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Expression, purification and use of the soluble domain of *Lactobacillus paracasei* β-fructosidase to optimise production of bioethanol from grass fructans

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ABSTRACT

Microbial inulinases find application in food, pharmaceutical and biofuel industries. Here, a novel *Lactobacillus paracasei* β-fructosidase was overexpressed as truncated cytosolic protein ($_{t}$fosEp) in *Escherichia coli*. Purified $_{t}$fosEp was thermostable (10–50 °C) with a pH optimum of 5; it showed highest affinity for bacterial levan (β[2-6] linked fructose) followed by nystose, chicory inulin, 1-kestose (β[2-1] linkages) and sucrose (K_m values of 0.5, 15, 15.6, 49 and 398 mM, respectively). Hydrolysis of polyfructose moieties in agriculturally-sourced grass juice (GJ) with $_{t}$fosEp resulted in the release of >13 mg/ml more bioavailable fructose than was measured in untreated GJ. Bioethanol yields from fermentation experiments with Brewer's yeast and GJ + $_{t}$fosEp were >25% higher than those achieved using untreated GJ feedstock (36.5[±4.3] and 28.2[±2.7] mg ethanol/ml, respectively). This constitutes the first specific study of the potential to ferment ethanol from grass juice and the utility of a novel core domain of β-fructosidase from *L. paracasei*.

1. Introduction

The use of plant biomass for the production of carbon–neutral biofuels continues to attract investment from research and commercial sectors (Larsen et al., 2008; Schmer et al., 2008). Industrially viable strategies to optimise the release of energy from cellulosic and lignocellulosic fractions of plant material (Hendriks and Zeeman, 2009 for recent review) continue to constitute a focal point for biofuel research (e.g. bioethanol from lignocellulosic biomass; Larsen et al., 2008). However, consideration of the complete spectrum of plant biomass that could be used for the production of bioethanol highlights additional reservoirs of carbon-energy; plant fructans comprise one of these sources.

In addition to sucrose and starch, fructans contribute to the pool of storage carbohydrates in plants (Ritsema and Smeekens, 2003). The simplest plant fructan is inulin which consists of a linear chain of β(2-1)-linked fructose monomers that extends from the fructosyl residue of a sucrose (α-β-1-2 linked glucose and fructose) starter molecule common to all plant fructans anabolised *in vivo*. Levan-type fructans also comprise a linear chain of fructose monomers; however these are linked via β(2-6) bonds. Finally, mixed-type fructans (gramminans) contain both β(2-1) and β(2-6)-linked fructose chains which can branch from the fructosyl and/or glucosyl residues of the sucrose starter molecule. Several fructan-containing plant crops including white clover (*Trifolium repens*), dandelion leaves (*Taraxacum* spp.), perennial ryegrass (*Lolium perenne* L.) and Jerusalem artichoke (*Helianthus tuberosus*) have been identified (see Kyazze et al., 2008). However, to date, few reports have been published on the production of bioethanol from plant fructans; those that are available tend to concentrate on the utilisation of *H. tuberosus* biomass (Nakamura et al., 1996; Szambelan et al., 2004). For example, it has been shown that inulin-type fructans derived from Jerusalem artichoke can be converted to ethanol by acidic hydrolysis followed by fermentation with *Saccharomyces cerevisiae* or via direct fermentation using *Kluyveromyces marxianus* strains (Negro et al., 2006). Similar research with a wider range of fructan-containing plant substrates is now required.

Given the total area (some 14 million hectares) of agricultural grassland in the UK (DEFRA, 2006), it is not surprising that perennial ryegrass has recently been identified as a possible substrate for the production of biofuels (Martinez-Perez et al., 2007). Unlike more specialist crops (e.g. wheat, sugar-beet) currently used for bioethanol production in Europe (Balat, 2007), ryegrass requires relatively few energy inputs (Donnison et al., 2009). Moreover, because of its fast establishment and robustness, ryegrass can be cultivated in marginal areas – an important consideration given the concerns surrounding use of arable land for non-food crops. The

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direct microbial fermentation of ryegrass biomass to hydrogen has recently been reported (Kyazze et al., 2008). However, the potential to produce bioethanol from ryegrass remains under investigation.

The polyfructose moieties present in agriculturally-sourced ryegrass (*L. perenne*) biomass (Udén, 2006; Conaghan et al., 2008) require enzymatic hydrolysis to support yeast growth and bioethanol production. For this purpose we looked to produce a novel β-fructosidase enzyme derived from *Lactobacillus paracasei*. Of all the epiphytic lactic acid bacteria commonly found on grass (including perennial ryegrass), the fructan fermenters are typically strains of the species *L. paracasei* ssp. *paracasei* (Muller and Lier, 1994). An extracellular (42 kDa) fructanhydrolase displaying hydrolytic activity on levan, inulin and sucrose has already been purified from *L. paracasei* (Muller and Seyfarth, 1997) and it had been postulated that this might be the only fructan-degrading enzyme in *L. paracasei* (Muller and Seyfarth, 1997). However, a recent study of fructooligosaccharide utilisation has highlighted the presence of a second fructanhydrolase in *L. paracasei* (Goh et al., 2007). This β-fructosidase protein, the subject of experimental work reported hereafter, is a cell-wall anchored protein with an estimated molecular weight of 139 kDa (Goh et al., 2007).

L. paracasei β-fructosidase (*fosE*) was expressed here as a truncated cytosolic protein ($_t$fosEp) in *Escherichia coli* (lacking *N*′ secretion signal sequence and peptidoglycan binding domains). Following biochemical determinations (thermostability, pH profile and substrate specificity), purified fractions of the $_t$fosEp were used (alongside two commercial inulinases) to pre-treat grass juice. Bioethanol fermentation experiments with enzymatically hydrolysed grass juice were then undertaken using an ethanol tolerant brewing strain of *S. cerevisiae* as proof of principle to see if the novel soluble domain had potential for application. It was hypothesised that addition of $_t$fosEp to grass juice feedstock would result in fructan hydrolysis, increased bioavailability of fermentable fructose monomers, yeast growth and ultimately the production of bioethanol.

2. Methods

2.1. Expression and characterisation of recombinant β-fructosidase

2.1.1. Bacterial strains, plasmid and reagents

Plasmid pET17b (Novagen) and *E. coli* strain BL21 (DE3) (Novagen) were used for the expression of recombinant truncated β-fructosidase protein ($_t$fosEp). Enzymes for DNA manipulation were purchased from Promega (Madison, WI, USA) unless stated otherwise, all other reagents were purchased from Sigma (Poole, Dorset, UK).

2.1.2. Construction of recombinant plasmids

DNA primers were supplied by Invitrogen (Paisley, UK): F1 = 5′-ACGTA**CATATG**GCTAGCGCTACAAGTGCTTCGTCTAC-3′; R1 = 5′-ACG TA**GGATCC**TCA*GTGGTGGTGGTGGTGGTG*TTTTTCAGTTAGTTGACCAG-3′. F1 and R1 were used to amplify a truncated ($_t$*fosE*) gene (UniProtKB/TrEMBL [Q27J21_LACPA]) from *L. paracasei* genomic DNA omitting the amino acid residues 1–40 and 749–1376 which encode a predicted gram positive *N*′ secretion signal peptide (SignalP v3.0) and peptidoglycan cell wall binding regions at the *C*′ terminus (Pfam consensus PF06458 and Prosite consensus PS50847). The tripeptide sequence, Met–Ala–Ser, was inserted prior to the fosEp truncation site to assist heterologous expression. Specifically alanine is a preferred second codon for gene expression in *E. coli* (Barnes et al., 1991). A poly-histidine tag (italicised in primer R1) was included for Ni^{2+}–NTA agarose protein purification. The PCR fragment was cloned into pET17b using *Nde*I and *Bam*HI restriction sites (emboldened in primer sequences).

2.1.3. Expression and purification of recombinant β-fructosidase

E. coli strain BL21(DE3) harbouring the $_t$*fosE*–pET17b construct was cultured in Terrific broth supplemented with 0.1 mg/ml sodium ampicillin and 20 g/l peptone. Cultures were grown (37 °C, 230 rpm) for 7 h and expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 18 h at 30 °C. Cells were harvested by centrifugation and sonicated in 0.1 M Tris-HCl (pH 8.1) containing 25% glycerol. The cytosolic fraction was isolated by removal of cell debris (20,000×*g*) and microsomes (150,000×*g*). The overexpressed $_t$fosEp was purified by affinity chromatography using Ni^{2+}–NTA agarose (Invitrogen). The purity of isolated $_t$fosE protein fractions was assessed by standard SDS polyacrylamide gel electrophoresis and protein identity confirmed by trypsin digestion and nano-LC/MS/MS analysis of a 100 kDa protein band (Pinnacle Laboratory; Newcastle University, UK). Protein concentrations were determined using the BioRad protein assay kit (BioRad, Hemel Hempstead, UK).

2.1.4. Biochemical characterisation of $_t$fosEp

Exo-inulinase activity was measured using a discontinuous assay system. One millilitre containing the saccharide of interest (dissolved in 0.1 M sodium acetate, pH 5) and the purified $_t$fosEp (≡1.62 μg protein/ml) was incubated for 30 min at 37 °C. A 0.1 ml volume from each reaction was then added to 0.9 ml 2,3,5-triphenyl tetrazolium chloride (1 mg/ml dissolved in 1 M NaOH), incubated at 37 °C for 15 min and the absorbance at 520 nm recorded. Fructose concentrations were determined relative to standards (0–6 mM fructose, in 0.1 M sodium acetate). The colorimetric reaction (Avigad et al., 1961) was found to be 20-fold more sensitive for D-fructose than D-glucose.

For thermostability determinations, stock solutions of the purified $_t$fosEp were heated for 10 min at temperatures ranging from 22 to 90 °C, cooled on ice for 5 min and assayed for residual exo-inulinase activity as described above using 10% (w/v) chicory inulin as substrate. The pH profile of the $_t$fosEp was determined using the exo-inulinase assay and 10% (w/v) chicory inulin dissolved in 0.1 M buffer solutions of sodium acetate (pH 3–6), sodium phosphate (pH 6.5–7.5), Tris–HCl (pH 8–8.5) or sodium bicarbonate (pH 9–11).

Substrate specificity for the $_t$fosEp was determined using chicory inulin (0.125–20% w/v), sucrose (0.025–2 M), 1-kestose (0.005–0.4 M), nystose (0.0045–0.35 M), raffinose (0.02–0.48 M), bacterial levan from *Zymomonas mobilis* (0.088–3.5% w/v), stachyose (0.04–0.4 M) and melezitose (0.04–0.4 M); all saccharides were dissolved in 0.1 M sodium acetate (pH 5) and assayed using the exo-inulinase system. For the determination of enzyme kinetic data, nominal molecular weights of 6200 and 12,000 Da were assumed for chicory inulin and bacterial levan, respectively.

2.1.5. Data processing and protein kinetics

DNA and protein sequences were processed using Chromas version 1.45, ClustalX version 1.8 and BioEdit version 5.0.6. Enzyme kinetics data were determined using the Michaelis–Menten equation: $v = (V_{max} \times [S]/(K_m + [S])$ and the Michaelis–Menten single substrate inhibition equation: $v = (V_{max} \times [S])/(k_m + ([S]^2/k_i) + [S]$. Curve-fitting was performed using ProFit 5.01 (QuantumSoft, Zurich, Switzerland). Enzyme activity is expressed as nkat/μg protein (equivalent to a reaction velocity of 60 nmol/min/μg protein).

2.2. Fermentation studies

2.2.1. Grass juice feedstock

Grass juice feedstock (hereafter GJ) extracted from the high-sugar perennial ryegrass *L. perenne* was supplied by the Institute of Biological, Environmental Research & Rural Sciences (IBERS, UK). Briefly, stands of *L. perenne* were maintained in 20 × 1.25 m plots under optimised management regimes (Wilkins et al., 2003). Vegetative