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Definition and characterization of enzymes for maximal biocatalytic solubilization of prebiotic polysaccharides from potato pulp

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ABSTRACT

Potato pulp is a high-volume co-processing product resulting from industrial potato starch manufacturing. Potato pulp is particularly rich in pectin, notably galactan branched rhamnogalacturonan I polysaccharides, which are highly bifidogenic when solubilized. The objective of the present study was to characterize and compare four homogalacturonan degrading enzymes capable of catalyzing the required solubilization of these pectinaceous polysaccharides from potato pulp in a 1 min reaction. An additional purpose was to assess the influence of the pH and the potential buffer chelating effects on the release of these polysaccharides from the potato pulp. The pH and temperature optima of two selected pectin lyases from *Emericella nidulans* (formerly known as *Aspergillus nidulans*) and *Aspergillus niger* were determined to 8.6 and 4.0, respectively, at ≥100 °C within 1 min of reaction. The optima for the two selected polygalacturonases from *E. nidulans* and *Aspergillus aculeatus* were determined to pH 4.4 and 46 °C, and pH 3.7 and ≥80 °C, respectively. The polygalacturonase from *A. aculeatus* was 4–42 times more heat-resistant at 50 °C than the other enzymes. The difference in pH optima of the pectin lyases and the exceptional thermal stabilities of some of the enzymes are proposed to be related to specific amino acid substitutions, stabilizing hydrogen bonding and structural traits of the enzymes. The K_M and V_{max} values ranged from 0.3–0.6 g/L and 0.5–250.5 U/mg protein, respectively. Phosphate buffer induced release of a higher amount of dry matter than Tris–acetate buffer at pH 6, indicating a chelating effect of the phosphate. Moreover, the phosphate had a higher chelating effect at pH 6 than at pH 4. The optimal conditions for a high yield of polysaccharides from potato pulp were therefore: 1% (w/w) potato pulp treated with 1% (w/w) enzyme/substrate (E/S) pectin lyase from *E. nidulans* and 1% (w/w) E/S polygalacturonase from *A. aculeatus* at pH 6.0 and 60 °C for 1 min.

1. Introduction

Potato pulp is a poorly utilized high-volume byproduct from industrial potato starch manufacturing; the pulp is currently used mainly as cattle feed and sold to farmers for less than 10 €/ton [1]. The potato pulp is mainly made up of the cell walls of the potato tuber. The main components in the potato pulp are pectin, cellulose, and hemicellulose but the pulp also contains some residual starch [1]. The monomers of destarched potato pulp are mainly galactose, arabinose, galacturonic acid (GalA) and rhamnose (Rha) indicating that potato pulp is made up of homogalacturonan and rhamnogalacturonan I with large galactan rich side chains [2]. Homogalacturonan generally consists of a backbone of α-1,4-linked D-galacturonic acid residues which can be methyl esterified at C-6 and/or O-acetylated at O-2 and/or O-3 [3]. The degree of methylation and acetylation in potato pectin has been reported to be 31 and 14%, respectively [4]. The rhamnogalacturonan I backbone is built of repeating disaccharide [→2)-α-L-Rha*p*–(1→4)-α-D-Gal*p*A-(1→] units with side chains consisting of mainly α-1,5-linked arabinose, β-1,4-linked galactose and/or arabinogalactan attached to the O-4 of the rhamnose residues [5]. Enzymatically solubilized polysaccharides from potato pulp have been shown to possess potentially beneficial properties as dietary fibers and prebiotics [6–8].

Pectin can be extracted from plant cell walls both chemically and enzymatically [9]. Chelating agents such as cyclohexanediaminotetraacetic acid (CDTA) are known to enhance the extractability of pectins from potato cell walls [10]. This effect is most likely a result of a perturbation of calcium–pectin interactions as low methyl-esterified pectins form gels with calcium ions according to the egg box model for pectin gelation [11].

Pectin lyase, polygalacturonase, pectin esterase, rhamnogalacturonase and endo-glucanase have been used to extract pectin from apple cell wall material [12]. In our exploratory study developing the proof of concept for a minimal enzymatic treatment to release bifidogenic polysaccharides from potato pulp [8], three fungal enzyme activities, pectin lyase, polygalacturonase and pectin

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Table 1
Origin, classification and properties of the applied enzymes.

Enzyme	Source	Molecular mass (kDa)	Protein concentration (g/L)	Family	EC no.	Reference
Pectin lyase (PL1)	*E. nidulans*	40	27.5 ± 0.6	PL1	4.2.2.10	[28]
Pectin lyase (PL2)	*A. niger*	38	17.2 ± 1.4	PL1	4.2.2.10	[41]
Polygalacturonase (PG1)	*E. nidulans*	38	56.4 ± 1.7	GH28	3.2.1.15	[28]
Polygalacturonase (PG2)[a]	*A. aculeatus*	39	–	GH28	3.2.1.15	[42]

[a] Solid enzyme preparation.

methyl esterase, were selected as the best for achieving high-yield solubilization of high-molecular weight polysaccharides [8]. Since the data revealed that the reaction conditions defined a "compromise" in reaction conditions for the different enzymes [8], we found it important to investigate if other enzymes would in fact provide for a better fit of optimal reaction conditions in relation to the minimal enzymatic polysaccharide solubilization from potato pulp. We also found it important to examine if the buffer selection might affect the enzyme catalyzed solubilization, e.g. via a chelation effect. Since enzymatic conversion of pectinaceous plant biomass and pectin reaction conditions at high temperatures give rise to lower substrate viscosity, and in turn easier mixing, better substrate solubility, and lower risk of contamination [13], it was an inherent purpose to conduct the reactions at relatively high temperatures. The objective of this study was to examine the pH and temperature optima of the enzymes in statistically designed experiments and to examine the abilities of the enzymes, individually and in different combinations, to catalyze the solubilization of polysaccharides in a one-step, minimal enzyme reaction. A particular effort was made to elucidate any possible relationships between structural/amino acid sequential traits of the enzymes and their pH–temperature optima. Also, the possible chelating effects of the buffers used for the enzymatic reactions were assessed and the carbohydrate composition and molecular sizes of the solubilized potato pulp polysaccharides were compared.

2. Materials and methods

2.1. Chemicals

Pectin from citrus fruits, isopropanol, pullulan, polygalacturonic acid (MW 25,000–50,000 g/mol), D-galactose, L-arabinose, L-rhamnose monohydrate, D-fucose, D-mannose and D-galacturonic acid monohydrate were purchased from Sigma–Aldrich (Steinhein, Germany). Dextran was from Pharmacia (Uppsala, Sweden) and D-xylose and D-glucose from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Riedel-deHaën (Seelze, Germany). All chemicals used were of analytical grade.

2.2. Pulp and enzymes

Fresh potato pulp was supplied by Lyckeby Stärkelsen (Kristianstad, Sweden). The pulp was stored frozen at −21 °C until use. The enzymes used are listed in Table 1. The pectin lyase (PL1) and the polygalacturonase (PG1), both from *Emericella nidulans*, were produced essentially as described in Ref. [14]. The *Pichia pastoris* clones transformed with the pectin lyase gene AN2569.2 and the polygalacturonase gene AN4372.2 were obtained from the Fungal Genetic Stock Center as described previously [15]. The pectin lyase (PL2) from *Aspergillus niger* was supplied by Danisco A/S (Brabrand, Denmark) and the polygalacturonase (PG2) from *Aspergillus aculeatus* was from Novozymes A/S (Bagsværd, Denmark).

2.3. Protein concentration in enzyme solutions

The protein concentration was determined by the BCA Protein Assay using BSA as standard (Thermo Fisher Scientific, Rockford, IL).

2.4. Reducing ends

The amount of reducing ends was determined as described in Ref. [8].

2.5. Determination of enzyme activity

The activities of the pectin lyases were measured on 1 g/L pectin from citrus fruits using a 1% (w/w) enzyme/substrate (E/S) dosage. The pectin and buffer were pre-heated to the specific temperature prior to addition of enzyme. The increase in absorbance was determined at 235 nm for 4 min at 40 °C in an Infinite200 microplate reader (TECAN, Salzburg, Austria) with the data collection controlled by the program Tecan i-control version 1.5.14.0 (TECAN, Salzburg, Austria). One unit of enzyme activity was defined as the amount of enzyme catalyzing the release of 1 μmol of unsaturated uronide per minute. The extinction coefficient used was 5.5 mM^{-1} cm^{-1} [16]. Polygalacturonase activity was measured on 2 g/L polygalacturonic acid using 0.01–0.5% (w/w) E/S. Samples were taken every minute for 4 min; enzyme inactivation was done by addition of NaOH (50 mM) and the amount of reducing ends was measured as described above. One unit of polygalacturonase activity was defined as the amount of enzyme catalyzing the release of 1 μmol of galacturonic acid per minute under the assay conditions. In all experiments control samples were made where distilled water was added instead of enzyme solution.

2.6. pH and temperature optimum

The temperature and pH optima of the enzymes were determined via randomized, quadratic central composite statistically designed experiments. Each design contained nine different combinations of pH and temperature and three replications of the center point. Substrate concentration of potato pulp of 1% (w/w) dry matter, enzyme concentration of 1% (w/w) enzyme/substrate (E/S), and a reaction time of 1 min were constant in all experiments. The following factor limits were used: PL1: pH 8–10 and 70–100 °C, PL2: pH 3–5 and 70–100 °C, PG1: pH 3–5 and 40–60 °C, and PG2: pH 3.5–5.5 and 50–80 °C. The applied buffer was McIlvaine buffer (mix of 0.2 M disodium hydrogen phosphate and 0.1 M citric acid) where the pH was adjusted at the specific temperature. For each reaction combination the enzyme activity was determined as described above.

2.7. Determinations of kinetic parameters

Michaelis parameters K_M and V_{max} were determined by measuring the initial reaction rate of the pectin lyases on pectin from citrus fruits and the polygalacturonases on polygalacturonic acid at substrate concentrations ranging from 0.125 to 2.25 g/L. The reaction rate was determined in McIlvaine buffer with the following pH at 40 °C: pH 8.9 (PL1), pH 4.1 (PL2), pH 4.4 (PG1) and pH 4.0 (PG2). The reaction rates were determined as described above. The K_M and V_{max} values were calculated from Hanes plots, $[S]/V_0$ versus $[S]$, where $[S]$ was the concentration of substrate and V_0 was the initial rate of the enzyme catalyzed reaction.

2.8. Thermal stability

The thermal stability of the pectin lyases was examined in McIlvaine buffer at pH 8.9 (PL1) and pH 4.1 (PL2). For PL1 the stability was tested at 40, 50, 60, and 100 °C and for PL2 at 30, 50, 70, and 100 °C. Furthermore the thermal stability of the pectin lyases was examined in water at 60, 65, 70 and 100 °C (PL1) and at 50, 55, 60 and 100 °C (PL2). The thermal stability of the polygalacturonases was tested in McIlvaine buffer at pH 4.4 and 30, 40 and 50 °C (PG1) and pH 4.0 at 50, 55 and 60 °C (PG2). The enzymes were heated at the specific temperature for up to 1 h and cooled on ice. For the pectin lyases the remaining activity was determined as described above with the following modifications: the substrate and enzyme were preheated separately at 40 °C for five min, they were then mixed so the final E/S was 1% (w/w). The increase in absorbance was determined at 235 nm for up to 4 min for PL1 and for up to 20 min for PL2. The remaining activity of the polygalacturonase was examined as described above with the following modification: for PG1 it was determined at 15 °C and for PG2 at 40 °C. Samples were taken after up to 5 and 10 min for PG2 and PG1, respectively. For each enzyme residual activity versus heating time was plotted semi-logarithmically and the deactivation rate constants (k_d) were determined as the slope (by linear regression) and used to estimate the enzyme half-life ($t_{1/2}$) in minutes calculated as: $t_{1/2} = \ln(2)/k_d$.

2.9. Removal of starch from potato pulp

The starch was removed from the potato pulp using the rationalized method described previously [2].

2.10. Acid hydrolysis and high-performance anion-exchange chromatography

Hydrolysis of polysaccharide and separation and quantification of the monosaccharides by HPAEC-PAD were accomplished as described in Ref. [2].