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Chelating agents improve enzymatic solubilization of pectinaceous co-processing streams

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ABSTRACT

This study investigates the hypothesis that loosening of the egg-box structure by presence of divalent ion chelating agents during enzymatic degradation of homogalacturonan (HG) can improve enzymatic polysaccharide solubilization on pectinaceous, agro-industrial co-processing streams. The influence of different levels of ethylene-diaminetetraacetic acid (EDTA), citric acid, oxalic acid, and phosphate was assessed in relation to enzymatic solubilization of isopropanol precipitatable oligo- and polysaccharides from sugar beet pulp, citrus peel, and two types of potato pulp. The two types of potato pulp were FiberBind 400, a dried commercial potato pulp product, and PUF, a dried calcium reduced product, respectively. The enzymatic treatment consisted of 1% (w/w) of substrate treated with pectin lyase from *Aspergillus nidulans* and polygalacturonase from *A. aculeatus* [each dosed at 1.0% (w/w) enzyme/substrate] at 60 °C, pH 6.0 for 1 min. Characterization of the released fractions demonstrated a significantly improved effect of chelating agents for polysaccharide solubilization from FiberBind 400, PUF, and citrus peel, whereas only low amounts of polysaccharides were solubilized from the sugar beet pulp. The results substantiated the importance of chelating agents during enzymatic extraction of pectinaceous polysaccharides. Lower levels of chelating agents were required for the calcium-reduced potato pulp substrate (PUF) indicating the significance of calcium cross-linking in HG in relation to the enzymatic solubilization yields. The effect of the chelating agents correlated to their dissociation constants (pK_a values) and calcium binding constants and citric acid and EDTA exerted highest effects. Maximum polysaccharide yield was obtained for FiberBind 400 where the enzymatic treatment in presence of citric acid yielded 22.5% (w/w) polysaccharides of the initial substrate dry matter.

1. Introduction

Large scale agro-industrial processes such as fruit juice production, potato starch manufacturing, and sucrose extraction from sugar beets (and many others) generate significant amounts of co-processing streams rich in pectin. Pectin encompasses a diverse and complex family of plant cell wall polysaccharides, and is a main constituent of the primary cell walls and the middle lamella of dicotyledonous plants, including citrus, potatoes, and sugar beet [1,2]. Pectin polysaccharides are principally based on four main types of backbone structures: homogalacturonan, xylogalacturonan, rhamnogalacturonan I, and rhamnogalacturonan II, as reviewed elsewhere [2,3] with homogalacturonan (HG) and rhamnogalacturonan I (RG I) being quantitatively the most dominant [3,4] and relevant for the present study. HG principally consists of an unbranched chain of α1,4-linked galacturonic acid (Gal*p*A) residues ([→4)-α-D-Gal*p*A-(1→]$_{n\geq1}$) which may be methyl-esterified and/or acetylated [3].

In the cell wall, negatively charged stretches of non-methylated and non-acetylated HG are stacked with similar neighboring HG chains via ionic crosslinks formed by divalent cations, mainly calcium [3,4]. These ionic cross-links form a continuous network in which stretches of the HG are locked in junction- or gel-zones usually described in terms of the "egg-box" model [5,6]. The RG I backbone consists of repeating diglycosyl units of rhamnose (Rha*p*) and galacturonic acid residues organized as [→2)-α-L-Rha*p*-(1→4)-α-D-Gal*p*A-(1→]$_{n\geq1}$ units. The α-L-Rha*p* residues are partly substituted with side chains of galactan, arabinan and/or different arabino-galactan side-chains at the O-4 (mainly) and/or at the O-3 (rarely) position [7]. The side chains may be of much higher molecular weight than the RG I backbone. Stretches of HG and RG I (and xylogalacturonan and rhamnogalacturonan II) are positioned in successive connections in the plant cell wall and the RG I structures are often referred to as ramified hairy regions [3].

Pectin is used in a range of different applications. Pectin – in this connection mainly understood as HG – derived from citrus peels

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and apple pomace already has a long history of use as a hydrocolloid promoting gelation and thickening in food applications [8], and pectic polysaccharide structures have also recently been proposed to be potentially suitable for e.g. advanced biomedical applications [9,10]. Reduced molecular weight citrus pectin fractions have also been reported to possess a variety of potentially health beneficial effects, including potential prebiotic effects in vitro, i.e. stimulating the growth of desirable bacteria such as bifidobacteria and lactobacilli [11,12], various positive effects on different types of cancer cells in vitro [13,14], and even clinical benefits on recurrent prostate cancer [15]. Analogously, enzymatically solubilized high-molecular weight galactan polysaccharides derived from the RG I fraction of potato pulp have been shown to act as fermentable dietary fibers in humans [16], to induce reduced weight gain in rats [17], and to exert bifidogenic prebiotic properties in vitro [18,19]. Analogously, arabino-oligosaccharides, i.e. RG I-side chain components, derived from sugar beet pulp have recently been shown to exert potential bifidogenic prebiotic properties in vitro [20,21].

The new prospects for advanced uses and the putative health effects of specific structural entities of pectin provide a large incentive for developing efficient, selective processes for liberation of the desirable pectin components from available low-value co-processing streams. A first requirement for upgrading the pectin from such streams, and thus provide a basis for producing these beneficial pectin components on a larger scale, is that the desirable part of the pectin is liberated from the fibrous plant cell wall matrix. Different selective enzymatic approaches have already been highly successful in this regard [22–26]. In order to particularly target the solubilization of the high-molecular weight galactan fibers of the ramified RG I region of potato pectin, we have recently developed a minimal enzymatic treatment procedure based on catalyzing the breakdown of the HG surrounding the RG I regions by use of a combination of mono-component pectin lyase (EC 4.2.2.10) attacking mainly α1,4 bonds of methoxylated Gal*p*A and polygalacturonase (EC 3.2.1.15) attacking mainly the bonds between non-methoxylated Gal*p*A residues [19]. However, considering that the HG in egg-box structures is known to partly retard the enzymatic attack on HG [6,27], and that calcium-induced pectin gelation to form the egg box structure is weakened in the presence of chelating agents [28], we wanted to test the hypothesis that presence of calcium chelating agents during enzymatic degradation of HG might enhance the enzymatic yields of enzymatically solubilized polysaccharides from potato pulp, and subsidiarily from other agro-industrial pectinaceous residues.

Chelating agents such as EDTA (ethylene-diamine-tetraacetic acid), CDTA (cyclohexane-diamine-tetraacetic acid), and oxalate have been used in the past to extract pectin in order to elucidate structural pectin components in plant cell wall materials [29–31]. Also the significance of the presence of calcium has been studied with respect to *promoting* pectin gelation by pectin methyl esterase activity (EC 3.1.1.11) [32], but the deliberate use of chelating agents to promote enzyme catalyzed degradation of HG has to our knowledge not been evaluated systematically.

The objective of this study thus was to examine the influence of chelating agents and enzyme treatment for solubilization of pectinaceous substances from various agroindustrial plant streams. In relation to further develop a minimal enzymatic treatment for solubilization of RG I-derived bifidogenic dietary fibers from potato pulp, in which the buffer originally used in lab experiments was 0.1 M phosphate [19,33], the first part of the study assesses the effect of the concentration of phosphate and the effect of the known chelating agent EDTA [34] on the enzymatic solubilization of high molecular weight pectinaceous polysaccharides from potato pulp substrates differing in calcium content. Subsequently, to obtain a more generic understanding of the prerequisite of (calcium) chelating agents for enzymatic polysaccharide solubilization from various types of genuine pectin rich biomasses, the influence of a series of relevant chelating agents, i.e. phosphate, EDTA, citric acid, and oxalic acid, were examined on potato pulp, citrus peel, and sugar beet pulp.

2. Materials and methods

2.1. Chemicals

Trifluoroacetic acid (TFA), D-(+)fucose, α-L-rhamnose, L-(+)arabinose, D-(+)galactose, D-(+)xylose, D-(+)mannose, D-(+)galacturonic acid, EDTA disodium salt dihydrate 99+%, oxalic acid 99+%, citric acid anhydrous ≥99.5%, monobasic sodium phosphate monohydrate, and sulfuric acid 72% (w/w), were purchased from Sigma-Aldrich (Steinheim, Germany). Dibasic sodium phosphate heptahydrate was purchased from Reidel-deHaën (Seelze, Germany). D-(+)Glucose was purchased from Merck (Darmstadt, Germany).

2.2. Substrates and enzymes

Dried citrus peel was supplied by Dupont Nutrition BioSciences ApS (Brabrand, Denmark) and stored at −21 °C until use. Dried sugar beet pulp was supplied by Nordic Sugar (formerly Danisco A/S) (Nakskov, Denmark) and stored at room temperature. Citrus peel and sugar beet pulp were milled to reach particles sizes ranging from 150 μm to 355 μm as obtained by sieving the milled material using stainless steel analytical sieves (Endecotts, London, UK). The commercial product FiberBind 400, produced from washed and dried potato pulp, and the calcium reduced dried potato pulp product, denoted PUF, were supplied as powders by KMC (Brande, Denmark). The calcium reduced potato pulp PUF product had been prepared according to a patented procedure involving swelling of the pulp in hot acid followed by washing [35]. According to the supplier (KMC, Brande, Denmark) the FiberBind 400 contained ~7000 ppm of calcium, whereas the calcium level in the PUF product was ≤200 ppm. Monocomponent endo-polygalacturonase from *Aspergillus aculeatus* was a gift from Novozymes A/S (Bagsværd, Denmark) (in our previous work [19,33] referred to as PG2). The pectin lyase from *Aspergillus nidulans* (now *Emericella nidulans)* was produced by fermentation of a *Pichia pastoris* clone transformed with the pectin lyase gene AN2569.2 [36]. The clone (in our previous work referred to as PL1 [19,33]) was obtained from the Fungal Genetic Stock Center, Carnegie Institution, Stanford, CA, USA [36]. *P. pastoris* fermentation was principally done according to [37] in 5 L fermentation as described in detail previously [38]. Dosing of the pectin lyase was done on the basis of protein content (22.0 g/L), whereas dosing of the monocomponent polygalacturonase, presented as a lyophilized powder, was directly on dry weight basis.

2.3. Enzymatic release of pectinaceous structures

The procedure for enzymatic release of pectinaceous structures was based on the minimal enzymatic treatment published recently [19]. 1% (w/w) of each plant cell wall substrate (excluding starch) was preheated at 60 °C for 5 min in milliQ water with/without the appropriate chelating agent and adjusted to pH 6.0. Pectin lyase [1.0% (w/w) Enzyme/Substrate (E/S)] and polygalacturonase [1.0% (w/w) E/S] were added and the samples incubated during shaking at 750 rpm in thermo mixers (Eppendorf, Hauppauge, NY, USA) at 60 °C for 1 min. The enzymatic reactions were halted by treatment at 100 °C for 10 min (thermo block; Eppendorf, Hauppauge, NY, USA) and subjected to centrifugation (15,000 × *g* for 10 min). The supernatant of each sample was then removed, filtered using a 0.2 μm syringe tip filter (Phenomenex, Torrance, CA, USA), and precipitated with 70% isopropanol for 30 min at room temperature.