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Development of complete hydrolysis of pectins from apple pomace

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ABSTRACT

Enzymatically extracted pectins have a more complex structure than those obtained by conventional methods. As a result, they are less susceptible to hydrolysis, which makes the precise determination of their composition difficult. The aim of the study was to develop a method of complete hydrolysis of enzymatically extracted apple pectins. Substrates were pectins isolated from apple pomace by the use of xylanase and multicatalytic preparation Celluclast and apple pomace. Hydrolysis was performed by a chemical method with 2 M TFA at 100 °C and 120 °C and a combined acidic/enzymatic method. After hydrolysis, the contents of galacturonic acid and neutral sugars were measured by HPLC. Complete hydrolysis of polygalacturonic acid occurred after 2.5 h incubation with 2 M TFA at 120 °C. The efficient hydrolysis of neutral sugars in pectins was performed with 2 M TFA at 100 °C for 2.5 h. Monomers most susceptible to concentrated acid were rhamnose, mannose and arabinose.

1. Introduction

Pectins are polysaccharides commonly used in food technology, which are obtained industrially by isolation from plant tissues (Mollea, Chiampo, & Conti, 2008; Willats, Knox, & Mikkelsen, 2006). Both sources and extraction conditions significantly influence the composition and degree of pectin polymerisation, resulting in differentiated physical and biological properties of the product (Baississe, Ghannem, Fahloul, & Lekbir, 2010; Chan & Choo, 2013; Yapo, Robert, Etienne, Wathelet, & Paquot, 2007). The neutral sugar profiles and galacturonic acid content are usually obtained by means of chromatographic techniques, preceded by the hydrolysis of the polymer. The latter process is complicated, because the reaction kinetics depends on pectin type. Acid-based hydrolysis is most commonly used for pectin depolymerisation (Arnous & Meyer, 2008; Garna, Mabon, Nott, Wathelet, & Paquot, 2006; Methacanon, Krongsin, & Gamonpilas, 2014; Yapo, 2009). The disadvantage of this method is that different glycoside bonds have different susceptibilities to acids, which results in their gradual hydrolysis (Voragen, Coenen, Verhoef, & Schols, 2009). The optimum choice of acidic hydrolysis conditions is very important. Both incomplete or excessive hydrolysis conditions result in errors during subsequent determination of galacturonic acid and neutral sugar contents. Acid hydrolysis of pectin is most commonly performed with H_2SO_4, TFA and HCl, usually at concentration of 1–2 M, at 100–121 °C for 1–3 h (Emaga, Ronkart, Robert, Wathelt, & Paquot, 2008; Garna et al., 2006; Methacanon et al., 2014). Too high a temperature and concentrated acids may cause the formation of furfural derivatives, which makes the precise determination of sugars impossible. The alternative to acid hydrolysis is enzymatic hydrolysis of pectin, which may be performed with multicatalytic preparations containing poligalacturonase, pectinesterase, cellulase, xylanase, β-glucanase, hemicellulase and arabanase (Garna, Mabon, Wathelet, & Paquot, 2004; Rumpunen, Thomas, Badilas, & Thibault, 2002). According to some researchers, the most effective type of pectin hydrolysis to its monomers is the combined acidic and enzymatic method (Garna et al., 2004, 2006). Unfortunately, none of these methods is adapted to enzymatically extracted pectins, which are characterised by more complex compositions and even several fold higher molecular masses, as compared to conventionally obtained pectins (Naghshineh, Olsen, & Georgiou, 2013; Yuliarti, Matia-Merino, Goh, Mawson, & Brennan, 2012). The sum of neutral sugars and galacturonic acid in enzymatically extracted pectins presented in the literature did not exceed 70% (Yuliarti et al., 2012; Zykwinska, Boiffard, Kontkanen, Thibault, & Bonnin, 2008), or barely reached 58%, as in the case of pectin extracted from butternut (Fissore et al., 2009). This indicates the significant underestimation of the samples, resulting most likely from the use of too aggressive or too mild hydrolysis techniques.

The main purpose of this research was to present a method of hydrolysis of enzymatically extracted pectins which enables their complete depolymerisation while maintaining physical and chemical properties of all obtained monomers. This allows the precise

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and unequivocal determination of the composition of these extremely complex polymers by HPLC techniques.

2. Materials and methods

2.1. Chemicals and reagents

Monosaccharides standards: L-(+)-arabinose 99%, D-(+)-fucose 99%, D-(+)-galactose 99%, D-(+)-glucose 99,5%, L-(−)-mannose 99%, L-(−)-rhamnose monohydrate 99%, D-(−)-xylose 99% and polygalacturonic acid 98% were from Sigma/Aldrich Chemical Co. Trifluoroacetic acid (TFA) 99% was from Merck (Germany), HPLC grade standard NaOH solution was purchased from Fluka/Sigma–Aldrich Chemical Co.

2.2. Enzymes

Pectins from apple pomace were extracted with xylanase (Pentopan®, Novozyme Co.) produced by *Thermomyces lanuginosus* and the multicatalytic preparation Celluclast® 1.5 L (Novozymes Co.) derived from *Trichoderma reesei* ATCC 26921. Both preparations are recommended for pectin extraction and allow to obtain pectins of particularly high molecular mass (Naghshineh et al., 2013; Yuliarti, Matia-Merino, Goh, Mawson, & Brennan, 2011).

The combined acidic/enzymatic hydrolysis of pectins was conducted with commercial multicatalytic preparations Energex® L and Viscozyme® L derived from *Aspergillus aculeatus* (Novozymes Co). According to the product data sheet, they are characterised by high activities of carbohydrases, including pectinase, arabanase, cellulase, β-glucanase, hemicellulase, and xylanase. Prior to application, a 5 ml sample of each preparation was subjected to ultrafiltration (5 h at 2000*g*) with the use of centricon tubes (VivaSpin 20 ml concentrator; cutoff 30 kDa) to eliminate carbohydrates and glycerol, as described by Garna et al. (2004). The retentate obtained was washed twice with 10 ml of redistilled water pH 4.5 (Energex preparation) or 5.0 (Viscozyme preparation) and finally diluted up to 5 ml with this water. Optimal pH values for the activity of Energex and Viscozyme preparation are 4.5 (Wikiera, 2004) and 5.0 (Rosset & Del Pino Beleia, 2014), respectively.

2.3. Standard solutions

A standard mixture of neutral sugars (SNS) containing 1 mg/ml glucose, galactose, arabinose, rhamnose, xylose, fucose and mannose was prepared from a 7 mg/ml stock water solution of every monosaccharide. SNS was subjected to the same conditions of hydrolysis as the experimental samples, as described in Sections 2.6 and 2.7.

2.4. Apple pomace

Apple pomace (Pektowin S.A., Jasło, Poland) is the solid remains after pressing for juice without the use of enzymes, utilised in commercial production of pectin. The pomace was subjected to leaching with distilled water at a ratio 1:20 (w/v), at 20 °C for 20 min with constant shaking (200 rpm). The leaching was repeated three times in order to obtain the concentration of refractometric index of leaching water below 1%. Next, the pomace was dried to constant mass (60 °C, 24 h) and its moistness was determined. Before extractions the pomace was ground to particles passing through a 40-mesh sieve (0.47 mm). Apple pomace subjected to hydrolysis was ground to particles that pass through a 60-mesh sieve (0.25 mm).

2.5. Pectin extraction

Pectin extraction from apple pomace (solid/liquid ratio 1 g/15 ml) was performed with the use of enzymatic preparations at pH 4.5, 50 °C for 10 h. The resulting pectins were: P_{cel} – obtained with Celluclast (applied preparation dose 50 μl/g apple pomace) and P_x – obtained with xylanase (50 U/g). After the extraction, samples were cooled to 20 °C and centrifuged (1300*g*, 10 min, 4 °C). Supernatants were filtered through cellulose paper. The precipitates were washed with distilled water, centrifuged and filtered. The supernatants were combined within the sample. Next, 96% ethanol (4 °C) was added. The volume of ethanol was adjusted to the final concentration of 70%. After an hour, the precipitated pectins were centrifuged (1200*g*, 20 min), washed twice with 70% ethanol, centrifuged again, and then dried to a constant weight (60 °C, 24 h) and their moistness was determined. Collected samples of pectins (P_x and P_{cel}) were ground to particles passing through a 60-mesh screen.

2.6. Chemical hydrolysis

Hydrolysis was performed in round bottom glass tubes with screw-caps (Schott tubes). Samples of P_{cel}, P_x, apple pomace and polygalacturonic acid (PGalA) standard (10 ± 0.1 mg) were treated with 2 M TFA (2 ml). Standard mixture of neutral sugars, (0.2 ml), was treated with 2 M TFA (1.8 ml). All samples were closed tightly, mixed and incubated with constant shaking (200 rpm) in an oil bath at 100 and 120 °C for 60, 90, 120, 150, 180 and 240 min. Next, the hydrolysed samples were cooled and volumes of 50 μl (pectins, pomace and PGalA hydrolysate) or 100 μl (SNS hydrolysate) were evaporated in Eppendorf tubes at 40°C. Dried samples were dissolved in deionised (Milli-Q™) water. The final concentrations of the prepared samples were: 0.2 mg/ml for pectin, apple pomace and PGalA hydrolysates, and 0.01 mg/ml for SNS.

2.7. Combined chemical and enzymatic hydrolysis

Hydrolysis was performed in round bottom glass tubes with screw-cap (Schott tubes). Combined hydrolysis was performed according to Garna et al. (2004) with some modifications. Samples of P_{cel}, P_x, apple pomace and PGalA (15 ± 0.1 mg) were treated with 3 ml of 0.2 M TFA. Standard mixture of neutral sugars (0.3 ml), was treated with 0.2 M TFA (2.7 ml). All samples were closed tightly, mixed and incubated with constant shaking (200 rpm) in a water bath at 80 °C for 72 h. Next, the pH of the samples was adjusted to 4.5 (for Energex) or 5.0 (for Viscozyme) with 1 M and 0.1 M NaOH (at constant shaking and pH monitoring), and their volume was made up to 25 ml with redistilled water (pH 4.5 or 5.0). In order to perform enzymatic hydrolysis, samples (10 ml) were incubated in closed test-tubes with 25 μl of Energex or Viscozyme for 24 h at 50 °C, with constant shaking (200 rpm). Hydrolysis was stopped by incubating in a boiling water bath for 10 min in order to inactivate the enzymes. The concentrations of the samples were: 0.6 mg/ml for pectin, apple pomace and PGalA hydrolysates, and 0.12 mg/ml for SNS. Before HPLC analysis, hydrolysates were diluted with deionised (Milli-Q™) water to a final concentration of 0.2 mg/ml.

2.8. Analytical methods

The water content in apple pomace and pectin preparations was calculated as the weight difference before and after drying at 105 °C until constant weight.

Neutral sugars (NS) and galacturonic acid (GalA) contents in hydrolysates were obtained by HPLC. Before every analysis,