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ScienceDirect
Food Chemistry 110 (2008) 239–247
FOOD CHEMISTRY
www.elsevier.com/locate/foodchem

Analytical Methods

Combined enzymatic and colorimetric method for determining the uronic acid and methylester content of pectin: Application to tomato products

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Received 17 April 2007; received in revised form 22 January 2008; accepted 26 January 2008

Abstract

A simple procedure for determining the galacturonic acid and methanol contents of soluble and insoluble pectins, relying on enzymatic pectin hydrolysis and colorimetric quantification, is described. Pectin samples are incubated with a commercial pectinase preparation, Viscozyme, then the galacturonic acid content of the hydrolyzed pectin is quantified colorimetrically using a modification of the Cu reduction procedure originally described by Avigad and Milner. This modification, substituting the commonly used Folin–Ciocalteau reagent for the arsenic containing Nelson reagent, gives a response that is linear, sensitive, and selective for uronic acids over neutral sugars. This method also avoids the use of concentrated acids needed for the commonly used *m*-phenylphenol method. Methanol, released by the action of the pectin methylesterase found in the Viscozyme, is quantified using alcohol oxidase and Purpald. This combined enzymatic and colorimetric procedure correctly determined the galacturonic acid and methanol content of purified, soluble citrus pectin. Application of the procedure to water insoluble pectins was evaluated with water insoluble material from apples and oranges. In both cases good agreement was obtained between this method and commonly used methods based on chemical pectin hydrolysis. Good agreement between these procedures was also found in the analysis of both soluble and insoluble pectins from several tomato products.

Keywords: Pectin; Tomato; Uronic acid; Methanol; Pectin methylesterase; Degree of esterification

1. Introduction

Pectins are a major constituent of plant cell walls and greatly affect the quality of many plant-derived food products. While tremendous variability in overall pectin composition exists across the plant kingdom, in many common fruits and vegetables galacturonic acid comprises >80% of the total pectin carbohydrate, with neutral sugars, primarily galactose, arabinose, rhamnose, and xylose, making up the remainder (Ridley, O'Neill, & Mohnen, 2001). Pectin galacturonic acid is present in three polymeric forms: homogalcturonan (HG), a linear polymer of α 1–4 linked galactuonic acids containing variable levels of methylesterification of the carboxyl group and acetylation on C-2; rhamnogalacturonan I (RG-1), a repeating disaccharide of galacturonic acid and rhamnose; and rhamnogalacturnon II (RG-II), a homogalacturonic backbone with numerous complex sidechains containing rhamnose and other neutral sugars. How these various polymers are attached to each other is still an open question (Vincken et al., 2003). HG the most abundant of the three polymers and the degree to which it is methylesterified has a large impact on the functional properties of the pectin.

During thermal processing of plant material both chemical and enzyme catalyzed reactions can occur that change pectin solubility, polymer size, and degree of methylesterification.. These changes can have important consequences for product quality. For example, reducing the degree of pectin methylesterification by activating endogenous pectin

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doi:10.1016/j.foodchem.2008.01.042

methylesterase (PME) activity, results in processed material with a firmer texture. This is because the lower degree of pectin methylesterification both increases Ca^{2+} cross-linking of the pectin chains and makes the pectin less susceptible to thermal cleavage by β-elimination (Stolle-Smits, Beekhuizen, Recourt, Voragen, & Van Dijk, 2000). Changes in pectins during processing can also affect quality in fluid products like juices and sauces. In tomatoes, hydrolysis of pectin by the combined action of PME and polygalacturonase (PG) will result in decreased pectin polymer size and a loss of fluid viscosity if these enzymes are not thermally inactivated at the time of homogenization in the so-called hot-break process. The heat input during the hot-break and the additional heating required to concentrate the juice to paste can cause other changes in the pectins, including solubilization and breakdown (Hurtado, Greve, & Labavitch, 2002; Xu, Shoemaker, & Luh, 1986). To follow the changes in pectin that occur during processing a simple method for quantifying the amounts of soluble and insoluble pectin, and assessing the degree of methyl esterification is thus desirable.

Several methods are available for quantifying and characterizing pectin. Most commonly a preparation containing the cell wall material is isolated, usually by ethanol precipitation, to separate the polymeric material from any monomeric galacturonic acid or other soluble small molecules that would interfere with subsequent the galacturonic acid analysis. This alcohol insoluble residue is then sequentially extracted with water, chelators, and finally high pH to produce fractions each containing a portion of the total pectic material. These fractions can then be hydrolyzed and analyzed for their methanol, uronic acid, and neutral sugar contents. Results are typically reported as the amounts of these constituent per amount of alcohol insoluble residue. Assuming that the pectin precipitation by the alcohol is quantitative, and that the yield of alcohol insoluble residue per amount of plant material is reported, the amount of pectin in the plant material can be calculated.

Quantifying the galacturonic acid content of the various fraction is most commonly done by first hydrolyzing the pectic material in hot concentrated acid, then quantifying the resulting anhydrogalacturonic acid residues colorimetrically with *m*-hydroxydiphenyl (Blumenkrantz & Asboe-Hansen, 1973). Since both the hydrolysis of the pectin and the reaction with *m*-hydroxydiphenyl occur in concentrated sulfuric acid, a rapid and simple procedure for quantifying the uronic acid content of pectin is possible (Ahmed & Labavitch, 1977). Improvements to reduce the interference by neutral sugars have been developed (Filisetti-Cozzi & Carpita, 1991). A different approach is to enzymatically hydrolyze the pectin to galacturonic acid using mixtures of pectin degrading enzymes. The galacturonic acid content can then be determined colorimetrically (McComb & McCready, 1952) or by anion exchange HPLC (Matsuhashi, Shin-ichi, & Hatanaka, 1992; Rumpunen, Thomas, Badilas, & Thibault, 2002; Garna, Mabon, Nott, Wathelet, & Paquot, 2006). Several different preparations of highly active pectin degrading enzymes are commercially available and have been shown to give complete pectin hydrolysis. In addition to eliminating the safety and disposal problems associated with the use of hot concentrated sulfuric acid, these enzymatic procedures have the advantage that they are specific for pectin and require mild conditions. Unlike acid hydrolysis, where the galacturonic acid released from the pectin can be further degraded, the galacturonic acid formed from enzymatic hydrolysis is stable.

Several methods are available for determining the degree of pectin methyl esterification. With pure pectins a simple titration procedure can be used (Schultz, 1965). With less pure preparations, such as alcohol insoluble residues, the methyl esters can be hydrolyzed under mild alkaline conditions and the amount of methanol produced determined. Chemical quantification of methanol can be done by first oxidizing it to formaldehyde, either with permanganate (Wood & Siddiqui, 1971) or enzymatically with alcohol oxidase (Klavons & Bennett, 1986), then be determining the formaldehyde with one of several colorimetric reagents (Anthon & Barrett, 2004). The methanol can also be quantified by GC (Bartolome & Hoff, 1972; Savary & Nunez, 2003). An alternative approach is to treat the partially methylesterified pectin with $NaBH_4$ which reduces the methylesterified galacturonic acids to galactose. The total galacturonic acid content with and without $NaBH_4$ reduction can then be determined and the methylester content calculated (Maness, Ryan, & Mort, 1990).

Our goal was to develop a simple method for determining the both amount and degree of methyl esterification of soluble and insoluble pectin, suitable for use with products such as tomato paste and juices. To eliminate the need for concentrated acids, we have used enzymatic pectin hydrolysis. Galacturonic acids can then be quantified with a modified version of the colorimetric procedure of Milner and Avigad (1967) in which uronic acids are quantified reductometrically at pH 4.8. In this assay neutral reducing sugars do not interfere, because at pH 4.8, only uronic acids and certain uncommon ketose sugars, but not most aldoses, will reduce copper. In the original procedure the reduced copper was quantified with the arsenomolybdate reagent of Nelson (1944). Here, we show here that the readily available Folin–Ciocalteau reagent works equally well, eliminating the need for arsenic in the assay. Since the mixtures of pectin degrading enzymes used to depolymerize pectin also contain pectin methylesterase activity, the amount of methanol in the hydrolyzed sample can be determined along with the galacturonic acid content to give the degree of methylesterification. In addition, we have examined the use of centrifugation through gel filtration media, a procedure commonly used to desalt protein solutions, as a method for separating pectin polymers from small molecules (e.g. methanol, galacturonic acid, and reducing sugars) that would interfere with the quantification of the pectin uronic acid and methylester content.