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Peptide Identification and Expression Analysis of Genes Involved in Inulin Metabolism in *Lactobacillus casei* AP

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Abstract

Background and objective: *Lactobacillus casei* AP are probiotic bacteria found in the gastrointestinal tracts of Indonesian breast-fed infants. *Lactobacillus casei* AP can degrade inulin through metabolic pathways that involve certain proteins. However, detailed information on how inulin is metabolized by this strain is limited. The aim of this study was to identify intracellular proteins that play roles in metabolism of inulin.

Material and methods: Bacteria were cultured on media containing commercial inulin or inulin extracted from dahlia tubers as the sole carbon source and protein identification was carried out using intracellular extraction. The bacterial intracellular proteins were isolated and separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then peptides were identified using electrospray ionization-liquid chromatography-mass spectrometry. Based on the identified proteins, corresponding genes involved in inulin metabolism were identified and their expression levels were quantified using quantitative real time-polymerase chain reactions.

Results and conclusion: Comparisons of intracellular proteins from *Lactobacillus casei* AP cultured in inulin and MRS control media showed a different protein band at 70 kDa in bacterial cells cultured in inulin or inulin extract. This protein was identified as a glycoside hydrolase (α-2,1 glycosidic) with a gene expression value of 1.55 or a fructan hydrolase (β-2,1 fructo-furanosidic) with a gene expression value of 2.68, compared to controls.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Inulins are fructans comprising glucose and fructose units with degrees of polymerization of 2-65. These linear polysaccharides of D-fructofuranose units are primarily linked by β-(2,1)-glycosidic bonds and include terminal glucose residues that are linked by α-(1,2)-glycosidic bonds [1]. The α-bond in inulin can be metabolized by digestive enzymes, whereas β-bonds cannot be metabolized. As a prebiotic compound, inulin is not structurally changed in the large intestine and is available for certain microflora of the gastrointestinal tract [2]. Members of lactobacilli and bifidobacteria are the major bacteria that are capable of degrading inulin in gastrointestinal tract [3]. Degradation of inulin by lactobacilli and bifidobacteria is associated with specific enzymes that hydrolyze β-(2,1)-fructosyl-fructose linkages to produce oligofructoses with low degrees of polymerization. Based on the published reports [4], fructooligosaccharides (FOS) metabolism varies between the microbial species. *Lactobacillus* (*L.*) *acidophilus* NCFM [5], *L. delbrueckii* [6], *L. plantarum* ST-III [7] and *L. paracasei* 1195 [8] transport FOS into bacterial cells using ATP-binding cassette transport system and degrade it via the actions of intracellular β-fructofuranosidases. In *L. paracasei* 1195, FOS metabolism is regulated by intracellular *fos*ABCDXE genes, which encode phosphor-transferase system (PTS) enzymes for fructose/mannose (*fos*ABCDX) and β-fructosidase (*fos*E) [9]. In contrast to

intracellular mechanisms, *L. paracasei* JCM 8130T, DSM 20020 [10], DSM 23505 [11] and *L. casei* IAM 1045 [12] degrade FOS outside the bacterial cells using extracellular enzyme β-fructosidase. The *L. casei* AP can degrade inulin as the sole carbon source [13,14]. Previous studies by Widodo et al. [15] showed that inulin metabolism by *L. casei* AP is facilitated by inulin influx via PTS with mannose-, fructose-, and sorbose-specific IID subunits and a putative protein ATP-binding cassette transporter. However, intracellular mechanisms, by which bacterial cells degrade inulin, have not been characterized. In this study, electrospray ionization (ESI)-LC-mass spectrometry (MS)/MS analyses were used to identify intracellular proteins that play roles in the metabolism of inulin in *L. casei* AP.

2. Materials and methods

2.1. Bacterial strains and media

The present *L. casei* AP strains were described in a previous report [13]. Inulin extraction from dahlia tubers (*D. pinnata* L.) was carried out according to Kusmiyati et al. [16]. Cells were grown in 1) De Man-Rogosa-Sharpe (MRS) broth (Merck, Germany), 2) modified MRS containing 1% (w v^{-1}) inulin extract from dahlia tubers (ieMRS), 3) modified MRS containing 1% (w v^{-1}) inulin (iMRS; Sigma-Aldrich, USA) and 4) modified MRS containing 1% (w v^{-1}) glucose (gMRS; Sigma-Aldrich, USA). All chemicals were in analytical grades.

2.2. Bacterial growth conditions

The *L. casei* AP cells were grown in MRS broth at 37°C for 24 h under microaerobic conditions and harvested using centrifugation (Eppendorf 5804 R, Germany) at 3000 ×*g* for 20 min at 4°C. Cell pellets were washed in 0.85% (w v^{-1}) NaCl solution and separately inoculated into MRS, ieMRS and iMRS media, followed by incubation at 37°C for 24 h. To assess bacterial growth and small-scale fermentation, cells were grown in media supplemented with 0.05 g l^{-1} of L-cysteine HCl and inulin or inulin extract at a final concentration of 10 g l^{-1}. Optical densities of the cultures were measured at 620 nm for each treatment condition.

2.3. Protein extraction and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The *L. casei* AP cells were grown in MRS, gMRS, ieMRS or iMRS media at 37°C. After 24 h of incubation, bacterial cells were centrifuged at 3000 ×*g* for 20 min at 4°C. After removal of supernatants, the harvested cell pellets were washed with acetate buffer (pH 5.0) and resuspended in the same buffer containing 40 µM of phenylmethanesulfonyl fluoride. Cell suspensions (pH 5) were sonicated with 40% duty cycles of impulses at 0.5 amplitude for 60 min using probes (Horn H22 D) immersed in the cell suspension to a depth of 2.5 cm. Then, crude extracts were centrifuged at 12,000 ×*g* for 15 min at 4°C and supernatants were extracted using 7-ml aliquots of 16% trichloroacetic acid. The trichloroacetic acid solutions were incubated at 4°C with agitation overnight and then centrifuged at 16,000 ×*g* for 20 min at 4°C using high-speed centrifuge (Sigma 3-30K, Germany). Pellets were washed with absolute ethanol and centrifuged at 16,000 ×*g* for 20 min at 4°C. Pellets were then dried, suspended in 50 mM tris-HCl (pH 7.3) and incubated overnight following addition of 1-ml aliquots of cold acetone. After incubation, solutions were centrifuged at 13,000 ×*g* for 20 min at room temperature and cell pellets were dried and suspended in 50 mM tris-HCl (pH 7.3). Concentrations of intracellular proteins were measured using Bradford method and microplate reader (Bio-Rad, USA) at 595 nm. Protein samples of equal concentrations were mixed with loading buffer containing 25% (w v^{-1}) of tris-HCl (4×, pH 6.8), 4% (w v^{-1}) of SDS, 20% (w v^{-1}) of glycerol, 2% (v v^{-1}) of β-mercaptoethanol and 0.001% (w v^{-1}) of bromophenol blue and boiled for 5 min before loading onto polyacrylamide gels. Electrophoresis was carried out on vertical 3 and 5% polyacrylamide slab gels containing 0.1% of SDS and tris-glycine buffer (pH 8.3) at a constant electric current of 10 mA [17]. Gels were stained with Coomassie brilliant blue G-250 to visualize the protein bands.

2.4. Peptide identification using electrospray ionization-liquid chromatography-mass spectrometry (ESI-LC-MS/MS)

Selected protein bands were excised from the SDS-PAGE gels according to Bringans et al. [18] and treated with trypsin. The resulting peptides were extracted and analyzed using ESI/MS and Shimadzu Prominence Nano HPLC system connected to 5600 Triple TOF Mass Spectrometer (AB Sciex, USA). Samples were injected into a 3.5-µm Agilent Zorbax 300SB-C18 column (Agilent Technologies, USA) and separated using a linear solvent gradient of water: acetonitrile in 0.1% (v v^{-1}) formic acid. To identify proteins, mass spectra were analyzed using Mascot Sequence Matching Software (Matrix Science, UK) and the MSPnr100 Database. Database searches were carried out using peptide masses in nonredundant NCBI Database and ProFound Search Program from Rockefeller University and ProteoMetrics (www.prowl.rockefeller.edu/cgibin/ProFound).

2.5. Candidate genes and primer design

Candidate genes of inulin metabolism were chosen based on the peptide sequences identified in ESI-LC-MS/MS analyses and were then analyzed in quantitative RT-PCR assays. Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) based on available DNA sequences of *L. casei* LevH1 and *L. paracasei* sugar multi-transport region (GenBank Assembly ID AB185852.1 and LN846901.1) (Table 1). Oligonucleotide synthesis was carried out by Genetika Science, Indonesia. Primer specificity was verified according to the numbers and sizes of bands after PCR amplification and gel electrophoresis.

Table 1. Primers used in this study

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)
Glycoside hydrolase	ATGGATGAAAAGAAACATTACAAGATG	GGCTGACTACCAGTGTTTGTTTGA	181
Fructan hydrolase	GACGCCATTCTGGCTTTCTA	CGTTTTGCCACCATCGAGGC	158

2.6. RNA isolation and cDNA synthesis

Total RNA was isolated using Easy-BLUETM Total RNA Extraction Kits (iNtRON Biotechnology, South Korea) according to the manufacturer instructions. However, an extra DNase I treatment step was carried out to eliminate contaminating DNA. The RNA concentrations were measured using NanoDrop 1000 Spectrophotometer at 260 nm (Thermo Science, USA). Then, cDNA synthesis was carried out using ReverTra Ace qPCR RT Kits (Toyobo, Japan) according to the manufacturer instructions. Briefly, 3-µg aliquots of total RNA and 3-µg aliquots of random primers (Invitrogen, USA) were mixed together, heated to 70°C for 3 min and set on ice. Synthesis of cDNA was carried out in a 50-mM tris-HCl solution (pH 8.3), containing 40 mM of KCl, 6 mM of $MgCl_2$, 10 mM of DTT and 0.3 mM of dNTPs. Reaction mixtures (30 µl) were initially incubated at 25°C for 5 min. Then, 300 U of Superscript II Reverse Transcriptase (Invitrogen, USA) were added to each mixture and incubated at 25°C for 5 min, followed by incubation at 42°C for 1 h. The enzyme was inactivated at 70°C for 15 min. Reaction mixtures were adjusted to 100 µl using ultrapure water and the cDNA was used in RT-PCR analyses.

2.7. Quantitative PCR (qPCR)

Quantitative PCR was carried out according to KAPA SYBRR FAST Universal One-Step qPCR (Merck, Germany) protocol. Briefly, PCR reaction mixtures included 10 µl of 2× Kappa SYBR Fast, 0.4 µl of 50× Kappa RT Mix, 0.4 µl of 10 mM dUTP, 0.4 µl of each 5 µM forward and reverse primers, 1 µl of 300-ng total RNA and 7.4 µl of RNase-free water. The RT-qPCR was carried out using one-step program of reverse transcription at 42°C for 5 min and enzyme inactivation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s and annealing at 55°C for 30 s. Cycle threshold values for glycoside hydrolase and fructan hydrolase genes were normalized to those of glyceraldehyde 3-phosphate dehydrogenase housekeeping genes [19]. Relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ equation [20]. Significant differences were identified using independent *t*-test and SPSS 16.0 software (IBM Analytics, USA).

3. Results and discussion

3.1. Bacterial growth on media containing inulin or inulin extract as the sole carbon source

In this study, bacteria were initially cultured in MRS broth for 24 h. After harvesting, bacteria were transferred into iMRS or ieMRS and their growth patterns were compared with each other (Figure 1). During small-scale fermentation (30 ml), growth patterns of *L. casei* AP in iMRS and ieMRS were similar to those of *L. casei* AP in MRS, indicating that *L. casei* AP could grow with inulin as the sole carbon source. Cultures reached the exponential growth phase after 4 h under all growth media conditions. However, Optical Density values for cells in control MRS were higher than those in iMRS and ieMRS during the exponential phase.

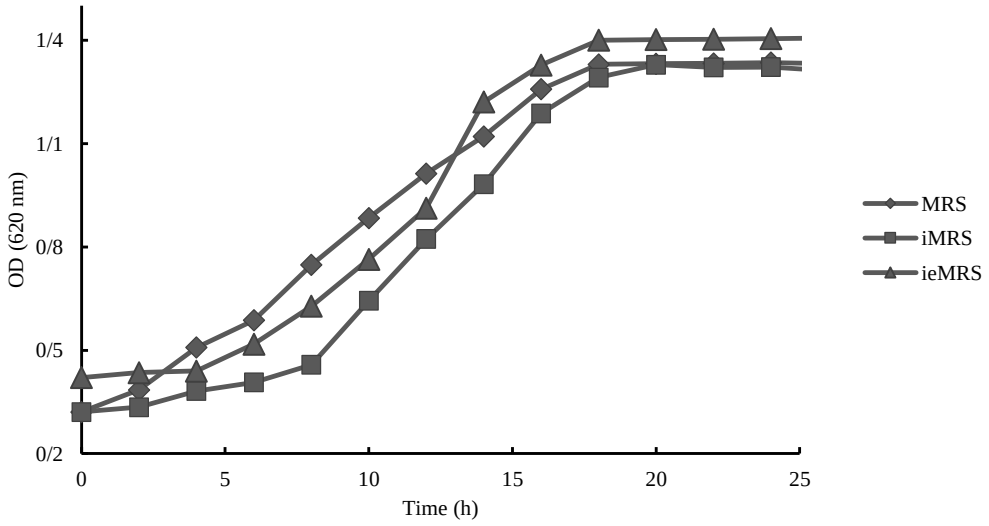

Figure 1. Growth curves of *Lactobacillus casei* AP in MRS broth, inulin (iMRS) and inulin extract (ieMRS), OD=Optical Density

3.2. Protein separation and peptide identification

A unique 70-kDa intracellular protein was detected in *L. casei* AP grown in inulin or inulin extract from dahlia tubers (Figure 2). Therefore, this protein seems to play an important role in the metabolism of inulin as the sole carbon source in *L. casei* AP cells. These findings are similar to previous findings by Warchol et al. [21], who associated a 70-kDa protein with β-fructofuranosidase and invertase activities in *Bifidobacterium infantis* ATCC 15697. These intracellular proteins catalyze metabolism of FOS, sucrose and inulin. Chen et al. [7] reported a 56.7-kDa β-fructofuranosidase protein encoded by *sac*A gene in *L. plantarum* ST-III. In the current study, amino acid sequences of inulin-associated 70-kDa peptide were identified using ESI-LC-MS/MS analyses. The amino acid sequence alignments showed 100% similarity between the 70-kDa *L. casei* AP protein and fructan hydrolase and glycoside hydrolase enzymes (Table 2). Glycoside hydrolase is a carbohydrate-active enzyme (CAZy) that hydrolyzes disaccharides into monosaccharides [21]. The presence of this intracellular protein in *L. casei* AP grown with inulin or inulin extract possibly allows metabolism of α-2,1 glycosidic bonds between glucose and fructose moieties of inulin [22]. In a study, Velikova et al. [23] identified a glycoside hydrolase in multiunit proteins of the GH32 protein family, including inulinase, levanase, invertase, fructan transferase and fructose transferase. These enzymes play important roles in hydrolysis of various fructooligosaccharides and differ in their fractal chain cutting regions. Furthermore, the 70-kDa protein from *L. casei* AP had 100% similarity to fructan hydrolase (Table 2). This enzyme hydrolyses β-2,1 fructofuranosidic bonds, resulting in release of terminal fructose residues [22]. Fructan hydrolase homologies with β-fructosidase, fructan β-fructosidase and sucrose-6-phosphate hydrolase. Of these enzymes, fructan hydrolase and fructan β-fructosidase play important roles in inulin metabolism in *L. pentosus* B235 [24], *L. paracasei* 1195 [8], *L. paracasei* subsp. *paracasei* 8700: 2 [25] and *L. casei* IAM 1045 [12].

In the present SDS-PAGE and amino acid sequencing analyses of inulin-supplemented bacteria, proteins with phosphotransferase and phosphoenol pyruvate-protein phosphotransferase PTS for mannose/fructose were identified (Table 2). These proteins may contribute to major PTS activities for inulin in *L. casei* AP. Relatively, a previous study on FOS metabolism in *L. plantarum* ST-III showed that β-fructofuranosidase (SacA) was involved in PTS of intracellular FOS metabolism [7]. Another study located mannose, fructose and sorbose-specific PTS IID subunits and an ATP-binding cassette transporter in cell walls [15]. Other similar studies demonstrated the highlighted enzymes in bacterial cell walls and intracellular compartments [9]. In the current study, an oligoendo-peptidase enzyme has been identified in *L. casei* AP (Table 2), which possibly provides amino acids for the bacterial growth and glycolysis. Inulin is a fructan polymer consisting of one glucose and many fructose units. The current data suggest that inulin metabolism in *L. casei* AP involves the transport of inulin into the bacterial cells via PTS/ PTSmanosa specific for mannose and fructose metabolisms, followed by intracellular hydrolysis by glycoside (α-2,1 glycosidic) and fructan (β-2,1 fructofuranosidic) hydrolases [15].

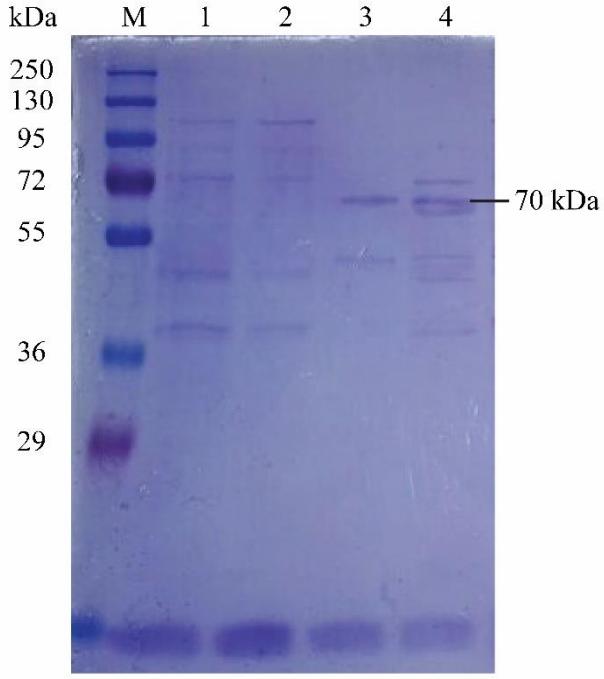

Figure 2. Intracellular proteins from *Lactobacillus casei* AP grown in glucose-MRS (1), MRS (2), iMRS (3) or ieMRS (4). M, protein marker.
MRS= Man-Rogosa-Sharpe
ieMRS= inulin extract from dahlia tubers

Table 2. Selected amino acid sequences and relevant protein names of 70-kDa proteins in *Lactobacillus casei* AP

Protein name	Accession no.	Identity (%)	Score	MW	Peptide sequence
Fructan hydrolase	BAD88632.1	100	110	70.2	R.FTVSRSDEQVQEGKYKISFSWDE.L
Glycoside hydrolase	KFL96866.1	100	102	70.6	R.LNFNIYDADTGRGYSP.L
Oligoendopeptidase	WP_082623122.1	79	80	67.9	K.ATMAALAPLGK.D
Phosphotransferase system	WP_079322000.1	73	47	70.4	K.MMGEGFGMDPVDK.Q
Pyruvate oxidase	WP_095761432.1	83	69	66.2	K.AGEPVVIDAK.I

3.3. Gene expression

In general, PCR analyses of glycoside and fructan hydrolase genes from *L. casei* AP grown in inulin extract resulted in PCR products of 181 and 158 bp, respectively (Figure 3). Presence of glycoside and fructan hydrolase genes suggests that they possibly contribute to inulin degradation in *L. casei* AP. Expression levels of glycoside and fructan hydrolases did not differ significantly between the bacterial cells grown with inulin and the control group (Figure 4). However, fructan hydrolase gene expression increased by inulin supplementation. The fructan hydrolase gene was expressed at higher levels than that the glycoside hydrolase gene was. Buntin et al. [26] reported that these genes were induced by fructans and repressed by their products. In the current study, feedback inhibition by fructose as the major product of glycoside and fructan hydrolases maybe contributed to low expression levels of these two genes. According to Goh et al. [9], higher fructan hydrolase expression levels reflect its ability to degrade carbohydrates with β-fructose bonds.

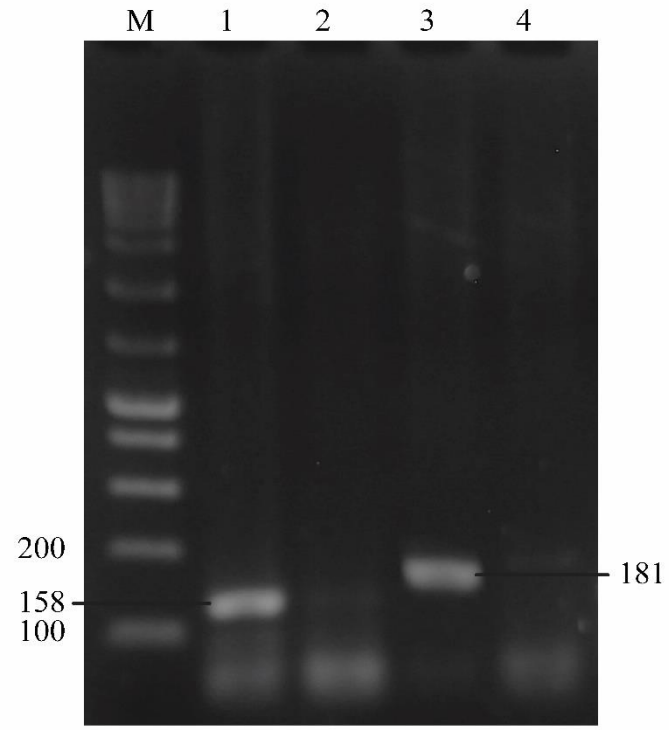

Figure 3. Amplification of fructan hydrolase and glycoside hydrolase genes in *Lactobacillus casei* AP. 1) fructan hydrolase gene expression in cells grown in ieMRS, 2) fructan hydrolase expression in cells grown in MRS media, 3) glycoside hydrolase gene expression in cells grown in ieMRS, and 4) glycoside hydrolase gene expression in cells grown in MRS. M, 100-bp DNA ladder.
MRS= Man-Rogosa-Sharpe, ieMRS= inulin extract from dahlia tubers

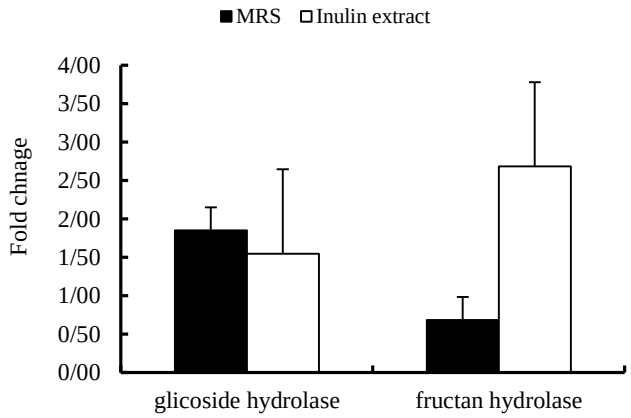

Figure 4. Fold changes in glycoside hydrolase and fructan hydrolase gene expression levels in *Lactobacillus casei* AP grown in MRS and ieMRS
ieMRS= inulin extract from dahlia tubers
MRS= Man-Rogosa-Sharpe

4. Conclusion

In conclusion, extraction of intracellular *L. casei* AP proteins revealed a 70-kDa protein in cells grown in the presence of inulin or inulin extract. This protein was identified as glycoside hydrolase (α-2,1-glycosidic) or fructan hydrolase (β-2,1-fructofuranosidic) and the increased expression levels of corresponding genes verified roles of these proteins in probiotic metabolism of inulin.

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6. Conflict of interest

The authors declare no conflict of interest.

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شناسایی پپتید و بیان ژن‌های درگیر در سوخت و ساز اینولین در لاکتوباسیلوس کازئی AP

نور کاسمی یاتی[۱]، سانارتی سانارتی[۱و۲]، تاتیک دوی واهیونگ سین[۱و۳]، ویدودو ویدودو[۱و۴*]

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چکیده

سابقه و هدف: *لاکتوباسیلوس کازئی* AP باکتری زیست یاری[۱] است که در مجرای معده ای-روده ای نوزادان اندونزیایی تغذیه شده با شیر مادر یافت می شود. *لاکتوباسیلوس کازئی* AP قادر است اینولین را از مسیر سوخت و ساز پروتئین های مشخصی تجزیه کند. اگرچه، جزییات اطلاعات اینکه چگونه اینولین توسط این گونه متابولیزه می شود، محدود است. هدف مطالعه حاضر شناسایی پروتئین های درون یاخته ای[۲] است که در سوخت و ساز اینولین نقش دارند.

مواد و روش ها: باکتری ها در محیط حاوی اینولین تجاری یا اینولین استخراج شده از غده های دالیا، به عنوان تنها منبع کربن کشت داده شدند و شناسایی پروتئین با استفاده از استخراج درون یاخته ای انجام شد. پروتئین های درون یاخته ای باکتریایی با ژل الکتروفورز سدیم دودسیل سولفات-پلی آکریلامید جدا و تفکیک شدند و سپس پپتیدها با طیف سنجی جرمی مایع-یونش الکترواسپری[۳] شناسایی شدند. بر اساس پروتئین های شناسایی شده، ژن های متناظر درگیر در سوخت و ساز اینولین شناسایی شدند و میزان بیان آنها به روش کمی واکنش های زنجیره ای پلیمراز-زمان واقعی اندازه‌گیری شد.

یافته‌ها و نتیجه‌گیری: مقایسه پروتئین های درون یاخته ای *لاکتوباسیلوس کازئی* AP کشت داده شده در اینولین و محیط کشت شاهد MRS، باند پروتئین متفاوتی در ۷۰ کیلو دالتون را در سلول باکتریایی کشت داده شده در اینولین یا عصاره اینولین نشان داد. این پروتئین به عنوان یک گلیکوزید هیدرولاز (۲ -α و ۱ گلیکوزیدیک) با میزان بیان ژن ۱/۵۵ یا یک فروکتان هیدرولاز (۲-βو۱ فروکتوفورانوزیدیک) با میزان بیان ژن ۲/۶۸ در مقایسه با شاهد شناسایی شد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- غده دالیا
- فروکتان هیدرولاز
- گلیکوزید هیدرولاز
- اینولین
- شناسایی پپتید

***نویسنده مسئول**

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[۱] Probiotic bacteria
[۲] Intracellular
[۳] Electrospray ionization-liquid chromatography-mass spectrometry