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Identification of Extensin Protein Associated with Sugar Beet Pectin

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Several studies have suggested that the emulsification properties associated with pectin obtained from sugar beet (*Beta vulgaris*) are due to the presence of a protein−pectin complex. Nevertheless, the identity of the protein has remained elusive. Pectin, extracted from sugar beet pulp by microwave-assisted extraction, and a commercial sample were both subjected to protease digestion with trypsin. The resulting peptides were separated from the pectin solution by ultrafiltration using a 3 kDa molecular weight cutoff (MWCO) membrane and analyzed using matrix-assisted laser desorption ionization with tandem time-of-flight mass spectrometry. The partial sequences derived from the mass spectrometry analyses of the resulting tryptic peptides are found to be highly consistent with extensin protein matched from the *B. vulgaris* Genetic Index database and also correspond to previously reported extensin peptides found in sugar beet cell suspension cultures. Further attempts were made to disassociate the protein from pectin using 1 M NaCl and a 100 kDa MWCO membrane; however, no peptides were observed following trypsin digestion of the permeate solution. This evidence suggests the existence of a complex between the pectin and extensin that is not due to ionic interactions. Trypsin digestion of commercial sugar beet pectin also produced the peptide profile observed with the microwave-assisted extracted pectin sample. Atomic force microscopy established that the number of rod-like elements decreased following protease treatment compared to the untreated sample.

KEYWORDS: Atomic force microscopy; *Beta vulgaris*; extensin; MALDI-TOF; mass spectrometry; pectin; sugar beet

INTRODUCTION

The root of the sugar beet plant (*Beta vulgaris*) has a high content of sucrose and is largely used in the United States, Europe, and other countries for the commercial production of sucrose. During the sugar extraction process a waste pulp residue with low commercial value is generated in large excess. It is estimated that in the U.S. industry alone about 2 million tons of dry sugar beet pulp is produced every year (*1*). The pulp is utilized for animal feed, but the energy consumed in the drying process of the pulp is making this alternative economically unfavorable. Consequently, other value-added commercial applications of the pulp are needed for the further utilization of the residue. Sugar beet pulp consists of polysaccharides, sugars, lignin, and proteins. Pectin is one of the extractable byproducts from sugar beet pulp with commercial interest, representing about 20% of the total waste product (*2*), and it has important commercial applications in the food industry as an emulsifier, thickener, stabilizer, and other potential nonfood industrial applications (*3*, *4*).

Structurally, pectins are a complex family of heteropolysaccharides composed of linear homogalacturonan chains [α-(1−4)-D-GalA] and rhamnogalacturonan regions containing the repeating disaccharide [-4)-α-D-GalA-(1−2)-α-L-Rha-(1-]. The rhamnogalacturonan regions are branched with neutral sugar (galactose and arabinose) side chains attached to the rhamnose residues, which increases the complexity of the structure. In the homogalacturonan region, the galacturonic acid is extensively methyl-esterified at the C6-carboxyl group and possesses variable degrees of acetylation at C2 or C3 (*1*).

Sugar beet pectin has poor gelling properties compared to other pectins from different sources, which may be related to the degree of acetylation of the homogalacturonan chain (*5*), the larger number of neutral side chains, and the presence of phenolic esters, mainly ferulic acid attached to galactose and arabinose (*2*, *6*). On the other hand, sugar beet pectin is a much better emulsifier compared to pectins from other sources (*6*, *7*), lending it to additional distinctive industrial applications. This enhanced emulsification property seems to be related to the hydrophobic structure given by the level of acetylation, the presence of esterified ferulic acid, and the high level of protein that is associated with sugar beet pectin (*5*, *7*). The amount of protein

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in sugar beet pectins fluctuates between 2 and 10%, depending mostly on the extraction method used (*2*, *8*, *9*).

The proteinaceous material associated with pectins has consistently shown an amino acid profile with a high content of hydroxyproline (*2*, *10*−*12*). A hydroxyproline-rich glycoprotein extensin was reported to be cross-linked with pectin in cotton (*11*). Extensin is a structural cell wall glycoprotein characterized by serine-(hydroxyproline)$_4$ sequences in which serine is substituted with single galactose residues and hydroxyproline is substituted with 2- and 3-linked arabinan chains (*13*). Oosterveld et al. (*12*) reported an arabinogalactan-protein (AGP) in pectin extracted from hops on the basis of the positive reaction test with the Yariv reagent. The AGP consisted of highly branched 3- and 3,6-linked galactan chains, decorated with single arabinose units, and a protein moiety, which was rich in cysteine, threonine, serine, alanine, and hydroxyproline. McKenna et al. (*14*) reported AGP in sugar beet pectin based on a 28% reduction of pectin's molecular weight following protease treatment. An atomic force microscopy (AFM) study found physical structural evidence suggesting the formation of pectin−protein complexes in sugar beet (*15*).

In this work, to investigate the protein associated with pectin, a proteomics approach was utilized. This approach involves the digestion of unknown proteins with trypsin. The resulting peptides were then analyzed by high-resolution mass spectrometry, which provides information on the peptide mass fingerprint (PMF) of the protein, and by doing tandem mass spectrometry (MS/MS), the amino acids intrinsically associated with the sequence of the selected peptides were analyzed. This set of information, PMF and MS/MS of the protein, was compared to public database libraries derived from cDNA nucleotide sequences of *B. vulgaris* using bioinformatics approaches. Although the databases are not complete for many plants, and only a portion of the sugar beet genome is known, this approach can reveal information about either homologous proteins or partial peptide sequences, if the specific protein is not fully included in the database. This is a powerful tool for the identification of proteins and has not been used previously for the analysis of protein associated with sugar beet pectin. Here, we report the direct identification of extensin associated with sugar beet pectin using this methodology.

MATERIALS AND METHODS

Sugar Beet Pectin. Pectin was obtained from partially dewatered sugar beet pulp, a gift from the American Crystal Sugar Co. (Moorehead, MN), using the microwave-assisted extraction method previously reported by Fishman et al. (*16*). Briefly, 1 g of sugar beet pectin was dissolved in 25 mL of an HCl solution (pH 1.0) and irradiated for 3 min at 60 °C under nitrogen with a pressure of 30 psi with 1200 W microwave power at 2450 MHz. After cooling at room temperature, the preparation was filtered and precipitated with a 2:1 ratio of 95% isopropanol in water. The precipitate was separated and vacuum-dried. Prior to use, pectin was dissolved in water and ultracentrifuged, and the supernatant fluid was lyophilized.

Commercial sugar beet pectin (beta pectin) used in this study was a gift from CP Kelco ApS (Skensved, Denmark).

Trypsin Digestion and Peptide Derivatization. Pectin was dissolved at a concentration of 1 mg/mL in ammonium bicarbonate buffer (50 mM, pH 7.5) and filtered with a 0.22 μm filter; 10 μL of a trypsin solution was added (Promega, Madison, WI) at a concentration of 0.5 μg/μL. The solution was incubated overnight at 37 °C and then filtered through a 3000 MWCO membrane. The filtrate containing the peptides resulting from the digestion was extracted and cleaned using a C18 resin pipet tips (ZipTip, Millipore Corp., Billerica, MA). The extracted peptides were recovered from the ZipTip with 10 μL of a matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/mL, in acetonitrile/water (50:50, TFA 0.1%)). Control analyses were conducted with boiled denatured trypsin or no trypsin in the pectin solution. In an additional experiment, the pectin solution, containing 1 M NaCl, was ultrafiltered with a 100 kDa MWCO membrane before trypsin digestion. Both permeate and retentate solutions were also digested with trypsin and treated as above for mass spectrometry analysis.

Mass Spectrometry. Matrix-assisted laser desorption/ionization mass spectrometry with automated tandem time-of-flight fragmentation of selected ions (MALDI-TOF/TOF) of trypsin-digested pectin solutions were acquired with a 4700 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) in the positive reflectron mode. Spectra were obtained by averaging 1000 and 2500 acquired spectra in the MS and MS/MS mode, respectively. Post source decay at 1 keV acceleration voltage was used for obtaining the MS/MS spectra of selected peptides. Conversion of TOF to mass (Da) for the monoisotopic ions, $[M + H]^+$, was based on calibration of the instrument with a peptide standard calibration kit (Applied Biosystems). MS and MS/MS spectra for the sample were combined and queried against the primary sequence database using the Mascot (Matrix Science, Inc., Boston, MA) search engine through GPS Explorer Software (Applied Biosystems). Additional database searches were conducted with lone MS/MS spectra of the selected peptides.

Guanidination of the lysine C-terminal peptides was performed with a ProteoMass guanidination kit (Sigma, St. Louis, MO) according to the manufacturer's instructions.

Atomic Force Microscopy. Sample solutions at approximately 1 mg/mL were serially diluted with distilled water, and 2 μL aliquots of 1 and 10 μg/mL concentrations were applied to the surface of freshly cleaved mica glued to magnetic stainless steel disks, air-dried, and mounted on the scanner tube of a scanning probe microscope (SPM), operating in the atomic force mode (Multimodal SPM-IIIa Nanoscope controller, Veeco Corp., Santa Barbara, CA). Imaging was done with TESP tips in the height mode for 1.0 and 2.5 μm square areas with nominal set points, as described in an earlier study (*16*).

RESULTS AND DISCUSSION

Mass Spectrometry and Protein Identification. The presence of proteinaceous material linked to pectins has been demonstrated by different approaches (*10*−*15*). However, to the best of our knowledge there has been no report of extensin associated with sugar beet pectin. The sugar beet pectin studied here was extracted and characterized as reported before, with a protein content of about 8.5% (*16*). Basically, a pectin solution of 1 mg/mL in NH_4HCO_3 buffer (50 mM, pH 7.5) was filtered with 0.22 μm filters to ensure that it was a cell wall free sample. This was confirmed by phase contrast microscopy inspection of the sample. The solution was then treated with trypsin enzyme and filtered with 3 kDa MWCO filters, and the filtrate was extracted with C18 resin for MALDI-TOF/TOF mass spectrometry analysis. The spectrum of the trypsinized sample produced a number of well-resolved mass to charge ratio (m/z) ion peaks, as shown in **Figure 1A**. In contrast, pectin solutions treated with denatured enzyme or without trypsin treatment did not exhibit these ion peaks (data not shown), which indicated that the peptide ions in **Figure 1A** were generated by proteolytic cleavage of the pectin solution.

Trypsin cleaves proteins selectively after lysine (K) or arginine (R) [for simplicity hereafter we use one letter for amino acid notation following the standard one-letter code (*17*)]; to provide an identification of the C-terminal region, the tryptic peptide products were derivatized with *O*-methylisourea using a guanidination kit. This reaction specifically converts K into homoarginine (annotated herein as K*), with an increase of monoisotopic mass of the peptide by 42.022 Da. Furthermore, the reaction increases the detection and fragmentation of the peptide for MS/MS analysis (*18*). As a consequence of this derivatization, all of the peptides in the spectrum displayed the corresponding mass displacement leading to the spectrum shown in **Figure 1B**, in which all peptides ended in K*. The guanidination kit includes a control peptide that after derivatization has a protonated ion,

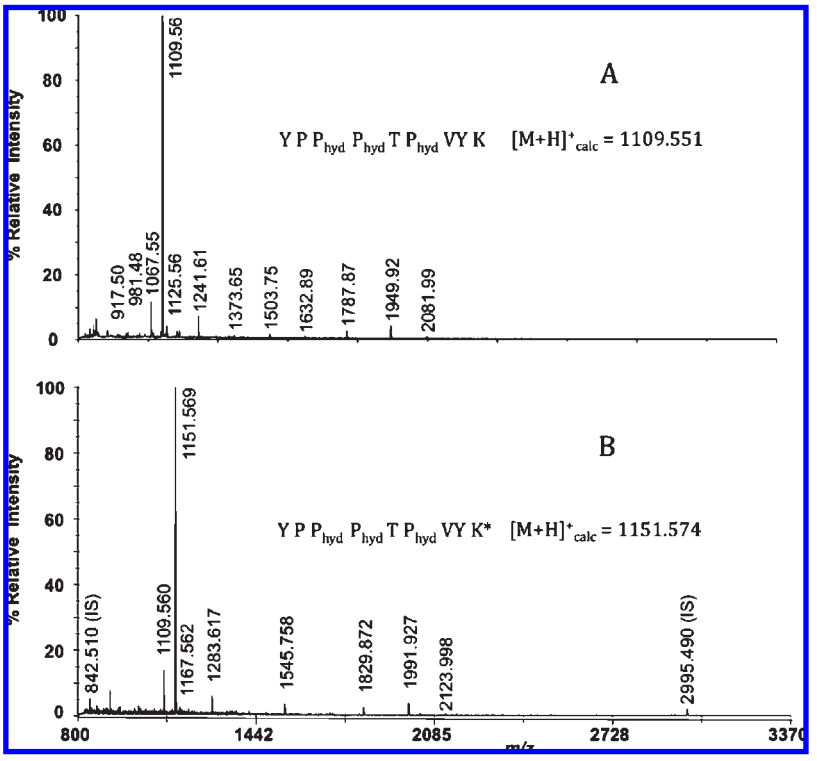

Figure 1. MALDI-TOF/TOF spectra of tryptic peptides extracted from digestion of pectin (1 mg/mL) and sequence matching the base ion peak: (**A**) underivatized peptides spectrum; (**B**) guanidated peptide spectrum (K* indicates homoarginine) with internal standard (IS) peptides from trypsin autolysis (*m*/*z* 842.510) and derivatized control peptide in the guanidation kit (*m*/*z* 2995.490).

$(M + H)^+$, at *m*/*z* of 2995.490, and because trypsin autolysis produced a small amount of a peptide with the $(M + H)^+$ ion at *m*/*z* 842.510, these two peaks were selected for internal calibration of the instrument for best mass accuracy.

The peptides shown in the spectrum of **Figure 1B** were analyzed with tandem MS/MS and the data processed for a combined search (MS and MS/MS) against the nonredundant protein database from the National Center for Biotechnology Information (NCBInr). The search resulted in a partial match to an extensin-like protein from Madagascar periwinkle (*Catharantus roseus*, accession no. BAA13175), with a protein score within the threshold of 95% confidence. The homologous protein identification was based fundamentally on the MS/MS spectrum of the peptide at *m*/*z* 1151.569. Databases from c-DNA nucleotide sequences do not contain hydroxyproline (P_{hyd}) because this amino acid is a posttranslational modification of P; consequently, the database search reported the sequence YPPPTPVYK with three possible hydroxylations of P on undefined positions for the peptide ion at *m*/*z* 1151.569. Further interpretation of the corresponding MS/MS spectrum leads to the sequence included in **Figure 2**, where the positions for the three P_{hyd} in the sequence are identified according to the corresponding *y* and *b* ions as shown. However, P_{hyd} has a residual mass of 113.048 Da, which is very close to the mass of 113.084 Da for L or I. The MS/MS data alone were not enough to distinguish between these three amino acids in the sequence, an aspect that was critical for further database searches. If one of the P_{hyd} is replaced in the sequence with L or I, the calculated $[M + H]^+$ mass for the peptide is 1151.610 Da versus the calculated $[M + H]^+$ mass of 1151.574 Da for the proposed sequence in **Figure 1B**. The experimental mass obtained for this peptide was 1151.569 Da, which was within the ~5 ppm error expected for the internally calibrated mass spectrometer (**Figure 1B**). Consequently, the peptide experimental mass confirmed the sequence YPP_{hyd} $P_{hyd}TP_{hyd}VYK$, because the difference resulting from one amino acid permutation is larger than the instrument error.

The Computational Biology and Functional Genomics Laboratory at Harvard University houses The Gene Index Project (http://compbio.dfci.harvard.edu/tgi/), and from this public access database the *B. vulgaris* Genetic Index (BvGI) database 2.0 was downloaded. Data-mining methods were employed to create a list of extensin-like protein sequences from the database, which were combined into a new data file and translated into proteins in all six reading frames using the Transeq program at EMBL-EBI using the Bacterial and Plant Plastid code table and trimming parameters (http://www.ebi.ac.uk/Tools/emboss/transeq/). The subsequent translated proteins were then aligned in MacVector version 10.0.2 and screened for the presence of extensin peptides similar to the sequence confirmed through the interpretation of the MS/MS spectrum in **Figure 2**. The BvGI sequence that matched YPPPTPVYK, was the frame 2 translation of the EST CV301320 and is shown in **Figure 3**. This protein is identified as extensin-like protein for its similarity with potato extensin protein (*Solanum tuberosum*, UniRef 100_Q06446 Cluster). The nucleotide sequence in the EST does not contain the stop codon and, consequently, is not a complete sequence. Also, the sequence starts with a G; however, the M at the sixth position seems more likely to be the beginning because the extensin sequence from the NCBInr database for Madagascar periwinkle also has the amino acids MASL at the start of the sequence.

The trypsin cleavage points of sugar beet extensin, marked with arrows on the sequence shown in **Figure 3**, predicted a number of peptides with sequence and mass that were compared against the tryptic peptides resulting from pectin digestion (**Figure 1**). From this comparison the matching peptide sequences and the position in the protein were grouped under **Table 1** (also shown underlined in **Figure 3**). The $[M + H]^+_{calcd}$ column in this table corresponds to the calculated mass of the peptide from the c-DNA sequence,

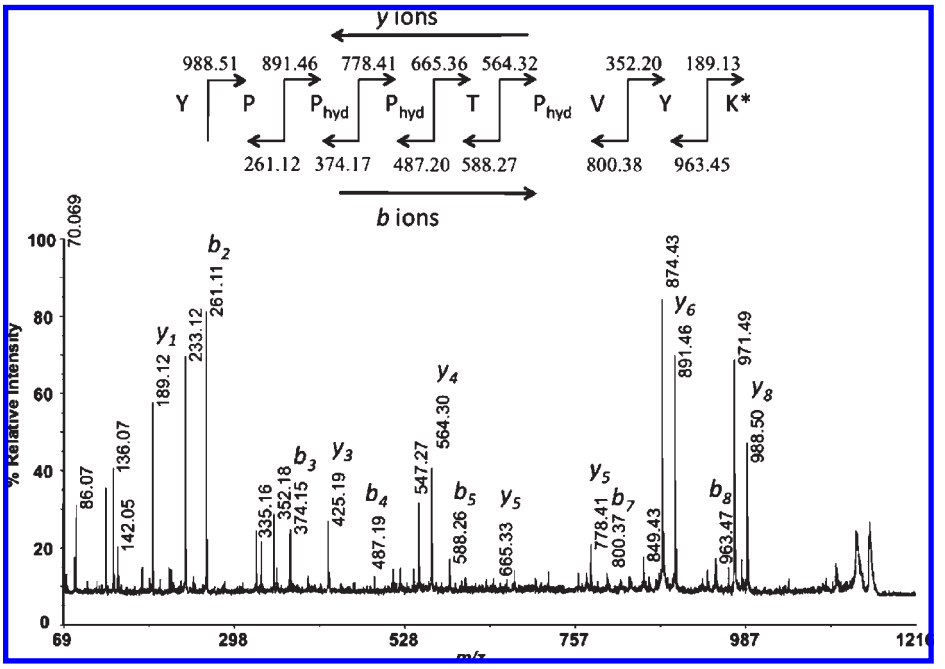

Figure 2. MS/MS spectrum of the precursor ion at *m*/*z* 1151.569 (**Figure 1B**) and the sequence with the calculated fragments showing the *b* and *y* ions, labeled on the spectrum, corresponding to the peptide extensin protein listed in **Table 1**.

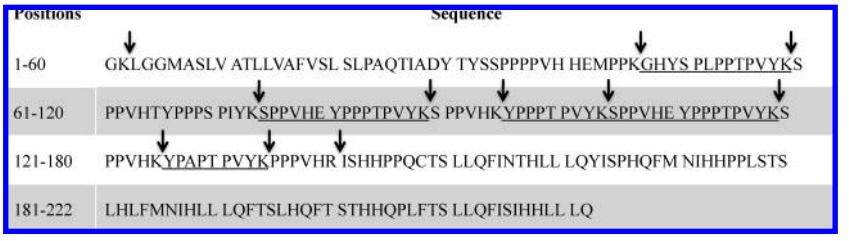

Figure 3. Extensin sequence from *Beta vulgaris* Genetic Index database. Trypsin cleavage points are marked with arrows, and matching peptides listed in **Table 1** are underlined.

Table 1. Identified Tryptic Peptides Corresponding to Extensin Protein from the BvGI Database (**Figure 3**)

sequence peptide[a]	position[b]	$[M + H]^+_{calcd}$[c]	$[M + H]^+_{und}$[d]	$[M + H]^+_{gua}$[e]	P_{hyd}[f]
GHYSPLPPTPVYK	47−59	1455.763	1503.75	1545.758	3
SPPVHEYPPPTPVYK	75−89/105−119	1707.874	1787.87	1829.872	5
YPPPTPVYK	96−104	1061.567	1109.56	1151.569	3
YPPPTPVYK (?)	96−104	1061.567	1125.56	1167.562	4
YPAPTPVYK	126−134	1035.551	1067.55	1109.560	2

[a] Tryptic peptide sequences for extensin protein from BvGI database. [b] Amino acid positions in the extensin protein sequence (see also **Figure 3**). [c] Calculated protonated peptide masses in daltons according to the c-DNA sequence. [d] Protonated peptide masses from the underivatized tryptic peptides from **Figure 1A**. [e] Protonated peptide masses from the guanidated tryptic peptides from **Figure 1B**. [f] Number of P_{hyd} in the peptides determine by the difference between the BvGI tryptic peptide sequence masses and the experimental masses determined from the spectra (**Figure 1**).

and the next two columns are the experimental masses from the underivatized peptides, $[M + H]^+_{und}$ (**Figure 1A**) and the C-terminal K guanidated peptides, $[M + H]^+_{gua}$ (**Figure 1B**). The differences between these masses and the c-DNA sequence provided the number of P_{hyd} present in the peptide and are listed in the far right column in **Table 1**.

Further confirmation for the matching sequences in **Table 1** was obtained by the MS/MS analyses of the guanidinated peptides. **Figure 4** is the MS/MS spectrum of the precursor ion at *m*/*z* of 1545.758, listed as the first entry in **Table 1**. The spectrum was an excellent ion match for the predicted protein sequence between positions 47 and 59. The fragmentation pattern allowed for the location of two of the three P_{hyd}, but there were no fragments for determining the position of the other P_{hyd}. Consequently, only one of the amino acids marked as $P^{\#}$ on the sequence in **Figure 4** corresponded to the third P_{hyd}. The peptide corresponding to the second entry can be found at two positions in the sequence, 75−89 and 105−119, with five P_{hyd}. The MS/MS spectrum of the precursor ion at *m*/*z* 1829.872 in **Figure 5** is not very informative, but it provides ions consistent with the predicted sequence, and the location of two P_{hyd} next to the S. The positions of the other three P_{hyd} were determined on the basis of the similarity with the peptide in **Figure 2**. The peptide at *m*/*z* 1991.927 in **Figure 1B** has an MS/MS spectrum almost identical to that of the ion at *m*/*z* 1829.872 (data not shown). The difference in mass between these two peptides is 162.055 Da, which is consistent with the mass for a hexose saccharide residue (162.053 Da). Furthermore, the small ion at *m*/*z* 2123.935 in **Figure 1B** is

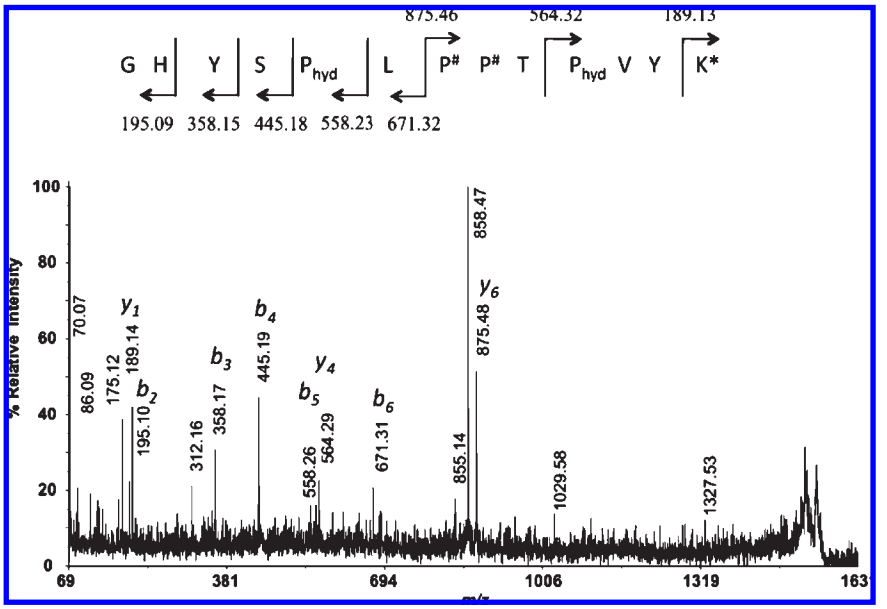

Figure 4. MS/MS spectrum of precursor ion at *m*/*z* 1545.758 and sequence showing the calculated fragments and labeled *b* and *y* ions from extensin peptide listed in **Table 1**.

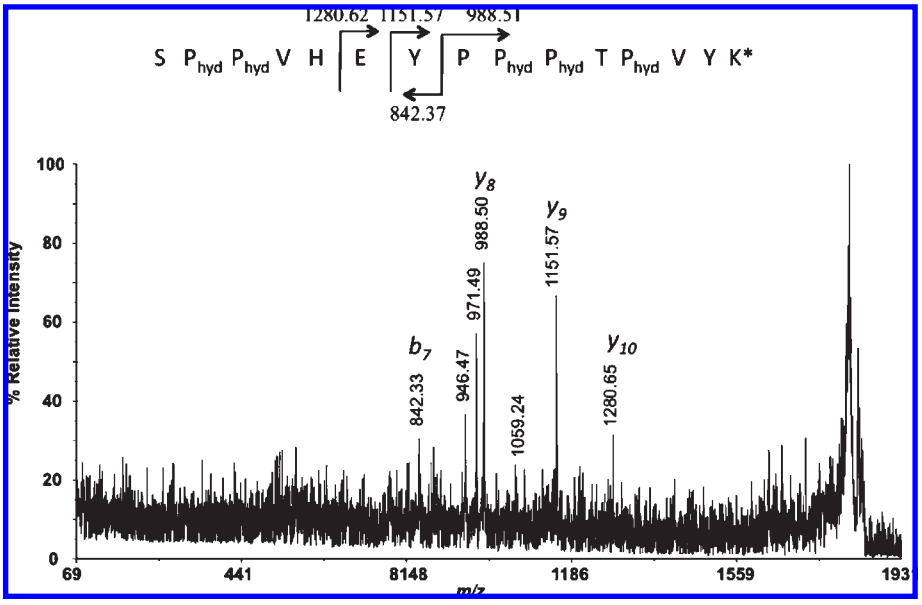

Figure 5. MS/MS spectrum of precursor ion at *m*/*z* 1829.872 and sequence showing the calculated fragments and labeled *b* and *y* ions from extensin peptide listed in **Table 1**.

132.071 Da from the previous peak at *m*/*z* 1991.927, consistent with the mass of a pentose residue (132.042 Da). The same peptides are observed also in the nonderivatized sample in **Figure 1A** at *m*/*z* 1949.92 and 2081.99. This suggests two possible residue glycosylations for the peptide at *m*/*z* 1829.872, which could be lost during pectin extraction.

The small ions at 1125.56 and 1167.562 in parts **A** and **B**, respectively, of **Figure 1** are 16 Da higher in mass from the preceding ions at *m*/*z* 1109.56 and 1151.569, suggesting an additional P_{hyd} in the sequence, but this could not be confirmed by MS/MS due to the low intensity of these ions and is indicated with a question mark in **Table 1**.

Glycosylation with a pentose sugar on the peptide at *m*/*z* 1151.569 seems to be producing the ion at *m*/*z* 1283.617 (**Figure 1B**, and also the corresponding peptide in **Figure 1A**). The MS/MS spectra of both ions are similar, but showed no evidence of the glycosylation site (data not shown). Finally, the last sequence in **Table 1** is also consistent with the corresponding MS/MS spectra, corresponding to the precursor ion at *m*/*z* 1109.560 as presented in **Figure 6**.

The MS and MS/MS spectra of the tryptic peptides from pectin digestion are highly consistent with the extensin protein sequence from the BvGI database. Previously, Li et al. (*19*) isolated and characterized extensin protein in sugar beet cell suspension cultures showing close homology with other members of the extensin family. Fractions of the isolated protein tryptic peptides were sequenced by Edman sequential methods, resulting in three complete peptide sequences with two having uncertainty in some of the amino acids. Two of the complete sequences are identical to those reported here in **Figures 2** and **5**, whereas the third matches the sequence at position 90−95 in the BvGI extensin (**Figure 3**), a peptide that was not found in the spectra in **Figure 1**.

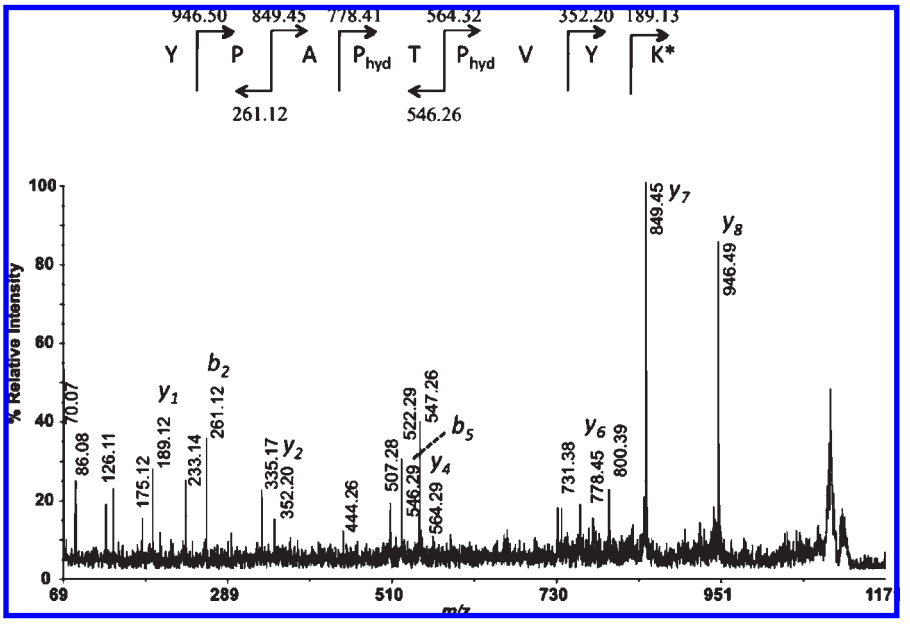

Figure 6. MS/MS spectrum of precursor ion at *m/z* 1109.560 and sequence showing the calculated fragments and labeled *b* and *y* ions from extensin peptide listed in **Table 1**.

Furthermore, the reported sugar beet extensin sequence lacked the characteristic pentamer $SP_{hyd}P_{hyd}P_{hyd}P_{hyd}$ found in other extensin proteins, but contained an interruption in which VHEYP or VHKYP was inserted in the middle of the tetrahydroxyproline block (*19*). The BvGI sequence shows, between positions 75 and 114 (**Figure 3**), two SPPVHEYPPP and one SPPVHKYPPP, confirmed to be $SP_{hyd}P_{hyd}VHEYPP_{hyd}P_{hyd}$ and $SP_{hyd}P_{hyd}VHKYPP_{hyd}P_{hyd}$, respectively, according to our MALDI-TOF/TOF-MS/MS analysis (**Figures 2** and **5**). The isolated extensin in sugar beet was a monomeric protein with an estimated molecular mass of 40 kDa for the deglycosylated protein, as determined by electrophoresis, and was about 40% glycosylated in the natural form (*19*). The protein sequence from the BvGI database is about 25 kDa, and the peptides identified here represent 27% of the sequence. The difference in molecular mass of the reported sugar beet extensin and those found in the BvGI sequences is an indication of an incomplete sequence.

It should be noted that there are two large tryptic peptides corresponding to the terminals of the BvGI sequence. It is difficult to predict the masses with the limitation in the sequence for the C-terminal peptide, but according to the c-DNA sequence the estimated $[M + H]^+$ masses are 4603 for the peptide at position 2−45 and 9640 Da for the peptide at position 151−222. Our cleaning procedure used a 3 kDa MWCO filter to eliminate most of the pectin; consequently, these peptides were very likely eliminated from the spectra presented in **Figure 1**, but even when a 10 kDa MWCO filter was used instead, there were no ion masses that suggested the presence of larger peptides, either in the reflectron or linear modes of operation of our MALDI-TOF/TOF instrument. It is very possible that these peptides remained associated with the pectin without being extracted through our procedure. In previous reports of sugar beet and cotton pectin digested with protease, only partial release of tryptic peptides was observed (*11*, *14*).

Because sugar beet extensin in the glycosylated form was estimated to have a molecular mass below 100 kDa (*19*), a pectin solution was prepared using the same conditions (1 mg/mL in 100 mM ammonium bicarbonate buffer, pH 7.5) but containing 1 M NaCl and ultrafiltered with a 100 kDa MWCO membrane in an attempt to disassociate the protein from the pectin. The permeate solution was digested with trypsin, but produced no peptides, whereas the retentate portion of the solution produced the peptide profile expected for extensin following trypsin treatment and 3 kDa MWCO ultrafiltration. In a recent study by Cannon et al., *Arabidopsis* cell culture extensin self-assembled into a network detected by AFM. They proposed that a positively charged, isodityrosine-cross-linked extensin network could interact with negatively charged carboxylate groups in pectin via ion pairs or salt bridges forming extensin pectates. These ionic interaction bridges were salt-elutable (*20*). The fact that we did not find that extensin protein was released from sugar beet pectin with a high concentration of salt suggests that either network entrapment or a covalent bond is responsible for the association between sugar beet pectin and extensin. Future research at our laboratory will elucidate the nature of this interaction.

The acidic conditions (pH 1) and temperature (60 °C) used for pectin extraction can hydrolyze glycosidic linkages; however, the duration is only 3 min. The molar mass and viscosity of the pectin extracted under these conditions were comparable to those of pectin extracted by others using whole sugar beet with conventional methods of extraction (*16*). Therefore, it is very unlikely that a significant deglycosylation takes place. To further prove that the sugar beet extensin protein identified is not a consequence of the extraction method, commercially available sugar beet pectin was used instead. The trypsin treatment of the commercial sugar beet pectin produced a MALDI-TOF/TOF spectrum with the same tryptic peptide profile as that observed for the tryptic digest of sugar beet pectin extracted using our procedure (data not shown). This provides additional evidence for the association of the extensin with pectin in sugar beet.

Atomic Force Microscopy. The pectin used in this study was well characterized previously by AFM (*16*). The image of a sample deposited on mica at a concentration of 6.25 μg/mL showed a structure with spherical or irregular-shaped particles surrounded by strands with a characteristic morphology, such as segmented, kinked, and/or loops attached to rods. Similarly, the solution of pectin used here, which was treated with denatured trypsin, produced images with the same structural characteristics as described before and shown in **Figure 7A**. Kirby et al. (*15*) has suggested that the spherical or globular structures observed by

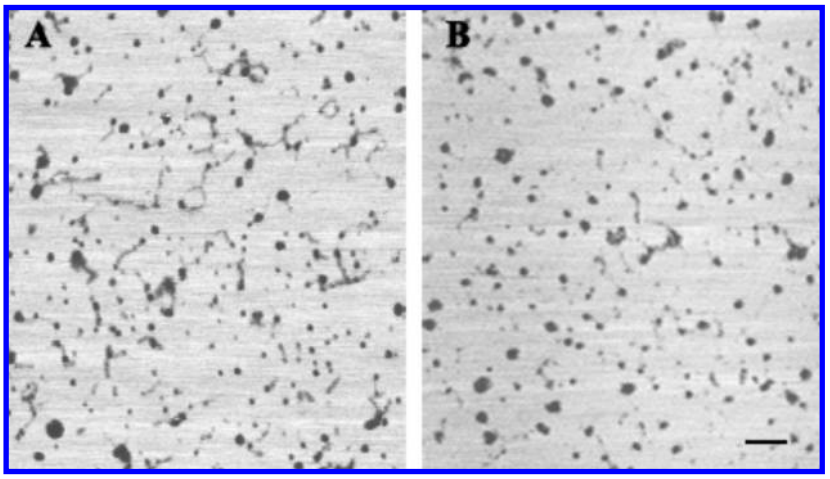

Figure 7. Atomic force microscopy image of sugar beet pectin solution at concentration of 10 μg/mL: (**A**) pectin control solution treated with denatured trypsin; (**B**) pectin treated with active trypsin. Inset scale bar is 100 nm.

AFM in sugar beet pectin are a protein complex with the pectin attached to one end. The protein structural evidence was from the characteristic physical appearance of proteins deposited on mica when scanned with AFM. The repeating hydroxyproline units in extensin predict a polyproline II rod-like molecule, and a kinked rod morphology was reported for rotary shadowed extensin precursors (*21*).

The AFM image obtained from the trypsin-digested solution shown in **Figure 7B** revealed circular or spherical particles without a distinguishable internal structure, but the number of connecting rod shapes or strands decreased. This observation possibly suggests that the rod structures occur where the protein is localized in sugar beet pectin. However, removal of arabinosides from extensin also resulted in a loss of the rod-like conformation in rotary shadowed replicas (*21*). Therefore, the rod-like structures reported here are probably a complex between extensin and pectin. Digestion of extensin would have disrupted the extensin network, which could have changed the global structure of the pectin network. It is also possible that the spherical particles still contain protein that is inaccessible to trypsin, so that no changes were produced in the spherical particle morphology.

AFM was not able to resolve pectin branching due to major differences in neutral sugar composition (*22*). Furthermore, the AFM structure of pectin changed, presumably due to enzymatic degradation during fruit ripening under modified atmosphere storage conditions (*23*). Therefore, it may not be possible to specifically localize extensin and pectin in the AFM structures reported here. However, AFM was an excellent method to detect disruption in the extensin network, and we have previously demonstrated its utility for pectin network analysis (*24*). This reinforces the need for further research to explore the nature of the complex extensin−pectin network. Because the sugar beet extensin complete sequence is not available, it is difficult to determine what percentage our identified peptides represent, but is very likely to be less than the 27% coverage determined from the sequence in **Figure 3**. Also, if we assume that the remaining protein is still associated with the pectin, then the protein content in the treated and the untreated samples is not expected to significantly change. For these reasons, the protein content was not determined.

We conclude from this study that extensin seems to be the main protein associated with sugar beet pectin. The use of MALDI-TOF/TOF mass spectrometry with the combination of bioinformatics has provided strong evidence for the identification of the resulting tryptic peptides. The protein identity associated with this peptide is derived from the existing information on the BvGI and the previous characterization of the protein by amino acid sequencing (*19*). AFM demonstrated changes in the physical structure of the rod-like elements that are related to trypsin enzyme activity, but no significant conclusion can be made concerning the localization of pectin and extensin in the rod and sphere network.

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