FINAL TECHNICAL REPORT FOR DE-FG02-96ER20215
The Structure of Pectin
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SUMMARY

At the beginning of this project we hypothesized that pectin, which is a major polysaccharide in primary plant cell walls, is composed of various distinct structural regions covalently linked together into a high molecular weight complex polymer. We also hypothesized that a considerable portion of xyloglucan, the major hemicellulose in most primary cell walls, is linked to the pectin.
Our goal was to determine if these interconnections exist and to characterize the exact nature of the interactions. It seems imperative that we have a complete knowledge of the structure of pectin to be able to propose realistic models of cell walls. There is a lot of interest in the biosynthesis of pectin. I do not think it will be possible to completely understand the biosynthesis of pectin without knowing the structure of pectin and thus the sequence of reactions needed to put each sugar or ester in its correct position in the polymer.
We made considerable progress in determining the detailed structure of pectin and within a year or so will be able to put forward a comprehensive model of it.

REPORT

At the start of the project we succeeded in characterizing the substrate specificity of a cloned endopolygalacturonase. This was necessary so that we could predict the nature of the fragments of pectin we produced using it.

Chen, E. M. W. and Mort, A. J. (1996) Nature of Sites Hydrolyzable by Endopolygalacturonase in Partially Esterified Homogalacturonans. *Carbohydr. Polymers* **29**, 129-136.

Thanks to support from a previous DOE grant we learned a lot about capillary electrophoresis of oligosaccharides and the use of fluorescent labeled oligosaccharides to study the mode of action of glycosyl hydrolases.

Mort, A. J. and Chen, E. M. W (1996) Separation of ANTS-Labeled Oligomers Containing Uronic Acids by Capillary Electrophoresis: Application to Determining the Substrate Specificity of Endopolygalacturonases. *Electrophoresis* **17,** 379-383.

Zhang, Z., Pierce, M. L. and Mort, A. J. (1996) Detection and Differentiation of Pectic Enzyme Activity *in vitro* and *in vivo* by Capillary Electrophoresis of Products from Fluorescent Labeled Substrate. *Electrophoresis* **17,** 372-378.

We gained more experience using high field 2D NMR spectroscopy to determine exact structures of oligosaccharides. We also provided more evidence that liquid HF can be used to cleave certain glycosidic selectively at low temperatures.

Nimtz, M., Mort, A. J., Domke, T., Wray, V., Zhang, Y., Qiu, F., Coplin, D. and Geider, K. (1996) Structure of Amylovoran, the Capsular Exopolysaccharide from the Fireblight Pathogen *Erwinia amylovora.* *Carbohydr. Res.* **287,** 59-76.

Nimtz, M., Mort, A. J., Domke, T., Wray, V., Zhang, Y., Qiu, F., Coplin, D. and Geider, K. (1996) Structure of Stewartan, the Capsular Exopolysaccharide from the Corn Pathogen *Erwinia stewartii.* *Carbohydr. Res*. **288,** 189-201.

Our continued efforts to prove the nature of the linkage between xyloglucan and pectin were thwarted by the presence of contaminating activities in commercial enzymes. We found that the linkage between xyloglucan and pectin could be broken by a commercial endoarabinanase. However, after a discussion with Gordon MacLachlan, we did his control experiment of seeing if the enzyme we used, "endoarabinanase" attacked xyloglucan. It did. Thus we could no longer assert that the linkage was through an arabinan. From then on we have tried to use cloned monospecific enzymes for all our work.

Fu, J., An, J., Thomas, J. and Mort, A. J. Half of the xyloglucan of cotton suspension culture cell walls is crosslinked to pectin. Not yet completed.

It was commonly accepted that the homogalacturonan region of pectin is interrupted about every 25 galacturonic acid residues with a rhamnose residue which would introduce a kink in the structure. If this were the case a complete digestion of saponified pectin with endoploygalacturonase (EPG) should yield significant quantities of glacturonic acid oligomers containing one rhamnose residue. Knowing the substrate specificities of our cloned EPG let us predict the exact nature of these oligomers. We found no such oligomers. Along with other evidence we could then assert that all of the rhamnose in pectin is in RGIi and RG II. The kinks do not exist.

Zhan, D., Janssen, P. and Mort. A. J. (1998) Scarcity of Complete Lack of Single Rhamnose Residues Interspersed Within the Homogalacturonan Regions of Citrus Pectin. *Carbo. Res.* **308**, 373-380.

We continued to develop methodology for CZE of oligosaccharides.

Mort, A.J., Zhan, D., Rodriguez, V. (1998) Use of Scavenger Beads to Remove Excess Labeling Reagents from CZE Samples. *Electrophoresis* **19**, 2129-2132.

Merz, J.M. and Mort, A.J. (1998) A Computer Controlled Variable Attenuator for Protection and Autoranging of a Laser Induced Fluorescence Detector for CZE. *Electrophoresis* **19**, 2239-2242.

We helped understand the role of pectin in plant pathogenesis.

Moerschbacher, B.M., Mierau, M., Graessner, B., Noll, U. and Mort, A.J. (1999) Small Oligomers of Galacturonic Acid are Endogenuous Suppressors of Disease Resistance Reactions in Wheat Leaves. *Journal Exp. Bot.* **50,** 605-612.

Mundodi, S. R., Anderson, J. A., Maness, N. O., Smith, M. W., Martin, B., Pierce, M. L. and Mort, A. J. (1998) Changes in Methanol Evolution and Pectin Methylesterification in Resistant and Susceptible Pepper Leaves Inoculated with *Xanthomonas campestris* pv. Vesicatoria. *J. Am. Soc. Hortic. Sci.* **123(6),** 980-986**.**

We contributed to a review on pectins and pectinases.

Prade, R., Zhan, D., Ayoubi, P. and Mort. A. J. (1999) Pectins, Pectinases and Plant-Microbe Interactions. *Biotechnol. Genet. Eng. Rev*. **16,** 361-391.

An excellent graduate student, along with my close colleague Rolf Prade from our microbiology department, cloned rhamnogalacturonase into the *pichia* yeast expression system so that would could very selectively degrade RGI.

Fu, J., Prade, R. and Mort, A. (2001) Expression and Action Pattern of *Botryotinia fuckeliana (Botrytis cinerea*) Rhamnogalacturonan Hydrolase in *Pichia pastoris*. *Carbohydr. Res.* **330**, 73-81.

I helped Ruth Stark's group by making oligomers from fruit cuticle using HF.

Fang, X., Feng, Q., Yan, B., Wang, H., Mort, A. J. and Stark, R. E. (2001) NMR Studies of Molecular Structure in Fruit Cuticle Polyesters. *Phytochemistry* **57,** 1035-1042.

We found that alkali can induce epimerization of galacturonic acid.

Zhan, D., Qiu, F. and Mort. A. J. (2001) L-Altruronic Acid Formed by Epimerization of D-galacturonic Acid Methyl Esters During Saponification of Citrus Pectin. *Carbohydr. Res.* **330,** 357-363.

I deglycosylated the arabinogalactan-peptide from wheat flour using HF.

Van den Bulck, K., Loosveld, A-M. A., Courtin, C. M., Proost, P., Van Damme, J., Robben, J., Mort, A., and Delcour, J. A. (2002) Amino Acid Sequence of Wheat Flour Arabinogalactan-Peptide, Identical to Part of Grain Softness Protein GSP-1, Leads to Improved Structural Model. *Cereal Chem.* **79**(3), 329-331.

Our expertise with pectin methyl esterification helped uncover differences between resistant and susceptible lines of wheat.

Wiethölter, N., Graeβner, Mierau, M., Mort, A. J. and Moerschbacher, B. M. (2003) Differences in the Methyl Ester Distribution of Pectins from Near-Isogenic Wheat Lines Resistant and Susceptible to the Wheat Stem Rust Fungus. *Molec. Plant-Microbe Int.* **16,** 945-952.

Our work on capillary zone electrophoresis allowed us to help determine the substrate specificity of two thermostable rhamnosidases.

Birgisson, Hakon; Hreggvidsson, Gudmundur O.; Fridjonsson, Olafur H.; Mort, Andrew; Kristjansson, Jakob K.; Mattiasson, Bo (2004) Two new thermostable a-L-rhamnosidases from a novel thermophilic bacterium. *Enzyme and Microbial Technology* **34**, 561-571.

A xylogalacturonan fragment we made during study of xylogalacturonan helped characterize a cell wall epitope.

William GT Willats, Lesley McCartney, Clare G Steele-King, Susan E Marcus, Andrew J Mort, Miranda Huisman, Gert-Jan van Alebeek, Henk A Schols, Alphons GJ Voragen, Angélique Le Goff, Estelle Bonnin, Jean-François Thibault and J Paul Knox. (2004) A xylogalacturonan epitope is specifically associated with plant cell detachment. *Planta* **218**, 673-681.

Our desire for pure enzymes for degradation of cell wall polysaccharides encouraged us to seek outside help in cloning them. First we collaborated with Rolf Prade and the with Chris Somerville. The DOE funded a separate grant for us to clone and characterize a wide range of enzymes from *Aspergillus nidulans.*

MORT , A.J., S. GAO, X. WU, and R. PRADE, *The need for pure enzymes that degrade cell wall polysaccharides and how to get them.* Plant Biosystems, 2005. **139**(1): p. 84-87.

Bauer, S., P. Vasu,, S. Persson,, A J. Mort, and Chris R. Somerville, (2006) Development and application of a suite of polysaccharide degrading enzymes for analyzing plant cell walls. Proc. Nat. Acad. Sci., **103**(30): 11417-11422.

We discovered that intercellular spaces of expanding cotton cotyledons, and by inference expanding leaves, exhibit rhamnogalacturonan lyase activity while they are actively expanding. This suggests that modification of the rhamnogalacturonan regions of pectin take place during cell expansion. The presence of seven genes for putative rhamnogalacturonan lyases in the Arabidopsis genome points to the importance of these enzymes.

Naran, R, ML Pierce, and AJ Mort, (2007) Detection and identification of rhamnogalacturonan-lyase activity in intercellular spaces of expanding cotton cotyledons. *The Plant Journal* **50:** 95-107

We found dramatic decreases in the levels of endopolygalacturonase in the intercellular spaces of cotton cotyledons during their expansion. We also found corresponding increases in the molecular weight of the homogalacturonan fractions during decreases in expansion rate.

Zhang, Z, ML Pierce, and AJ Mort. (2007) Changes in homogalacturonans and enzymes degrading them during cotton cotyledon expansion. *Phytochemistry* **68:** 1094-1103

We helped Rith Stark's group characterize lime fruit cuticle by generating oligomers from it with HF.

Isolation and identification of oligomers from partial degradation of lime fruit cutin. (2008) Shiying Tian, Xiuhua Fang, Xiaofang Cheng, Feng Qiu, Andrew J. Mort, Weimin Wang, Hsin Wang, and Ruth E. Stark. :Journal of Agricultural and Food Chemistry **56:** 10318-10325

Dr. Zheng isolated and characterized an oligosaccharide that gave the first indication that the well known 5-linked arabinanin RG I is linked to a O-3 of a single galactose residue on 0-4 of the rhamnose residues in rhamnogalacturonan.

Yun Zheng, Andrew Mort (2008) Isolation and Structural Characterization of a Novel Oligosaccharide from the Rhamnogalacturonan of *Gossypium hirsutum* L. Carbohydrate Research **343:** 1041-1049

We isolated a variety of xylogalacturonan fragments from watermelon fruit pectin and completely characterized one of them by NMR and MS. The structures of the oligomers told us how endopolygalacturonase activity is affected by the xylose residues in xylogalcturonan. Since most xylogalacturonan has, on average one Xyl per two GalA it should be completely resistant to normal plant endopolygalacturonases! No, homologues to the fungal xylogalacturonases have been found in Arabidopsis.

Andrew Mort , Yun Zheng, Feng Qiu, Manfred Nimtz, Gianna Bell-Eunice. (2008) Structure of xylogalacturonan fragments from watermelon cell wall pectin. Implications for the action pattern of endopolygalacturonase on xylogalacturonan. Carbohydrate Research **343:** 1212-1221

We helped Dr. Ken Gross's group characterize substrate specificities of tomato galactosidases and found that one of them can remove the single galactose linked to O-4 of rhamnose in RG.

Ishimaru M, Smith DL, Mort AJ, Gross KC (2009) Enzymatic activity and substrate specificity of recombinant tomato beta -galactosidases 4 and 5. Planta **229:** 447-456

We contributed to finding substrate specificities of multiple glycanases

Squina FM, Mort AJ, Decker SR, Prade RA (2009) Xylan decomposition by Aspergillus clavatus endo-xylanase. Protein Expression & Purification **68:** 65-71

Liu Z, Bhattacharyya S, Ning B, Midoro-Horiuti T, Czerwinski EW, Goldblum RM, Mort A, Kearney CM. (2010) Plant-Expressed Recombinant Mountain Cedar Allergen Jun a 1 Is Allergenic and Has Limited Pectate Lyase Activity. *Int Arch Allergy Immunol;153:347-358.*

Squina, Fabio M.; Santos, Camila R.; Ribeiro, Daniela A.; Cota, Junio; de Oliveira, Renata R.; Ruller, Roberto; Mort, Andrew; Murakami, Mario T.; Prade, Rolf A. Substrate cleavage pattern, biophysical characterization and low-resolution structure of a novel hyperthermostable arabinanase from Thermotoga petrophila. Biochemical and Biophysical Research Communications (2010), 399(4), 505-511.

Wang, H., Squina, F., Segato, F., Mort, A., Lee, D., Pappan, K., & Prade, R. (2011). High- temperature enzymatic breakdown of cellulose. *Appl. Environ. Microbiol., 77*, 5199-5206.

Santos CR, Squina FM, Navarro AM, Oldiges DP, Leme AFP, Ruller R, Mort AJ, Prade R, Murakami MT (2011) Functional and biophysical characterization of a hyperthermostable GH51 α-L-arabinofuranosidase from Thermotoga petrophila. *Biotechnol Lett*, **33**, 131-137.

Anamika Ray, Sayali Saykhedkar, Patricia Ayoubi, Steven D. Hartson, Rolf Prade and Andrew J. Mort , (2012) *Phanerochaete chrysosporium* produces a diverse array of extracellular enzymes when grown on sorghum. *Appl.Microbiol.and Biotechnol.* **93**,2075-2089.

Saykhedkar S, Ray A, Ayoubi-Canaan P, Hartson SD, Prade R, Mort AJ. (2012) A time course analysis of the extracellular proteome of *Aspergillus nidulans* growing on sorghum stover. *Biotechnol Biofuels*, **5**, 52.

Segato F, Damasio ARL, Goncalves TA, Murakami MT, Squina FM, Polizeli MdLTM, Mort AJ, Prade RA. (2012) Two structurally discrete GH7-cellobiohydrolases compete for the same cellulosic substrate fiber. *Biotechnol Biofuels*, **5**, 21.

To determine the exact chemical shifts of methyl esterified GalA residues and the residues adjacent to them we isolated a singly methyl esterified GalA oligomer and subjected it to a complete NMR analysis. We corrected previously published literayture assignments and at the same time characterized further the mode of ction of endopolygalacturonases around esterified residues.

Andrew Mort, Gianna Bell-Eunice, and Xiangmei Wu (2013) Characterization of a Methyl-Esterifed Tetragalacturonide Fragment Isolated from a Commercial Pectin with a Medium Degree of Methyl-esterification. Carbohydr. Res. **380,** 108-111.

We helped Dr. Ken Gross's group characterize substrate specificities of another tomato galactosidase.

Eda M, Ishimaru M, Tada T, Sakamoto T, Kotake T, Tsumuraya Y, Mort AJ, Gross KC. (2014) Enzymatic activity and substrate specificity of the recombinant tomato β-galactosidase 1. *J Plant Physiol*, **171**, 1454-1460.

In our quest to detrmine which, and how, regions of pectins are linked together we isolated an oligosaccharide which assert represents thelink between homogalacturonan and rhamnogalacturomnan. Reviewers of the manuscript raised an alternative origin for the oligomers, so we did not get this resut published in a well reckognized journal. We have now generated an oligosaccharide by a combination of HF solvolysis and pectate lyase digestion which unquestionably shows a linear likage between HG and RG. A manuscript describing this oligomer is in preparation.

Xiangmei Wu, Andrew Mort (2014) Structure of a Rhamnogalacturonan fragment from Apple Pectin: Implications for Pectin Architecture. International Journal of Carbohydrate Chemistry. 2014, 347381/347381-347381/347387

To find out how the 1-4 linked galactan is linked to pectin we isolated an oligomer of RG with a disaccharide remnant of the galactan sidechain attached. Characterization of this oligomer by 2 D NMR and mass spectrometry shows exactly how the galactan chains are linked.

Xiangmei Wu, Andrew Mort, Isolation and characterization of a rhamnogalacturonan oligomer with galactan side chains isolated from apple pectin. Submitted to Carbohydrate Research.

We helped characterize hydroxyproline rich cell wall glycoproteis in Arabidopsis roots.

Chen Y, Ye D, Held MA, Cannon MC, Ray T, Saha P, Frye AN, Mort AJ, Kieliszewski MJ: Identification of the Abundant Hydroxyproline-Rich Glycoproteins in the Root Walls of Wild-Type Arabidopsis, an ext3 Mutant Line, and Its Phenotypic Revertant *Plants* 2015, 4:85-111

Articles in books made possible by DOE funding

Mierau, M., Graessner, B., Mort, A. J. and Moerschbacher, B. M. (1996) Pectins and Pectinases in Stem Rust Infected Wheat. In *Pectins and Pectinases,* Visser, J., ed., Elsevier, Amsterdam, pp 687-692.

Yu, L. and Mort, A. J. (1996) Partial Characterization of Xylogalacturonans from Cell Walls of Ripe Watermelon Fruit: Inhibition of Endopolygalacturonase Activity by Xylosylation. In *Pectins and Pectinases,* Visser, J., and Voragen, A.G.J., eds., Elsevier Science B.V., Amsterdam, pp. 79-88.

Prade, R. A., Zhan, D., Ayoubi, P. and Mort, A. J. (1999) Pectins, Pectinases and Plant-Microbe Interactions. In *Biotechnology and Genetic Engineering Reviews,* Harding, S. E., ed., Intercept Limited, Andover, pp 361-391.

Mort. A. J. (2002) Interactions Between Pectins and Other Polymers. In *Pectins and their Manipulation*, Seymour, G. B. and Knox, J. P. eds., Blackwell Publishing, CRC Press, Boca Raton, FL, pp. 30-51.

Mort, A. J. and Pierce, M. L. (2002) Preparation of Carbohydrates for Analysis by Modern Chromatography and Electrophoresis. In *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, El Rassi, Z. ed., Elsevier, Amsterdam, pp. 3-38**.**

Mort, AJ. (2006) Chemistry in the Determination of Cell Wall Structure. In *The Science and Lore of the Plant Cell Wall Biosynthesis, Structure and Function* (Hayashi, T., ed.): Brown Walker Press. Pgs. 48-54.

With a few more cleaning up experiments we will be able to submit manuscripts describing the linkage between RG and xylogalacturonan, between arabinan and RG, and between xyloglucan and RG. Experiments are well underway to finding the molecular weights of the various regions of pectins. With this information in hand we will be able to put forward a comprehensive model of pectin.

Over these last twenty years five post docs supported by DOE have gained experience with carbohydrate chemistry. Two of them went on to be valued members of the Complex Carbohydrate Research Center at the University of Georgia.
Nine graduate students were supported in whole or in part. All have gone on to become valued members of the scientific community. One is continuing his graduate career at the CCRC.

Last, but not least, in large part because of my long-standing support from the DOE, I was awarded the Stevens Endowed Chair in Agricultural Biotechnology. This is a very well endowed chair, so I will be able to complete the work supported for so long by the DOE.

Thank you.