csl*) genes in insect cells reveals that *CslA* family members encode mannan synthases. *The proceedings of the National Academy of Science, USA,* 2005, Vol. 102, pp. 2221-2226
38) Scheible, W.R., Pauly, M.: Glycosyltransferases and cell wall biosynthesis: Novel players and insights. *Current opinions in Plant biology*, 2004, Vol. 7, pp. 285-295
39) Zykwinska, A.W., Ralet, M.C., Garnier, C.D., Thibault, J.F.: Evidence for *in vitro* binding of pectin side chains to cellulose. *Plant Physiology*, 2005, Vol. 139, pp. 397-407,
40) Schols, H.A., Bakx, E.J., Schipper, D., Voragen, A.G.J.: A xylogalacturonan subunit present in the modified hairy regions of apple pectin, *Carbohydrate Research* 1995, Vol.279, pp.265-279
41) Hyasi, T., Ogawa, K., Mitsuishi, Y.: Characterization of the adsorption of xyloglucan to cellulose, *Plant Cell Physiology* 1994, Vol.35, pp. 1199-1205
42) Levy, I., Shani, Z., Shoseyov, O.: Modification of polysaccharides and plant cell wall by endo-1,4-β-glucanase and cellulose-binding domains, *Biomolecular Engineering* 2002, Vol.19, pp. 17-30
43) Hyaschi, T.: Xyloglucans in the primary cell wall. Annual Review: *Plant Molecular Biology*, 1989, Vol. 40, pp. 139-168
44) Fry, S.C.: Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship, *Physiologia Plantarum*, 1989, Vol.75, pp. 532-536
45) Baba, K., Sone, Y., Misaki, A., Hayashi T.: Localization of Xyloglucan in the Macromolecular Complex Composed of Xyloglucan and Cellulose in Pea Stems. *Plant Cell Physiology*, 1994, Vol.35, pp. 439-444
46) Talbott, L.D., Ray, P.M.: Changes in molecular size of previously deposited and newly synthesised pea cell wall matrix polysaccharides. Effects of auxin and turgor, *Plant Physiology* 1992, Vol. 98, pp. 369-379
47) Thompson, J.E, Fry, S.C.: Density-labelling of cell wall polysaccharides in cultured rose cells: comparison of incorporation of ^{2}H and ^{13}C from exogenous glucose, *Carbohydrate Research* 2001, Vol. 332, pp. 175-182

48) Cumming, C. M., Abdel-Massih, R.M., Brett, Ch.T.: Biosynthesis and cell-wall deposition of a pectin-xyloglucan complex in pea, *Planta* 2005, Vol. 222, pp. 546-555,
49) Keegstra, K., Talmage, K., Bauer, W., Albersheim, D.: The structure of plant cell walls. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components, *Plant Physiology* 1973, Vol.51, pp. 188-196,
50) Darvill, J.E., McNeil, M., Darvill, A.G., Albersheim P.: Structure of plant cell walls. XI. Glucuronoarabinoxylan, a second hemicellulose in primary cell walls of suspension cultured sycamore cells. *Plant Physiology* 1980, Vol. 66, pp. 1135–1139
51) Spellman, M.W., McNeil, M., Darvill, A.G., Albersheim, P., Dell, A.: Structure of plant cell walls. Characterization of a structurally complex heptasaccharide isolated from the pectic polysaccharide rhamnogalacturonan II, *Carbohydrate Research,* 1983, Vol. 122, pp.131-153
52) Prade, R.A., Zhan, D., Ayoubi, P., Mort, A.J.: Pectins, pectinases and plant-microbe interactions, *Biotechnology and Genetic Engineering Review* 1999, Vol.16, pp. 361-391,
53) McCann, M.C., Roberts, K.: Architecture of the primary cell wall. *The cytoskelet basis of plant growth and form*, 1991, Vol.100, pp.109-129
54) Femenia, A., Rigby, N.M., Selvendran, R.R, Waldron, K.W.: Investigation of occurrence of pectic-xylan-xyloglucan complexes in the cell walls of cauliflower stem tissues, *Carbohydrate research* 1999, Vol.39, pp. 151-164
55) Brett, CT, Baydoun, EA-H, Abdel-Massih, R.M: Pectin-xyloglucan linkages in type I primary cell walls of plants. *Plant Biosystems*, 2005, Vol. 139, pp. 54-59
56) Kerr, E.M., Fry, S.C.: Pre-formed xyloglucans and xylans increase in molecular weight in three distinct compartments of a maize cell-suspension culture. *Planta,* 2003, Vol. 217, pp. 327-339
57) Popper, Z.A., Fry, S.C.: Widespread occurrence of a covalent linkage between xyloglucan and acidic polysaccharides in suspension-cultured angiosperm cells. *Annals of Botany*, 2005, Vol. 96, pp. 91-99
58) Popper, Z.A., Fry, S.C.: Xyloglucan-pectin linkages are formed intra-protoplasmically, contributes to wall-assembly, and remains stable in the cell wall. *Planta* 2008, Vol. 227, pp. 781-794
59) Ait-Mohand, F., Farkaš, V.: Screening for hetero-transglycosylating activities in extracts from nasturtium (*Tropaeolum majus*). *Carbohydrate Research,* 2006, Vol. 341, pp. 577-581
60) Fry, S.C.: The growing plant cell wall: Chemical and metabolic analysis, *The Blackburn press*, New Jersey, 2001, ISBN-13: 978-1-930665-08-8
61) Wallner, S.J., Walker, J.E.: Glycosidases in cell-wall degrading extracts of tomato furits, *Plant Physiology*, 1975, Vol. 55, pp. 94-98
62) Webb, E.C.: Enzyme Nomenclature, recommendations of the nomenclature committee of the international union of biochemistry and molecular biology on the nomenclature and classification of enzymes. *Academic press*., 1992, San Diego
63) Gross, K.C., Sams, C.E.: Changes on cell-wall neutral sugar composition during fruit ripening- a species survey, *Phytochemistry,* 1984, Vol. 23, pp. 2457-2461

64) Willats, W.G., McCartney, L., Mackie, W., Knox, J.P.: Pectin: cell biology and prospects for functional analysis, *Plant Molecular Biology*, 2001, Vol.47, pp. 9-27
65) Simpson, S.D., Ashford, D.A., Harvey, D.J., Bowles, .J.: Short chain oligogalacturonides induce ethylene production and expression of the gene encoding aminocyclopropane-1-carboxylic acid oxidase in tomato plants, *Glycobiology*, 1998, Vol. 8, No.6, pp. 579-583
66) Dumville, Jo. C., Fry, S.C.: Uronic acid-containing oligosaccharins: Their biosynthesis, degradation and signalling roles in non-diseases plant tissues, *Plant Physiology and Biochemistry*, 2000, Vol. 38, pp. 125-140
67) Minic, Z., Rihouey, C.,. Do, C.T, Lerouge, P, Jouanin, L.: Purification and characterization of enzymes exhibiting beta-D-xylosidase activities in stem tissues of *Arabidopsis*, *Plant Physiology,* 2004, Vol. 135**,** pp. 867–878
68) Li, S.C., Han, J.W., Chen, K.C., Chen, C.S.: Purification and characterization of isoforms of β-galactosidases in mung bean seedlings, *Phytochemistry,* 2001, Vol. 57, pp. 349–359
69) Esteban, R., Dopico, B., Munoz, F.J., Romo, S., Martin I., Labrador, E.: Cloning of a *Cicer arietinum* beta-galactosidase with pectin-degrading function, *Plant Cell Physiology,* 2003, Vol. 44, pp. 718–725
70) Rose, J.K., Braan, J., Fry, S.C., Nishitani, K.: The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: current perspectives and a new unifying nomenclature, *Plant Cell Physiology,* 2002, Vol. 43, pp. 1421–1435
71) Edwards, M., Bowman, Y.J., Dea, I.C., Reid, J.S.: A beta-D-galactosidase from nasturtium (*Tropaeolum majus* L.) cotyledons. Purification, properties, and demonstration that xyloglucan is the natural substrate, *Journal of Biological. Chemistry,* 1988, Vol. 263, pp. 4333–4337
72) Sampedro, J., Sieiro, C., Revilla, G., Gonzalez-Villa, T., Zarra, I.: Cloning and expression pattern of a gene encoding an alpha-xylosidase active against xyloglucan oligosaccharides from *Arabidopsis*, *Plant Physiology,* 2001, Vol. 126, pp. 910–920
73) Suzuki, M., Kato, A., Nagata, N., Komeda, Y.: A xylanase, AtXyn1, is predominantly expressed in vascular bundles, and four putative xylanase genes were identified in the *Arabidopsis* genome, *Plant Cell Physiology,* 2002, Vol. 43, pp. 759–767
74) Gamborg, O.L., Shyluk, J.P.: Nutrition, media and characteristics of plant cell and tissue cultures. *Plant Tissue Culture Methods and Applications in Agriculture*, Academic Press, 1981, New York, pp. 21-44
75) Van der Heijden, R., Verpoorte, R., den Hoopen, H.J.G.: Cell and tissue cultures of *Catharanthus roseus*. *Plant cell, tissue and organ culture,* 1989, Vol. 18, pp. 231-280,
76) Heyser, J.W., Dykes, T.A., DeMott, K.J., Nabors, M.W.: High frequency, long term regeneration of rice from callus cultures. *Plant Science letters*, 1983, Vol. 29, pp. 175-182
77) Novák, F.J.: *Explanátové kultury a jejich využití ve šlechtění rostlin*. Academia, 1990, Praha
78) Lindsey, K.: *Plant Tissue Culture Manual*, Kluwer Academic Publisher, 1991, ISBN 0-7923-1115-9, pp. 1-24

79) Murashige, T., Skoog, F.: A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Plant Physiology,* 1962, Vol. 15, pp. 473
80) Elias, B.A., Givan, C.V.: Density gradient and differential centrifugation methods for chloroplast purification and enzyme localization in leaf tissue. *Planta*, 1978, Vol.142, No.3, pp. 317-329
81) Dische, Z.: Quantitative and qualitative colorimetric determination of heptoses. *Journal of Biological Chemistry,* 1953, Vol. 204, pp. 983-987
82) Dische, Z.: Color reactions of carbohydrates. In *Methods in Carbohydrate Chemistry*, 1962, Vol. 1. Academic Press, New York, 478-512
83) Blumenkratz, N., Asboe-Hansen, G.: New method for quantitative determination of uronic acids, *Analytical Biochemistry*, 1973, Vol. 54, pp. 484-489
84) Cruikshank, R.H., Wade, G.C.: Detection of pectic enzymes in pectin-acrylamide gels. *Analytical Biochemistry*, 1980, Vol. 107, pp. 177-181
85) Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature,* 1970, Vo.227, pp. 680-685
86) Wray, W., Boulikas, T., Wray, V.P., Hancock, R.: Silver staining of protein in polyacrylamide gels. *Analytical Biochemistry*, 1981, Vol. 118, pp. 197-203
87) Merril, C.R.: Gel-staining techniques. In Deutscher MP (Ed) Guide to protein purification. *Methods in Enzymology*, 1990, Vol. 182. Academic press Inc.
88) Kussmann, M., Nordhoff, E., Rahbek-Nielsen, H., Haebel, S., Rossel-Larsen, M., Jakobsen, L., Gobom, J., Mirgorodskaya, E., Kroll-Kristnsen, A., Palm, L., Roepstorff, P.: Matrix-assisted laser desorption/ionization mass spectrometry sample preparation techniques designed for various peptide and protein analytes. *Journal of Mass Spectrometry*, 1997, Vol. 32 (6), pp. 593-601
89) Edman, P.: Method for determination of the amino acid sequence in peptides. *Acta Chemica Scandinavica,* 1950, Vol. 4, pp. 283-93
90) Jensen, O.N., Wilm, M., Shevchenko, A., Mann, M.: Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. *Methods in Molecular Biology: 2-D Proteome Analysis Protocols*, 1999, chapter 52, 513-530. Humana Press Inc., Totowa
91) Shevchenko, A., Wilm, M., Vorm, O., Mann, M.: Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Analytical Chemistry*, 1996, Vol. 68(5), pp. 850-858
92) Korwar, A.M., Santhakumari, B., Kulkarni, M.J.: Identification of ripening specific proteins in tomato by intact tissue MALDI-TOF-MS, *Electronic Journal of Food and Plant Chemistry*, 2008, Vol. 3 (1), pp. 10-13
93) Bradford, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 1976, Vol. 72, pp. 248-254
94) Sulová, Z., Lednická, M., Farkaš, V.: A colorimetric assay for xyloglucan-endotransglycosylase from germinating seeds. *Analytical Biochemistry*, 1995, Vol. 229, pp. 80-85
95) Kosík, O., Farkaš V.: One-pot fluorescent labelling of xyloglucan oligosaccharides with sulforhodamine. *Analytical Biochemistry*, 2008, Vol. 375, pp. 232-236

96) Kohn, R., Furda, I.: Interaction of calcium and potassium ions with carboxyl groups of pectin. *Collection Czechoslovak of Chemical Communications,* 1967, Vol. 32, pp. 4470-4484
97) Koller, A., Neukom, H.: Detection of oligogalacturonic acids by thin-layer chromatography. *Biochimica et Biophysica Acta*, 1964, Vol. 83, pp. 366-367
98) Heinrichová, K., Simon, L.M.: Preparation of oligo-(D-galactosiduronic) acids by the action of endo-D-galacturonanase. *Biologia* 1991, Vol.46, pp. 1081-1087
99) Somogyi, M.: Notes on sugar determination. *Journal of Biological Chemistry*, 1952, Vol. 195, pp. 19-23
100) Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K. , Matthews, K.J.: Xyloglucan endotransglycosylase a new wall-loosening enzyme activity from plants. *Biochemistry Journal*, 1992, Vol. 282, pp. 821-828
101) Redgwell, R.J., Fry, S.C.: Xyloglucan endotransglycosylase activity increases during kiwifruit (*Actinidia deliciosa*) ripening. *Plant Physiology,* 1993, Vol. 103, pp. 1399-1406,
102) Schubert, E.: Differentiation of the polygalacturonases of *Aspergillus niger*, *Nature* , 1952, Vol. 169, pp. 931 – 932
103) Anton, G.E., Barrett, D.M.: Kinetic parameters for the thermal inactivation of duality-related enzymes in carrots and potatoes. *Journal of Agricultural and Food Chemistry*, 2002, Vol. 50, pp. 4119-4125
104) Kačuráková, M., Capek, P., Sasinková, V., Wellner, N., Ebringerová, A.: FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. *Carbohydrate Polymers*, 2000, Vol.43, pp. 195-203
105) Černá, M., Barros, A.S., Nunes, A., Rocha, S.M., Delgadillo, I., Čopíková, J., Coimbra, M.A.: Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives. *Carbohydrate Polymers*, 2002, Vol. 51, pp. 383-389
106) Wellner, N., Kačuráková, M., Malovíková, A., Wilson, R.H., Belton, P.S.: FT-IR study of pectate and pectinate gels formed by divalent cationts. *Carbohydrate Research*, 1998, Vol. 308, pp. 123-131
107) Kačuráková, M., Wilson, R.H.: Developments in mid-infrared FT-IR spectroscopy of selected carbohydrates. *Carbohydrate Polymers*, 2001, Vol. 44, pp. 291-303
108) Coimbra, M.A., Barros, A., Barros, M., Rutledge, D.N., Delgadillo, I.: FTIR spectroscopy as a tool for analysis of olive pulp cell wall polysaccharide extracts. *Carbohydrate Polymers*, 1999, Vol. 317, pp. 145-154
109) Izydorczyk, M. S., Biliaderis, C. G.: Cereal arabinoxylans: Advances in structure and physicochemical properties, *Carbohydrate. Polymers,* 1995, Vol. 28, pp. 33-48
110) Zheng, Z., Mort, A.: Isolation and structural characterization of a novel oligosaccharide from the rhamnogalacturonan of *Gossypium hirsutum* L. *Carbohydrate Research*, 2008, Vol. 343, pp. 1041-1049
111) Coenen, G.J., Bakx, E.J., Verhoef, R.P., Schols, H.A., Voragen, A.G.J.: Identification of the connecting linkage between homo-or xylogalacturonan and rhamnogalacturonan type I. *Carbohydrate Polymers*, 2007, Vol. 70, pp. 224-235
112) Rexová-Benková, L., Markovič, O.: Pectic enzymes. *Advances in Carbohydrate Chemistry and Biochemist*ry, 1976, Vol. 33, pp. 323-385

113) Heinrichová, K.: Isolation, characterization and mode of action of exo-d-galacturonase from carrot. *Collection of Czechoslovak Chemical Communications*, 1977, Vol. 42, pp. 3214-3221
114) Stratilová, E., Dzúrová, M., Mislovicová, D.: Multiple forms of carrot exopolygalacturonase. In: Pectin and Pectinases, *Progress in Biotechnology,* 1996, Vol. 14 (Visser J. and Voragen A.G.J., eds.). Elsevier Science, Amsterdam, pp. 807-814
115) Stratilová, E., Markovič, O., Dzúrová, M., Malovíková, A., Capek, P., Omelková, J.: The pectolytic enzymes of carrots. *Biologia, Bratislava*, 1998, Vol. 53, pp. 731-738
116) Stratilová, E., Dzúrová, M., Malovíková, A., Omelková, J.: Oligogalacturonate hydrolase from carrot roots. *Zeitschrift fuer Naturforsch*, 2005, Vol. 60c, pp. 899-905
117) Flodrová, D., Dzúrová, M., Lišková, D., Ait Mohand, F., Mislovičová, D., Malovíková, A., Voburka, Z., Omelková, J., Stratilová, E.: Pectate hydrolases of parsley (Petroselinum crispum) roots. *Zeitschrift fuer Naturforsch*, 2007, Vol. 62c, pp. 382-388
118) Markovič, O., Janeček, Š.: Pectin degrading glycoside hydrolases of family 28: sequence-structural features, specificities and evolution, *Plant Engineering*, 2001, Vol. 14, No.9, pp. 615-631
119) Hadfield, K.A., Bennet, A.B.: Polygalacturonases: many genes in search and function. *Plant Physiology*, 1998 , Vol. 117, pp. 337-343
120) Torki, M., Mandaron, P., Mache, R., Falconet, D.: Characterization of a ubiquitous expressed gene family encoding polygalacturonase in *Arabidopsis thaliana*. *Gene,* 2000, Vol. 242, pp. 427-436
121) Pressey, R., Avants, J.K.: Modes of action of carrot and peach exopolygalacturonases. *Phytochemistry*, 1975, Vol. 14, pp. 957-961
122) García-Romera, Fry S.C.: The longevity of biologically active oligogalacturonides in rose cells cultures: degradation by exo-polygalacturonase. *Journal of Experimental Botany,* 1995, Vol. 46, pp. 1853-1857
123) Rayle, D.L., Clealand, R.E.: The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiology*, 1992, Vol. 99, pp. 1271-1274

7. ABREVIATIONS

AA	Acryl Amide
AceA	Aceric Acid
A-F-F-A	Ferulic Acid Esters
Api	Carbohydrate-Active Enzymes (database)
APS	Ammonium Persulphate
Ara	Arabinose
AX	Arabinoxylan
CCRC	Complex Carbohydrate Research Centre
CEOs-SR	cellulose oligosaccharides labelled with sulforodamine
CESA	Cellulose Synthase Superfamily
CMC	Carboxymethyl cellulose
CSC	Cellulose Synthase Complex
CSL	Cellulose Synthase-Like (genes) - superfamily
CSL(A,B,H)	Cellulose Synthase-Like (genes) - subfamily
DEAE	Diethylaminoethyl Cellulose
DP	Degree of Polymerization
ER	Endoplasmic Reticulum
FAE	Ferulic Acid Esters
FPLC	Fast Protein Liquid Chromatography
FTIR	Fourier Transform Infrared Spectroscopy
Fuc	Fucose
Gal	Galactose
GalA	α-D-galacturonic acid
GH	Glycosyl Hydrolase
GPC	Gel Permeation Chromatography
GTs	Glycosyl Transferases
HG(A)	Homogalacturonan (polygalacturonic acid)
HEC	Hydroxyethyl cellulose
HPLC	High Performance Liquid Chromatography
[^{3}H]XXXG	tritium labelled xyloglucan-heptasaccharide
IEF	Isoelectric Focusing
KDO	2-keto-3-deoxy-D-*manno*-octulosonic acid
KOR	κ-opioid receptor
LAOs-SR	Laminarin oligosaccharides labelled with sulforodamine
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass Spectrometry
NDP	Nucleotide Diphosphate
NMR	Nuclear Magnetic Resonance
OGAs	Oligogalacturonides
OGH	Oligogalacturonate Hydrolase
OGH6	OGH with preference for hexagalacturonate
OGH10	OGH with preference for decagalacturonate

PGs	Polygalacturonases
PME	Pectin Methyl Esterase
RG I, II	Rhamnogalacturonan I, II
Rha	Rhamnose
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
SR	Sulforodamine
TEMED	Tetramethylethylendiamin
TLC	Thin Layer Chromatography
UDP	Uridine Diphosphate
XG	Xylogalacturonan
XET	Xyloglucan endotransglycosylase
XGOs-SR	Xyloglucan oligosaccharides labelled with sulforodamine