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REPORT

Novel enzymatic catalysts for fructooligosaccharides production from cane sugar

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ABSTRACT

Fructooligosaccharides (FOS) are soluble fibers with prebiotic effects in humans and animals. Inulin-type FOS [fructosyl-fructose β (2→1) linkages] are currently in the market, while levan-type FOS [fructosyl-fructose β 2→6) linkages] are not commercially available. The work was attempted to produce modified enzymes capable to yield short-chain FOS with different types of linkages. The genes encoding the enzymes 1) β-fructosidase (BfrA, EC 3.2.1.26) from the bacterium *Thermotoga maritima* and 2) sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) from the plant *Schedonorus arundinaceus* were modified by directed mutagenesis and expressed constitutively in the yeast *Pichia pastoris*. Three BfrA mutants (W14Y, W14Y–N16S and W14Y–W256Y) enhanced 4 fold the ratio of fructosylation/hydrolysis activities. The reaction with sucrose (1.75 M) yielded 37 % (w/w) FOS with a predominant composition of 6-kestose and neokestose. On the other hand, 1-SST synthesized 1-kestose and nystose in ratio 9:1, with their sum representing 55-60 % (w/w) of total carbohydrates. The culture supernatants from the recombinant *P. pastoris* clones expressing either BfrA or 1-SST were submitted to ultrafiltration (concentration, dialysis) and lyophilization. The resulting water-soluble powders displayed high specific activity (> 8 000 U/g), high protein purity (> 50 %) and remained stable after 1-year storage at 4 °C. The enzymatic catalysts BfrA and 1-SST provide attractive alternatives for cane sugar conversion into short-chain FOS of the levan- and inulin-type, respectively. This work received the Annual Award of the Cuban Academy of Sciences for the year 2019.

Keywords: Fructooligosaccharides, FOS, β-fructofuranosidases, fructosyltransferases

RESUMEN

Catalizadores enzimáticos novedosos para la producción de fructooligosacáridos a partir del azúcar de caña. Los fructooligosacáridos (FOS) son fibras solubles con acción prebiótica en Seres humanos y animales. En la actualidad se comercializan FOS tipo inulina (enlaces fructosil-fructosa β [2→1]), pero no hay disponibilidad de FOS tipo levana (enlaces fructosil - fructosa β[2→6]). Objetivo: obtener enzimas modificadas productoras de FOS de cadenas cortas y diferentes tipos de enlaces. Los genes que codifican las enzimas β-fructosidasa (BfrA, EC 3.2.1.26) de la bacteria *Thermotoga maritima* y sacarosa: sacarosa 1-fructosiltransferasa (1-SST, EC 2.4.1.99) de la planta *Schedonorus arundinaceus* se modificaron mediante mutagénesis dirigida y se expresaron de forma constitutiva en la levadura *Pichia pastoris*. Tres mutantes BfrA (W14Y, W14Y–N16S y W14Y–W256Y) incrementaron 4 veces la relación de actividades transferasa/hidrolasa. La reacción con sacarosa (1,75 M) rindió 37 % (w/w) FOS con una distribución mayoritaria de 6-kestosa y neokestosa. Por otro lado, 1-SST produjo 1-kestosa y nistosa en proporción 9:1, cuya suma representó de 55 % a 60 % (w/w) del contenido de carbohidratos. Mediante procesos de concentración, diálisis y liofilización del sobrenadante de cultivo de los clones recombinantes de *P. pastoris*, se desarrollaron preparados de BfrA y 1-SST en forma de polvo soluble en agua, de alta actividad específica (> 8000 U/g), elevada pureza proteica (> 50 %) y estable durante 1 año de almacenamiento a 4 °C. Los catalizadores enzimáticos BfrA y 1-SST constituyen alternativas atractivas para la conversión del azúcar de caña en FOS de cadenas cortas del tipo levana e inulina, respectivamente. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2019.

Palabras clave: Fructooligosacáridos, FOS, β-fructosidasas, fructosiltransferasas

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Introduction

Fructooligosacharides (FOS) comsumption is highly beneficial both for humans and animals [1]. The synthesis of natural prebiotics occurs by enzymatic processes from sucrose. The first fructosylation cycle can generate 1-kestose (αglu [1,2] βfru [1,2] βfru), 6-kestose (αglu [1,2]βfru [6,2] βfru) or neokestose (βfru [2,6] αglu [1,2] βfru),depending on the type and origin of the enzyme used for catalysis. The trisacharide can extend through successive transfructosylation reactions. The ingestion of 1-kestose, 6-kestose or neokestose differentially stimulates the resident intestinal flora, particularly bifidobacteria and lactobacilli [2, 3].

FOS manufacturers carry out sugar cane or sugar beet conversion into FOS by using customized

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catalysts which are not available in the market, mainly β-fructofuranosidases (EC 3.2.1.26) or fructosyltransferases (EC 2.4.1.100) isolated from fungi of the genera *Aspergillus* o *Aureobasidium*. Those enzymes synthesize exclusively lineal FOS series bearing β (2→1) bonds. The FOS molecules produced show an approximate percentage rate of tri- (1-kestose), tetra- (nystose) and penta-sacharide (fructosyl nystose) of 37:53:10 at the time of maximal total FOS yield (approximately 55-60%, w/w). Moreover, there is no β (2→6)-bound FOS available in the market due to the lack of adequate enzymatic catalysts for its production [4].

In this setting, the β-fructosidase enzyme (BfrA, EC 3.2.1.26) of the thermophilic bacterium *Thermotoga marítima* is characterized by the successful total inversion of sucrose at high concentrations of 600-800 g/L at 60-70 ºC. Lower FOS yields are obtained at this initial phase of the reaction [5, 6]. The availability of the 3D structure of BfrA and the identification of the amino acids forming the active site of the enzyme which interact with sucrose as ligand [7, 8] make possible establishing a rational design of genetic mutations to increase the ratio of transferase/hydrolase enzyme reactions. The increase in the fructosylation reaction (acceptor molecule for the fructosyl residue: sucrose or fructan) in detriment of the hydrolysis reaction (acceptor molecule for the fructosyl residue: water) leads to higher FOS yields.

The sucrose:sucrose 1 fructosyltransferase enzyme (1-SST, EC 2.4.1.99) starts the synthesis of fructans in plants, with 1-kestose as the main reaction product. Plant fructosyltransferases, including 1-SST, are glycoproteins which evolved from vacuolar invertases through gradual point mutations, favoring the synthesis reaction of FOS with a decrease or loss of the ancestral hydrolytic activity [9, 10].

The production of the BrfA and 1-SST enzymes for biotechnological purposes from their natural sources is not feasible, neither technically or economically. Other recombinant and more adequate host organisms for enzyme production have being traditionally considered, as the generally regarded as safe (GRAS) yeast *Pichia pastoris*. This non-saccharolytic yeast has the capacity for secreting and glycosylating heterologous proteins efficiently. An additional advantage is the absence of contaminant sucrose- or fructan-transforming activity in culture supernatants [11]. BfrA and 1-SST are enzymes of food industrial applications. Therefore, it is recommended to explore the use of a host organism provided with a constitutive expression instead of the traditional expression system by induction with methanol. Such reagents are troublesome to handle due to their flammable nature and also toxic for human health.

Hence, considering these backgrounds this work was aimed to develop novel enzymatic biocatalists to easily convert sucrose into short-chain FOS molecules of different bonds. More precisely, mutants of the abovementioned bacterial β-fructosidase enzyme were obtained and expressed in *P. pastoris* with an increase in the fructisylation/hydrolysis activity ratio, mainly yielding β (2→6)-bound FOS. A *P. pastoris* was generated able to secrete high yields of the vegetal fructosyltransferase specialized in 1-kestose synthesis. Enzymatic preparations were also obtained, highly active and very stable, able to be applied for the commercial production of short-chain FOS.

Materials and methods

Microorganisms and culture media

The strains X-33 (wildtype) and GS115 (auxotrophic to His [4]) of *P. pastoris* (Invitrogen) were used as expression hosts.

P. pastoris cells were grown at 30 ºC in culture media YPG (1 % [w/v] Yeast extract, 2 % [w/v] peptone, 2 % [v/v] glycerol) or YNB-G (1.34 % [w/v] yeast nitrogenated base without amino acids, 2 % [w/v] glucose) supplemented or not with L-histidine (100 g/L).

The discontinuous phase fermentation medium was composed of 5 % (v/v) glycerol, 0.5 % (w/v) yeast extract, 2.2 % (w/v) $(NH_4)_2SO_4$, 1.82 % (w/v) K_2HPO_4, 0.75 % (w/v) $MgSO_4$ and 0.05 % (w/v) $CaCl_2$, supplemented with vitamins and traces as recommended by Cregg and Madden [12]. The feeding medium consisted of 50 % (v/v) glycerol. As required, glycerol was replaced by cane sugar as the sole carbon source.

Obtention of mutant variants of the β-fructosidase enzyme (BfrA) of *T. maritima* and its expression in *P. pastoris*

Polymerase chain reactions were run for enzyme active site-directed mutation at positions W14Y, W14Y-N16S and W14Y-W256Y, by using the *Pfu* DNA polymerase (Promega, EE. UU). The plasmid pALS263 was used as template, carrying the β-fructosidase (BfrA) gene of *T. maritima* MSB8 (GenBank Accession No. AJ001073), codon-optimized for its expression in *P. pastoris* [6]. The PCR *Eco*R I-*Kpn* I (87 bp) restrictase digestion products were used to replace the equivalent fragment in the pALS263 fragment, and the respective pALS266 and pALS267 constructs were obtained. The same was done with the same enzymes' digestion product 689bp-long fragment, to introduce the W256Y mutation in the pALS266, generating the pALS268 construct. The presence of the designed mutations was corroborated by sequencing of the respective plasmid constructs, further checking the fusion of the BfrA gene to the factor α signal peptide of *Saccharomyces cerevisiae* and that coincidence of the six histidines in the His6x tag in the same reading frame. The empty vector pGAPZαC (negative control) and plasmids pALS263, pALS266, pALS267 and pALS268 were linearized by *Avr*II and subsequently electroporated in the *P. pastoris* X-33 strain. Transformants were selected in plates containing solid YPG medium supplemented with zeocin (100 µg/mL).

Expression of multiple copies of the 1-SST of *Schedonorus* arundinaceus in *P. pastoris*

A DNA fragment of 1668 bp coding for the 548 aa. mature region of the 1-SST of *S. arundinaceus* was amplified by PCR, using a full length complementary DNA as template [10], and primers 5´GGGGC-GAATTCCGCCGACGGCGGGTTC´3 and 5´TG-GTTCTAGATACAAGTCGTCGTTCGTG´3 with bases substituted to create flanking sites *Eco*R I and *Xba* I, respectively. The amplified product Sa1-SST

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without stop codon was then *Eco*R I-*Xba* I digested and cloned into the respective sites in the expression vector pGAPZαC (Invitrogen, Carlsbad, CA, USA), generating the pALS214 construct. The constitutive expression cassette of Sa1-SST was then multimerized through repetitive cycles of cut and self-ligation of the 2.82 kb-long *Bgl* II-*Bam*H I fragment from the pALS214 plasmid. Multimers of ~17 kb were extracted from agarose gels and inserted into the *Bam*H I site in the pAO815 vector resistant to ampicillin (Invitrogen, Carlsbad, CA, USA).

The pALS227 construct was confirmed as bearing the six tandem copies of the Sa1-SST and the *His4* gene flanked by the 5´AOX1 and 3´AOX1 regions, the tandem repeats generated through double homologous recombination events. Single copy clones of the *Avr* II-linearized pALS214 plasmid were eletroporated into the X-33 wild strain. Transformants were selected in YPG plates with zeocin (100 µg/mL).Constitutive clones bearing six copies were linearized by *Bgl* II digestion from plasmid pALS227 and the 22.3 kb fragment electroporated into the GS115 stain auxotrophic for his4. Transformants were selected in plates containing YNB-G medium without histidine supplementation. Cell electroporation was done according to the instructions in the Manual for expression in *Pichia* (Invitrogen-Thermo Fisher Scientific). Clones bearing up to nine copies of the 1-SST transgen were obtained through retransformation assays from recombinant yeast.

Fed-batch culture of *P. pastoris* clones

Recombinant yeast clones were grown in an INFORS HT bioreactor of 7.5 L volume (5 L working volume) filled with 3.5 L of fermentation culture medium with temperature control at 30 ºC and pH 5.5. The pH value was adjusted by adding ammoniac or phosphoric acid as required. Dissolved oxygen was kept into the medium at 20 % by the automatic variation in the stirring speed (500-900 rpm) and an air flow of 1-2 vvm. In the phase of incremented culture, 1.5 L of medium was added (50 % [w/v] of sugar cane sucrose and 0.5 % [w/v] of yeast extract) at a constant growing flow of 5.4-8.2 mL/h per liter. Operation conditions during the incremented culture were: 30 ºC, pH 5.5, stirring at 900 rpm and aeration at 2 vvm. Fermentation was run for three days.

Enzyme assays

A unit of BfrA activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute at the initial speed of reaction with 120 mM of sucrose, in 100 mM sodium acetate buffer, at pH 5.5 and 60 ºC. The global β-fructosidase activity of the variants was estimated as the kinetics in the release of glucose, concomitant to reactions of hydrolysis (fructose release) and transfructosylation (FOS synthesis). A unit of 1-SST activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute at the initial reaction speed with 1.46 M sucrose (500 g/L), 100 mM sodium acetate at pH 5.5 and 30 ºC. Reducing sugars in the reaction mixtures were quantified through the colorimetric method of the dinitrosalicilic acid (DNSA), using calibration curves of glucose for sucrose substrate and fructose for rafinose and fructans.

Carbohydrate analysis

Carbohydrates in the reaction mixtures of 1-SST were analyzed by High Performance Liquid Chromatography (HPLC) in an Aminex HPX-42C (0.78 × 30) cm column (Bio-Rad; CA, USA) equipped with a refractive index detector. The temperature of the column was kept at 85 º C. Water was used as mobile phase at a constant flow of 0.5 mL/min. Samples (20 µL) were diluted properly prior to injection. Solutions with defined amounts of fructose, glucose, sucrose, 1-kestose, nystose and fructosylnistose were used as standards. A mix of fructose, rafinose and stachyose was used as alternative standard as required. The program package BioCrom version 3.0, developed at the CIGB (Havana, Cuba), was used for sugars quantification.

Oligosaccharides obtained by the reactions of the native and mutated BfrA were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD). A Dionex DX-500 IC system was used equipped with a GP50 pump, an ED50 electrochemical detector, a U3000 automatic injector and a CarboPac PA200 column (3 mm × 250 mm, catalog number 062896, Thermo Scientific; USA), at 30 ºC. The product was eluted by applying a sodium acetate gradient with 100 mM NaOH at 0.5 mL/min as follows: sodium acetate 5-100 mM in 25 min, 100-400 mM in 60 min and 10 to re-equilibrate (5 mM sodium acetate). Standard curves of FOS (1-kestose, 6-kestose, neokestose, 1,1-kestose, 1,6-kestose, 6,6-kestose and 1,1,1-kestopentose) were used for the quantitative analysis by HPAE-PAD. Glucose, fructose and sucrose were quantified by HPLC with a Waters 600 E Plus system controller (Waters Corp.; Milford, MA, USA) equipped with a diffraction detector index (Waters 410) and a column Prevail Carbohydrate ES (250 × 4.6 mm) at 30 °C. Sugars were eluted with a mobile phase consisting on acetonitrile and water 70:30 (v/v) at a range flow of 1 mL/min. Samples were properly diluted prior to injection.

Time progression of FOS production

The effects of sucrose concentration and enzyme dose on FOS production were examined through time-course reactions using a crude 1SST preparation consisting on the ultrafiltrated culture supernatant of the clone PGFT6 x – 308. All reaction mixes were prepared in 100 mM acetate buffer, pH 5.5. Reactions per lots were run in a 1-L container under agitation at 250 rpm or a 7.5-L bioreactor under agitation at 250 rpm. The 0.5 mL samples were extracted at fixed intervals and incubated into boiling water for 5 min to stop the reaction prior to HPLC analysis.

Results and discussion

Construction of BfrA mutants and its constitutive expression in *P. pastoris*

A template was used, consisting on the codon-optimized gene coding for the intact β-fructosidase (BfrA) protein of the hyperthermophilic bacterium *T. maritima* strain MSB8. Three amino acids (W14, N16 and W256), strictly preserved at the ctive site of sucrose hydrolases enzymes were replaced by their counterparts in vegetal enzymes showing

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frutosiltransferase activity. The native BfrA and the mutants (W14Y, W14Y-N16S and W14Y-W256Y) were fused to the signal peptide of factor α from *S. cerevisiae*, and further expressed in *P. pastoris* under the control of the strong and constitutive promoter of glyceraldehyde 3P dehydrogenase (GAP). Neither the constitutive expression of the native BfrA nor the three mutants inhibited cell growth. In all cases, the recombinant yeast secreted an active thermostable enzyme. The sucrase activity was detected both in the 74-76 % culture supernatant and in the 24-26 % intact biomass [13].

Increase in the fructosylation/hydrolysis activity ratio in BfrA mutants

The initial speeds of the hydrolase (V_F) and transferase (V_{G-F}) activities of each mutant variant were compared with that of the native BfrA enzyme in reactions at high sucrose concentration (1.75 M) and 50, 60 and 70 °C. The three mutants (W14Y, W14Y-N16S and W14Y-W256Y) showed a similar two- to three-fold increase in the transferase/hydrolase ratio as compared to the native enzyme, regardless the temperature. In all the cases, it was shown that hydrolysis remained as the predominant reaction, with more than 80 % of the fructosyl residues transferred to water [13].

Yields and composition of FOS products from the native BfrA and mutant enzymes

BfrA and the three mutant variants were able to produce a FOS mix mainly composed of 6-kestose [13]. The W14Y mutant showed the highest specific activity (841 U/mg) and 88 % (w/w) of FOS global yield, being an attractive candidate to produce FOS with β(2→6) bonds in high temperature (50-70 %) reactions [13]. Similarly, 6K and NK have been reported as the main FOS products of mesophilic yeast fructosidases with preponderance of sucrase activity [14]. Despite the prebiotic properties of 6K, NK and 1K, the low yields of total FOS with BfrA and yeast β-fructosidases (below 16 %, w/w) make those enzymes commercially unprofitable for converting sucrose into short-chain fructans. The enzyme reaction was performed at 1.75 M sucrose, sodium acetate (pH 5.5) a 60 ºC for 22 h. The products quantitated in the reaction mix were: glucose (G), fructose (F), sucrose (S), 1-kestose (1K), 6-kestose (6 K), neokestose (NK), 1,1-kestose (1,1 N), 1,6-kestose (1,6 N), 6,6-kestose (6,6 N) and 1,1,1-ketopentose or fructosylnistose (FN). The total FOS production contained tri-, tetra- and pentasaccharides. All the values stood for three replicates, with standard deviation lower than 10 %.

Constitutive expression of the 1-SST gene from *S. arundinaceus* in *P. pastoris*

The cDNA of the 1-SST gene from *S. arundinaceus* (Sa1-SST) codes for a precursor protein of 654 amino acids, with a N-terminus sequence of 106 amino acids (pre and propeptides), which are excised during protein vacuolar trafficking. The mature Sa-SST protein (548 amino acids) was fused to fused to a yeast signal peptide and expressed in *P. pastoris* under the control of the constitutive GAP promoter. The single-copy clone (PGFT1x) was grown in saline medium supplemented with glycerol, yielding 102 g of biomass/L (dry weight) and displaying 3955 U/L recombinant enzyme (Sa1-SSTrec) activity at 72 h of culture. The volumetric productivity was 55 U/L/h, the constitutive expression of the transgene not inhibiting the yeast cell growth [15].

Higher Sa1-SSTrec yields through increased gene dose and using sucrose as carbon source

The *pGAP-1SST* expression cassette was multimerized in vitro to increase the transgene transcription rate and, thereby, the enzyme yield. Multimers containing three and six cassettes were cloned into vectors bearing different selection markers, and they were further integrated by homologous recombination into specific regions of the *P. pastoris* genome. It was determined by Southern blot and qPCR that clones PGFT1×, PGFT6× and PGFT6×-308 bear 1, 6 and 9 intact copies of the transgene. The increase in the transgene dosage up to 9 copies proportionally increased the yield and the secretion of the recombinant enzyme. In fact, the constitutive expression of the *1-SST* gene provided the yeast with saccharolytic activity. The substitution in the culture medium of glycerol by cane sugar, a cheaper carbon source, increased the yield of the Sa1-SSTrec up to 60 U/mL in the PGFT6×-308 clone. The glucose released the activity of the recombinant enzyme on sucrose in the culture medium had a stimulatory effect in the transcription of the GAP promoter [15].

Sa1- SSTrec converts sucrose into 1-kestose and nystose

Sa1-SSTrec was purified by ion exchange chromatography from the culture supernatant of clone PGFT6x-308, yielding an active and stable glycoprotein. Sa1-SSTrec showed no invertase activity on sucrose, but it was able to hydrolyse the 1-kestose product. The production process of FOS by Sa1-SSTrec was assessed under optimal operation conditions in a 7.5-L stirred tank bioreactor [15]. The enzyme produced equimolar ratios of glucose and trisaccharide (GF_3). The release of free fructose indicated the optimal moment to heat inactive the enzyme, reaching a FOS value ($GF_2 + GF_3$) of approximately 360 g/L, up to 55-66 % (w/w) of final carbohydrate content, with up to 100 g of remaining sucrose substrate [15].

The products synthesized by Sa1-SSTrec were structurally characterized by HPAEC-PAD. The chromatographic analysis of the reaction mix at the optimal yield of total FOS (120 min) revealed that GF_2 and GF_3 corresponded to 1-kestose and nystose at a 9:1 ratio. The absence of peaks consistent with 6-kestose or neokestose evidenced that Sa1-SSTrec only bound fructose residues by β (2→ 1) bonds.

Similar total FOS yields of 55-60 % have been obtained with more thermostable fungal frucotsyltransferases, but at three times lower GF_2 ratios, due to the increase in GF_3 and the formation of GF_4 [4, 16, 17]. Sucrose is the donor substrate and preferential acceptor of Sa1-SSTrec. The 1-kestose synthesis as single trisaccharide and the absence of fructosyl transfer to water, even at a lower sucrose concentration of 50-100 mM, denotes the high specialization of Sa1-SST on its biological function as initiator of fructan synthesis in a perennial monocothledoneous plant.

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Obtention of enzymatic preparations of BfrA and 1-SST for commercial purposes

The recombinant yeasts were grown in saline medium with cane sugar as the carbon source in fed-batch fermentations with controlled parameters: pH 5.5, 28 ºC for 72 h. The culture supernatants containing the BfrA and 1-SST enzymes were subjected to a simultaneous process of concentration and dialysis in 100 mM sodium acetate buffer (pH 5.5) and subsequently lyophilized. The enzyme preparations' powder soluble in water showed a high enzymatic activity (>8000 U/g) and high protein purity (> 50 %). Besides, they were stable for one year upon storage at 4 and 25 ºC.

Conclusions

FOS-producer companies tend to convert sucrose with custom-made catalysts, which are not available in the market, containing fungi enzymes. Advantageously, in this work, novel catalysts were produced through the extracellular secretion in *P. pastoris* of the 1-SST enzyme from the plant *S. arundinaceus* and mutated variants of the β-fructosidase enzyme from the *T. marítima* bacterium. The recombinant enzymes are presented in the form of water soluble powder which constitute stable catalysts for the production of different types of short-chain FOS, prebiotic sugars of high commercial value.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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