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(54) Title: IMPROVED ENDOINULINASES

(57) Abstract: A modified endoinulinase is provided, comprising modified wild-type *T. purpuregenus endoinulinase*, or a functional fragment thereof, in which an amino acid residue at each one of one or more positions corresponding to 128, 316, 344, 350 or 504 of wild-type *T. purpuregenus* endoinulinase is substituted, wherein: (i) a tyrosine residue corresponding to Y128 is substituted with H, K or R; a glutamate residue corresponding to E344 is substituted with K, H or R; and a threonine residue corresponding to T504 is substituted with M, S or Y; and optionally an alanine residue corresponding to A316 is substituted with T, S, C or M; (ii) a tyrosine residue corresponding to Y128 is substituted with H, K or R; a glutamate residue corresponding to E344 is substituted with K, H or R; a threonine residue corresponding to T504 is substituted with M, S or Y; and a glutamine residue corresponding to Q350 is substituted with L, G, A, V or I; or (iii) a tyrosine residue corresponding to Y128 is substituted with H, K or R.

IMPROVED ENDOINULINASES

FIELD OF THE INVENTION

The present invention relates in general to improved endoinulinases and their use in hydrolysis of inulin to form fructooligosaccharides.

BACKGROUND OF THE INVENTION

Fructooligosaccharides (FOSs) are naturally occurring fructose polymers that have been shown to provide beneficial health effects as functional food component. FOSs are a type of soluble dietary fibers with low caloric value, has no cariogenic properties, promote the intestinal health by stimulating the growth/activity of beneficial bacteria and support the immune system (Fanaro et al., 2005), A series of clinical studies showed that up to 20 g/day of inulin and/or FOSs is well tolerated (Carabin and Flamm, 1999). The application of FOS with controlled molecular size (degree of polymerization (DP) of 3 to 6) would result in an increased colonic persistence of the prebiotic effect and reduce the risk of chronic disease of distal intestinal region(van de Wiele et al., 2007; Wichienchot et al., 2006). Commercial production of FOS currently rely on the enzymatic hydrolysis of inulin by endo-inulinases from various sources (Chi et al., 2009; Kango and Jain, 2011; Singh et al., 2016). Inulinases are fructofuransyl hydrolases that target the β-2,1 linkage of inulin in an exo or endo fashion to hydrolyze it into fructose, glucose or FOS. Inulinases from different microorganisms have been produced, purified, cloned and characterized. Recombinant inulinases and engineered host strains have many potential industrial applications, however, efficient production processes of highly processive enzymes are required (Wang et al., 2016). Enzymes that are easily produced in high yields usually have low specific activity, such as the endo-imulinase gene product from *Pseudomonas sp.* expressed in *E.coli* (Wang et al., 2016; Xu et al., 2016; Yun et al., 1999). Therefore, there is a need for the combination of high yield production of an inulinase with high specific activity.

SUMMARY OF INVENTION

In one aspect, the present invention provides a modified endoinulinase comprising modified wild-type *T. purpuregenus* endoinulinase, or a functional fragment thereof, in which an amino acid residue at each one of one or more positions

corresponding to 128, 316, 344, 350 or 504 of wild-type *T. purpuregenus* endoinulinase is substituted, wherein: (i) a tyrosine residue corresponding to Y128 is substituted with H, K or R; a glutamate residue corresponding to E344 is substituted with K, H or R; and a threonine residue corresponding to T504 is substituted with M, S or Y; and optionally an alanine residue corresponding to A316 is substituted with T, S, C or M; (ii) a tyrosine residue corresponding to Y128 is substituted with H, K or R; a glutamate residue corresponding to E344 is substituted with K, H or R; a threonine residue corresponding to T504 is substituted with M, S or Y; and a glutamine residue corresponding to Q350 is substituted with L, G, A, V or I; or (iii) a tyrosine residue corresponding to Y128 is substituted with H, K or R.

In another aspect, the present invention is directed to an isolated nucleic acid molecule comprising a nucleic acid sequence encoding any one of the modified endoinulinases of the present invention or a functional fragment thereof as defined herein.

In an additional aspect, the present invention provides an expression vector comprising any one of the isolated nucleic acid molecules defined herein operably linked to a promoter.

In a further aspect, the present invention is directed to a cell comprising any one of the isolated nucleic acid molecules defined herein or any one of the expression vectors defined herein.

In yet another aspect, the present invention provides a method of producing a modified endoinulinase or functional fragment thereof, the method comprising (i) transfecting a cell with any one of the isolated nucleic acid molecules defined herein or any one of the expression vectors defined herein; and separating said modified endoinulinase from said cell, thereby obtaining a modified endoinulinase or functional fragment thereof.

In still another aspect, the present invention is directed to a method for producing fructooligosaccharides comprising contacting inulin with any one of the modified endoinulinase of the invention.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows scheme of a plasmid construct (pHIS-MAL-reTP (TP-optimized); 8101 bp) used for the expression of the fungal endoinulinase in *E.coli.* To express the fungal enzyme, a gene encoding the *T. purpuregenus* endoinulinase

was codon optimized for expression in *E.coli* and cloned into a modified pMAL vector to result in a fusion protein containing a 6Xhis tag and a maltose binding protein at the amino terminus. All of the sequence features are derived from the commercial vector pMAL_C2T with the addition of 6 histidine residues (6XHis) directly upstream and in frame with the malE gene.

Figs. 2A-C show expression, purification and activity of the recombinant enzymes. (**A**) An SDS-PAGE analysis of the expression and amylose column purification procedure. P-insoluble fraction. S-soluble fraction. F-Flow through. W-Wash. M-molecular marker. 1-10 elution fractions after the addition of 10mM maltose. TP- *T. purpuregenus* endoinulinase. (**B**) SDS-PAGE analysis of the purification process of reTP variants R3:5-12G after expression in E. coli. (1) protein size marker (from the top (kD) 180, 140, 100, 75, 60, 45, 35, 25, 20). (2) crude soluble cell lysate. (3) purified endoinulinase eluted from an amylose column. (4) pooled active fractions eluted from a superdex 200 gel filtration column. Arrow, expected Mw of reTP variant R3:5-12G, WT TP endoinulinase with Y128H, A316T, E344K and T504M substitutions. (**C**). Inulin hydrolysis activity of the purified enzyme, as determined using DNS, at 55°C, with 27nM reTP at the indicated substrate concentrations, K_M= 0.78±0.14 mM (4±0.39 mg/ml), k_{cat}=850±52s^{-1}, k_{cat}/K_M=1.15*10^6.

Fig. 3 shows the partitioning of the recombinant enzyme after expression in *E. coli*: SDS-PAGE showing the partitioning of the recombinant enzyme between the soluble and the insoluble fraction after cell lysis. Over 80% of the expressed wt enzyme ended up as inclusion bodies. 1, molecular weight marker; 2, TPwt insoluble fraction; 2, TPwt soluble fraction.

Figs. 4A-C deptic analysis of wild type and the recombinant endo-inulinase expression and activity. (**A**) an SDS-PAGE analysis of wt and the recombinant endo-inulinase. Soluble cell lysate after 48h of expression at 30°C and analysis by SDS-PAGE indicated the improvement of soluble expression, as evident by the increase in soluble protein expression of the evolved variants compared to reTP. (1) protein size marker (2) cell lysate from cells harboring the empty expression vector. (3) cell lysate from cells expressing reTP (4) cell lysate from cells expressing variant R1:1-7B (Y128H) (5) cell lysate from cells expressing variant R2:1-8G (Y128H, E344K and T504M). (6) cell lysate from cells expressing variant R3:5-12G (Y128H, A316T,

E344K and T504M). **Figs. 4B-C** show a graphs representing the activity of *E. coli*-expressed protein: (**B**) To evaluate inulin hydrolysis activity, 10µl of the soluble cell lysate of each variant was incubated with 1% (*w/v*) inulin at 55°C for 30min and the formation of reducing sugars was evaluated using DNS. (**C**) Activity in crude lysates was measured using 10µl of the soluble protein fraction incubated with 1% inulin in Na acetate buffer, pH 5.5 for 20min at 55°C and the activity was estimated using the DNS method to quantify the formation of reducing sugars. WT, wild type *T. purpuregenus* (TP) endoinulinase; R2:1-8G, WT TP endoinulinase with Y128H, E344K and T504M substitutions; R3:5-12G, WT TP endoinulinase with Y128H, A316T, E344K and T504M substitutions.

Fig. 5 shows TLC assays comparing inulin hydrolysis products generated by reTP and its evolved variants. (**A**). Samples were tested after 1h and after 24h of incubation with 6% inulin as substrate. Lanes 1: no enzyme, lanes 2: reTP and lanes 3, 4, 5: reactions with the evolved variants. Lanes 6: FOS standards mixture: Fructose, Sucrose, GF2, GF3, GF4, GF5 and GF6. Lane 7: reaction with no enzyme added, after a 24h incubation. Lane 8: reaction with the optimized reTP endoinulinase, after a 24h incubation. Lanes 9-11: reaction with the evolved variants after a 24h incubation: Lane 9: R1:1-7B-11 (Y128H). Lane 10: (R2:1-8G (Y128H, E344K and T504M). Lane 11: R3:5-12G (Y128H, A316T, E344K and T504M). Lanes 12: Standards mixture.

Figs. 6A-B shows the effect of pH on the activity and stability of reTP and its variants. (**A**) The optimal pH was determined by measuring enzyme activity over a pH range (3.5-8.2). (**B**) pH stability was tested by pre-incubating the enzymes without substrate for 1h at 4°C, in buffers with a range of pH values (3.5-8.2). Residual activity was then measured at the optimal pH and at a set temperature of 55°C.

Figs. 7A-B shows the effect of temperature on the activity and the stability of reTP and its evolved variants. (**A**) Temperature optimum was determined by measuring inulin enzymatic hydrolysis rates over a temperature range of 4-70°C. (**B**) Thermal stability was tested by pre-incubating the enzymes without substrate for 1h at the designated temperature (4-70^{0}C). Residual activity was then measured at the optimal pH and at a set temperature of 55°C.

Fig. 8 shows increased enzyme activity upon addition of Mg^{+2}. The effect of Mg^{+2} on the inulinase activity was determined by expressing and purifying the

endoinulinase variants with and without the addition of Mg^{2+}; after purification, enzymatic activity was measured in the presence of 1.5% (*w/v*) inulin.

Fig. 9 shows a structure homology model of endo-inulinase from *T. purpuregenus*. The solved structure of homologous endo-inulinase from *A. ficuum* (pdb 3RWK, in blue) was used as a template to create a 3D model of endo-inulinase from *T. purpuregenus* using the SWISS-MODEL server (https://swissmodel.expasy.org/). The residues that were mutated and fixated in the evolution experiments are labeled; Residues Y128 and A316 are within the active site, while Q350, E344 and T504 are located at the surface.

DETAILED DESCRIPTION OF THE INVENTION

Inulinases are fructofuransyl hydrolases that target the β-2,1 linkage of inulin and hydrolyze it into fructose, glucose and fructooligosaccharides (FOS). Inulinases from different microorganisms have been characterized, purified and produced for industrial applications; however, the high yield production of enzymes with high specific activity is still required to fulfill the growing industrial demand.

Here we used directed enzyme evolution to increase the yield and stability of an endo-inulinase enzyme cloned from the filamentous fungi *Talaromyces purpureogenus* (*Penicillium purpureogenum*, ATCC4713), the amino acid sequence of which is as set forth in SEQ ID NO: 1.

A gene encoding the *T. purpureogenus* endoinulinase lacking its 25 amino acid long signal peptide (Onodera et al., 1996) was first codon-optimized for expression in *E. coli*. The optimized gene is denoted herein as reTP (recombinant endoinulinase from *T. purpureogenus*) and its nucleotide sequence is as set forth in SEQ ID NO: 2. The amino acid sequence of the wild-type *T. purpureogenus* endoinulinase lacking its 25 amino acid long signal peptide is set forth in SEQ ID NO: 3.

To improve the functional yield and the catalytic properties of reTP, a random genetic library was constructed. Thus, four different improved *T. purpureogenus* inulinase variants were found by the inventors, in all of which a tyrosine residue corresponding to Y128 is substituted with histidine (Y128H). In three variants two additional mutations are found: a glutamate residue corresponding to E344 is substituted with lysine (E344K) and a threonine residue corresponding to T504 is substituted with methionine (T504M). In one variant, a fourth mutation is added to the

three mutations described above, wherein an alanine residue corresponding to A316 is substituted with threonine (A316T), and in another variant a fourth mutation is added to the three mutations described above, wherein a glutamine residue corresponding to Q350 is substituted with leucine (Q350L).

It has thus been found in accordance with the present invention that these mutations introduced into the sequence of wild-type *T. purpuregenus* endoinulinase improves several properties of the enzyme. For example, a clear increase in soluble protein expression was achieved, from <20% solubility in the recombinant wild-type *T. purpuregenus* endoinulinase (reTP) to approximately 80% in one of the variants. In addition, variants demonstrated an up to 5-fold improvement in hydrolysis activity in cell lysates compared to reTP, as compared with unmodified wild-type *T. purpuregenus* endoinulinase. Furthermore, variants exhibited altered pH optimum above pH 5, such as pH 5.4, which provides reduced spontaneous uncontrolled inulin degradation during the enzymatic reaction, which occurs preferentially at pH<5 and at temperatures exceeding 60ºC.

It was further found in accordance with the present invention that *E.coli* bacteria expressing the codon-optimized gene encoding for wild type *T. purpuregenus* endoinulinase having the nucleotide sequence set forth in SEQ ID NO: 2, produce wild-type *T. purpuregenus* endoinulinase having functional activity which is approximately 5-fold higher than the activity previously reported for the enzyme purified from *P. purpurogenum*. The term "functional activity" as used herein refers to the activity of the enzyme as measured in a crude extract, e.g. in terms of units or concentration of substrate hydrolyzed/second (or units or concentration of product formed/second).

The catalytic values and the high yields of the evolved variants are superior to any commercially available enzyme.

It is highly likely that substitutions at these positions of the mutant proteins or functional fragment thereof with an amino acid residue belonging to the same class of amino acids, i.e. a conservative substitution, would result in new mutants having the same properties as the original mutants identified above.

The location of a certain amino acid residue in the proteins or fragments thereof disclosed herein is according to the numbering of the wild type *T. purpureogenus* inulinase as depicted in SEQ ID NO: 1 and is designated by referring

to the one-letter code of the amino acid residue and its position in the wild type *T. purpureogenus* inulinase. Thus, for example, the tyrosine at the position corresponding to position 128 of the wild-type *T. purpureogenus* inulinase, also referred to herein as Y128, would be referred to as Y128 also in an inulinase fragment or in a homologous inulinase of a different size according to alignment algorithms well known in the art of protein chemistry, such as (MUSCLE (**MU**ltiple **S**equence **C**omparison by **L**og-**E**xpectation) or MAFFT (**M**ultiple **A**lignment using **F**ast **F**ourier **T**ransform).

A substitution of an amino acid residue at a certain position with another amino acid residue is designated by referring to the one-letter code of the amino acid residue, its position as defined above and the one-letter code of the amino acid residue replacing the original amino acid residue. Thus, for example, a substitution of Y128 with histidine would be designated Y128H.

Methods for growing bacterial cells and for harvesting secreted proteins from the cells are well-known in the arts (Choi and Lee, 2004). As an unlimiting example, *E.coli* cells may be grown in a suitable growth medium, such as Lysogeny Broth (LB) medium comprising glucose. The bacteria is then harvested and lysed in a suitable lysis buffer and disrupted, for example by sonication. The secreted protein is then isolated and purified from a clarified lysate. In case the protein of interest is tagged for the purpose of facilitating isolation, it is purified on a column that specifically binds the tag, washed and eluted. For example, clarified lysate containing a recombinant protein comprising the protein of interest and a maltose-binding protein is loaded onto an amylose column. The recombinant protein is then eluted with maltose-supplemented column buffer. Protein-containing elution fractions are collected, concentrated and optionally fractionized using a size exclusion column.

Methods for assessing the functionality of an inulinase or a fragment thereof are well known in the art. For example, as disclosed herein below in the examples, the activity may be evaluated by quantifying the amount of reducing ends produced by the inulinase or a fragment thereof using the 3,5-dinitrosalicylic acid (DNS) assay (ε=45 OD/M). An inulinase variant or a fragment with a specific activity, functional expression, yield and/or stability similar or equal to – or better than – that of wild-type inulinase would be considered as a functional inulinase variant or fragment thereof.

Thus, in one aspect, the present invention provides a modified endoinulinase comprising modified wild-type *T. purpuregenus* endoinulinase, or a functional fragment thereof, in which an amino acid residue at each one of one or more positions corresponding to position 128, 316, 344, 350 or 504 of wild-type *T. purpuregenus* endoinulinase is substituted, wherein: (i) a tyrosine residue corresponding to Y128 is substituted with H, K or R; a glutamate residue corresponding to E344 is substituted with K, H or R; and a threonine residue corresponding to T504 is substituted with M, S or Y; and optionally an alanine residue corresponding to A316 is substituted with T, S, C or M; (ii) a tyrosine residue corresponding to Y128 is substituted with H, K or R; a glutamate residue corresponding to E344 is substituted with K, H or R; a threonine residue corresponding to T504 is substituted with M, S or Y; and a glutamine residue corresponding to Q350 is substituted with L, G, A, V or I; or (iii) a tyrosine residue corresponding to Y128 is substituted with H, K or R.

In certain embodiments the modified endoinulinase or functional fragment thereof has improved functional activity or solubility as compared with reTP endoinulinase, or altered pH optimum from about pH 5 of the unmodified wild type *T. purpuregenus* endoinulinase to above pH 5 of the reTP.

In particular embodiments, the improved activity is up to fivefold improvement in hydrolysis activity in cell lysates compared to reTP as measured in a lysate of the bacteria expressing the enzyme; for example, the functional activity of the modified endoinulinase is between about 15-20μM/s as compared with about 5μM/s for reTP.

In particular embodiments, the improved solubility of the modified endoinulinase or functional fragment thereof is the presence of more than 50%, 60%, 70%, 80%, 90%, 95% or 99% of the activity, e.g. 80%, in a soluble fraction of lysed bacteria, as shown by an increase in soluble protein expression from <20% solubility in the reTP to approximately 80% in one of the mutants named R3:5–12G (Y128H, A316T, E344K, and T504M).

In particular embodiments, the pH optimum is shifted to a range between pH 5.1 to pH 6, for example pH 5.4.

In certain embodiments, (i) the tyrosine residue corresponding to Y128 is substituted with H (Y128H); (ii) the glutamate residue corresponding to E344 is substituted with K (E344K); (iii) the threonine residue corresponding to T504 is

substituted with M (T504M); and (iv) the alanine residue corresponding to A316 is substituted with T (A316T).

Thus, in particular embodiments the modified endoinulinase comprises modified wild-type *T. purpuregenus* endoinulinase, or a functional fragment thereof, in which the following substitutions have been made: (a) Y128H; (b) Y128H, E344K and T504M; (c) Y128J, E344K, T504M and Q350L; or (d) Y128J, E344K, T504M and A316T.

In certain embodiments, the modified endoinulinase or functional fragment thereof as defined in any one of the above embodiments has an amino acid sequence, not including the signal peptide, which is at least 80, 85, 90, 95, 96, 97, 98, or 99% identical to the sequence of wild-type *T. purpuregenus* endoinulinase of SEQ ID NO: 3.

Alternatively, the modified endoinulinase or functional fragment thereof as defined in any one of the above embodiments has no other modifications made to the amino acid sequence of the wild-type *T. purpuregenus* endoinulinase of SEQ ID NO: 3.

For practical purposes, the endoinulinase may be provided as a fusion protein containing a tag useful for separating it from the cell extract by specific binding to a ligand-containing substrate or for improving solubility. For example, any one of the improved endoinulinases of the present invention may be provided as a fusion protein with a 6Xhis tag and/or a maltose binding protein at the amino terminus.

In another aspect, the present invention is directed to an isolated nucleic acid molecule comprising a nucleic acid sequence encoding any one of the modified endoinulinases of the present invention or a functional fragment thereof as defined herein above.

In certain embodiments, the isolated nucleic acid molecule is optimized for expression in *E. coli*. Methods for optimizing expression of foreign DNA in *E.coli* cells are well known in the art e.g. (Burgess-Brown et al., 2008).

In certain embodiments, the isolated nucleic acid molecule encodes for a fusion protein containing a tag useful for separating it from the cell extract by specific binding to a ligand-containing substrate. For example, the nucleic acid sequence encoding any one of the improved endoinulinases of the present invention may be

fused to sequences encoding a 6Xhis tag and/or a maltose binding protein (for example as set forth in SEQ ID NO: 4).

In particular, the isolated nucleic acid molecule comprises a nucleic acid sequence as set forth in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8, encoding for the improved *T. purpuregenus* endoinulinase variant (a), (b) (c) and (d) described above, respectively.

Furthermore, the present invention is directed to an isolated nucleic acid molecule comprising a nucleic acid sequence codon-optimized for expression in *E. coli*, wherein said nucleic acid sequence is as set forth in SEQ ID NO: 2 encoding wild-type *T. purpuregenus* endoinulinase (reTP).

In an additional aspect, the present invention provides an expression vector comprising any one of the isolated nucleic acid molecules defined herein above operably linked to a promoter.

In a further aspect, the present invention is directed to a cell comprising any one of the isolated nucleic acid molecules defined herein above or any one of the expression vectors defined herein above.

In certain embodiments, the cell is selected from a bacterial, fungal, mammal or plant cell, such as an *E.coli* cell.

In yet another aspect, the present invention provides a method of producing a modified endoinulinase or functional fragment thereof, the method comprising (i) cultivating a cell defined herein above; and separating said modified endoinulinase from said cell, thereby obtaining a modified endoinulinase or functional fragment thereof.

The cell used in the method may be a cell selected from a bacterial, fungal, mammal or plant cell, such as an *E.coli* cell.

In certain embodiments, more than 50%, 60%, 70%, 80%, 90%, 95% or 99% of the activity, e.g. 80%, of the modified endoinulinase or functional fragment thereof obtained by the method of the present invention is in a soluble fraction, and its specific activity is about five-fold higher, as compared with unmodified endoinulinase.

In certain embodiments, the modified endoinulinase expressed in the method is *T. purpuregenus* endoinulinase, in which (i) Y128H; (ii) Y128H, E344K and T504M; (iii) Y128J, E344K, T504M and Q350L; or (iv) Y128J, E344K, T504M and

A316T. In certain embodiments, the modified endoinulinase or functional fragment thereof obtained by the method of the present invention as defined in any one of the above embodiments has an amino acid sequence which is at least 80, 85, 90, 95, 96, 97, 98, or 99% identical to the sequence of wild-type *T. purpuregenus* endoinulinase of SEQ ID NO: 1. In particular, no other modifications are made to the amino acid sequence as compared with the wild-type *T. purpuregenus* endoinulinase.

Methods for separating, isolating and enriching proteins expressed in isolated cells, such as *E.coli* cells are well known in the art. For example, the modified endoinulinase may be expressed as a fusion protein having a 6Xhis tag and/or a maltose binding protein and separated on a Ni-containing and/or maltose containing column (Uhlen, 2008).

In still another aspect, the present invention is directed to a method for producing fructooligosaccharides comprising contacting inulin with any one of the modified endoinulinase of the invention as defined herein above.

In certain embodiments, the modified endoinulinase used in the method is *T. purpuregenus* endoinulinase, in which (i) Y128H; (ii) Y128H, E344K and T504M; (iii) Y128J, E344K, T504M and Q350L; or (iv) Y128J, E344K, T504M and A316T.

In certain embodiments, the method is for producing fructooligosaccharides with extended product distribution as compared with distribution of fructooligosaccharides obtained using unmodified wild-type *T. purpuregenus* endoinulinase, such as a mixture of IOS with a DP ranging between DP3 and DP6, or DP2–8 using the R3:5-12G.

The proteins encoded by the nucleic acid molecules of the invention are not limited to those defined herein by specific amino acid sequences but may also be variants of these proteins or have amino acid sequences that are substantially identical to those disclosed above. A “substantially identical" amino acid sequence as used herein refers to a sequence that differs from a reference sequence by one or more conservative or non-conservative amino acid substitutions, deletions, or insertions, particularly when such a substitution occurs at a site that is not the active site of the molecule, and provided that the polypeptide essentially retains its functional properties. A conservative amino acid substitution, for example, substitutes one amino acid with another of the same class, *e.g.*, substitution of one hydrophobic amino acid with another hydrophobic amino acid, a polar amino acid with another polar amino

acid, a basic amino acid with another basic amino acid and an acidic amino acid with another acidic amino acid. One or more amino acids can be deleted from the peptide, thus obtaining a fragment thereof without significantly altering its biological activity.

The term “variant" as used herein refers to polynucleotides or polypeptides modified at one or more base pairs, codons, introns, exons, or amino acid residues, respectively, yet still retain the biological activity of a polypeptide of the naturally occurring sequence.

The present invention further relates to an isolated nucleic acid molecule comprising a polynucleotide sequence encoding a protein variant that has an amino acid sequence that is at least 80%, at least 85 %, at least 90 %, or at least 95, 96, 97, 98, or 99% identical to the amino acid sequence encoded by one of the DNA sequences of SEQ ID NO:4 or SEQ ID NO: 8 as long as each protein variant has equal or substantially similar activity to the protein to which it is similar.

For purposes of clarity, and in no way limiting the scope of the teachings, unless otherwise indicated, all numbers expressing quantities, percentages or proportions, and other numerical values recited herein, should be interpreted as being preceded in all instances by the term "about." Accordingly, the numerical parameters recited in the present specification are approximations that may vary depending on the desired outcome. For example, each numerical parameter may be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

The term "about" as used herein means that values of 10% or less above or below the indicated values are also included.

The invention will now be illustrated by the following non-limiting examples.

EXAMPLES

Material and Methods

Reagents

Inulin from dahlia tubers (average degree of polymerization 10 (DP-10)), fructose, glucose and sucrose were purchased from Sigma-Aldrich (St Louis, MO, USA). Fructo-oligosaccharides (FOS) with DP between 3-7 were purchased from Elictyl (Crolles, France). Acetonitrile for HPLC was purchased from J.T. Baker (Avantor, Center Valley, PA, USA). Double-distilled water (DDW) for HPLC was prepared on an Arium®Pro ultrapure water systems (Sartorius, Goettingen, Germany). Nitrogen

gas 99.999% was acquired from Oxygen & Argon Works Ltd (Caesarea, Israel). All other chemicals were of analytical grade.

Gene cloning

A gene encoding the endo-inulinase from TP with the 25 amino acids signal peptide removed(Onodera et al., 1996) was codon optimized for expression in *E. coli* using the OptimumGene™ algorithm and synthesized by GenScript®. The synthetic gene was cloned into a modified pMAL-c4x (NEB) vector containing an additional 6Xhis tag upstream to the MBP reading frame using *EcoRI* and *HindIII* restriction sites, for expression with a maltose binding protein (MBP) fusion tag resulting in a pMAL_TP vector (**Figure 1**). The cloned gene was transformed into E. coli DH5-α cells and the correct sequence was verified by sequencing.

Expression optimization.

pMAL_TP was transformed into *E. coli* BL21 cells and plated on LB agar plates supplemented with 100 μg/ml ampicillin and 1% glucose. A single colony was used to inoculate 3 ml of LB containing 100 μg/ml ampicillin and 1% glucose. The overnight culture was used as a starter for protein expression. Expression conditions: fresh terrific broth (TB) supplemented with 100 μg/ml ampicillin was inoculated 1:100 with the overnight culture and grown at 25, 30 or 37°C until OD_{600} ~0.6. Enzyme over-expression was induced by adding IPTG to a final concentration of 0.4 mM and cultures at 25, 30 or 37°C were shaken at 250 RPM for 40, 24 or 4h respectively. Cells were harvested by centrifugation, resuspended in lysis buffer (50mM NaOAc pH 5.4, 300mM NaCl , 100 μg/mL lysozyme, 0.5 unit/mL DNase, 0.1% tritonX-100, 1:500 protease inhibitor cocktail (sigma P8849) and shaken for 1h at 950rpm at room temperature. After centrifugation, the clarified cell lysate was used for activity test and analysis by SDS-PAGE. The best expression conditions were growth at 37°C, following the lowering of the temperature to 20°C before adding of IPTG.

Expression and purification of the optimized TP gene and its evolved variants.

LB medium (5 ml) containing 100 μg/ml ampicillin and 1% (*w/v*) glucose was inoculated with a single colony of *E. coli* BL21 cells harboring pMAL_reTP and cultures were grown overnight at 37°C. The next day the culture was used to inoculate 500 ml of fresh TB supplemented with 200 μg/mL ampicillin and 1mM $MgSO_4$, and

the culture was grown at 30°C until OD_{600} ~1.2. The temperature was lowered to 20°C and enzyme expression was induced by adding IPTG to a final concentration of 0.4 mM and the culture was grown with shaking at 250 RPM for 40 h. Cells were harvested by centrifugation, resuspended in the corresponding lysis buffer (50 mM NaOAc pH 5.4, 300 mM NaCl, 100 µg/mL lysozyme, 0.5 unit/mL DNase, 0.1% tritonX-100, 1:500 protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA, P8849) and disrupted by sonication. The clarified lysate was loaded onto an amylose column (NEB) equilibrated with column buffer (50 mM NaOAc pH 5.4, 150 mM NaCl). The fusion protein was eluted with maltose (10 mM) supplemented column buffer (**Figure 2A**). Protein containing elution fractions were collected, concentrated and loaded on a Superdex-200 (GE, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) column pre-equilibrated with column buffer. Active fractions were pooled and used for activity tests (**Figure 2B**). All purification steps were performed at room temperature, except the size exclusion step that was performed at 4°C. The purity of the fusion enzymes and their concentrations were validated by 12% SDS-PAGE and OD_{280} respectively.

Enzyme kinetics

Enzyme reactions were carried out using various substrate concentrations (inulin from dahlia purchased from Sigma-Aldrich (St Louis, MO, USA, I3754) dissolved in 50 mM sodium acetate pH 5.5 and 150 mM NaCl, with enzyme concentration adjusted to result in a linear production of reducing ends during the incubation period (with mixing) in 96 wells microplates at 55°C. Samples were taken at regular intervals and the reactions were stopped by heating the reaction mixtures at 95°C. The activity of the enzyme was evaluated by quantifying the amount of reducing sugars produced using the DNS assay (ε=45 OD/M). The absorbance was measured at 535 nm using infinite M2000 pro, TECAN plate reader. The endoinulinase reTP and its evolved variants were used to study their kinetic parameters (K_M and k_{cat}). For the determination of the reaction rate, different inulin concentrations were used, ranging from 0-45 mM (0-8%). The initial reaction rates were corrected for the background rate of spontaneous hydrolysis in the absence of enzyme. Kinetic parameters were obtained by fitting initial rates directly to the Michaelis –Menten equation [$v_0= k_{cat}[E]_0[S]_0/([S]_0+K_M)$] with GraphPad prism

(GraphPad Software, Inc. La Jolla, CA, USA). Data points and errors were obtained from at least three independent measurements.

Library construction and screening

Genetic libraries originating from the optimized endoinulinase gene were constructed using GeneMorph II Random Mutagenesis Kit (Agilent Technologies, La Jolla, CA, USA) adjusted to produce an average of 2 non-synonymous mutations per gene. The estimated theoretical diversity of the library is ~6.5e^{10} individual variants based on the following calculation: $\binom{N}{m} \times m^{19}$ where N is the number of amino acids (500), m is the average number of mutations (2) and 19 is the number of possible substitutions for a single amino acid. Following the mutagenic PCR, libraries were cloned back into the modified pMAL vector as described for the pMAL_reTP. The cloned libraries were transformed into BL21 cells and plated on LB plates supplemented with 100 µg/ml ampicillin and 1% (*w/v*) glucose. In each round of screening, approximately 600 randomly chosen single colonies were picked and grown overnight in 96 deep-well plates containing 500 µl of LB supplemented with 100 µg/mL ampicillin and 1% (*w/v*) glucose, at 37°C with shaking. The overnight cultures were used to inoculate (at 1:20 dilution) fresh 500µl TB supplemented with 200 µg/mL ampicillin in 96 deep-well plates. Cells were grown at 30 °C with shaking for about 4 h, to an OD_{600}=0.6-1.0, IPTG was then added (final concentration 0.4 mM) to induce expression of the endoinulinase variants. Following overnight incubation at 20°C, the cells were pelleted and freezed at -80°C. Cells were resuspended in lysis buffer (50 mM NaOAc pH 5.4, 150 mM NaCl, 100 µg/mL lysozyme, 0.5 unit/mL benzonase, 0.1% triton X-100, 1:500 protease inhibitor cocktail for 1h shaking at 960 RPM at 25°C). The lysates were clarified by centrifugation, diluted in activity buffer, and assayed for hydrolysis of inulin using the 3,5-dinitrosalcylic acid (DNS) assay(Miller, 1959) One unit of inulinase activity was defined as the quantity of enzyme required to liberate 1 µmol of fructose equivalent from inulin per minute at 55°C, and specific activity was defined as units per mg protein. In each round, variants with top activities were selected to serve as parents for the next round, where their genes were shuffled and mutated using GeneMorphII kit.

pH optimum and stability of reTP and its evolved variants

The effect of pH on reTP and its evolved variants was evaluated by incubating the enzymes for 1h at 4°C, with pH solution ranging from 3.5 to 8.2, followed by endoinulinases activity measurements using the DNS assay, in 100mM potassium phosphate, 150 mM NaCl, pH 5.4 at 55°C. Enzyme concentration was set to be 18.2 ng/µl for reTP, R2:1-8G and R3:5-12G, and 4.5ng/µl for variant R1:1-7B. 50 mM sodium acetate buffer, with 150 mM NaCl, was used for obtaining pH values 3.5-5.5, while 100 mM potassium phosphate buffer, with 150 mM NaCl was used for pH 5.5-8.2.

Temperature optimum and thermostability of optimized reTP and its evolved variants

Temperature optimum was measured by performing endoinulinase assay at temperature ranging from 4 to70°C, 2% (*w/v*) inulin (dissolved in 50 mM sodium acetate buffer (pH 5.4), 150 mM NaCl), was incubated for 30 min with optimized reTP and its evolved variants. Enzyme concentration was set to be 18.2 ng/µl for reTP, R2:1-8G and R3:5-12G, and 4.5 ng/µl for R1:1-7B. **b.** Thermal stability was tested by pre-incubating the enzyme variants at temperatures ranging between 4-70°C for 1h. Residual activity was than measured by following the thermal activity analysis.

Effects of metal ions and inhibitors on reTP and its evolved variants inulinase activity

The effect of metal ions and inhibitors on the inulinase activity was determined by adding different elements in the concentration of 1 mM (for K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Al^{2+}, Zn^{2+}, Ni^{2+}, EDTA, DTT, SDS) or 0.1% (*w/w*) for Triton X-100, to the activity buffer 50 mM sodium acetate buffer (pH 5.4), 150 mM NaCl. With 2% (*w/v*) inulin, 18.2 ng/µl of reTP and the evolved variants. Results are presented as residual activity compared to the control (no metal/inhibitor added).

Preparation of fructooligosaccharides standard solutions

Mixed standard stock solutions containing inulin-type FOS (DP3-DP7) were prepared in double-distilled water. The concentrations of DP3-DP7 were about 2 mg/mL. The standard stock solutions were stored at 4°C before use. Working standard

solutions were prepared by dilution in 50 mM sodium acetate buffer (pH 5.4), 150 mM NaCl.

TLC analysis of hydrolysis products

The products of inulin hydrolysis by purified endoinulinases were analyzed by thin-layer chromatography (TLC). The enzyme reaction contained purified enzyme (0.25 μM) with 2 or 6% inulin at 50mM sodium acetate buffer (pH 5.4), 150 mM Nacl, for 1 or 24h at 48°C. 1 μl of each sample was spotted on TLC plate (Silica gel from Mercury). First running solution: Butanol/ Ethanol/ DDW (5:5:3, v/v/v) and the second running solution: Acetone/DDW (9:1, v/v). Inulo-oligosacharides were detected using 0.3% (*w/v*) of N-(1-naphthyl) ethylenediamine and 5% (v/v) sulfuric acid in methanol. Color development was initiated by placing the plates in an oven at 150°C for about 5 min until bends were observed.

HPLC-CAD Analysis

Chromatographic analysis was performed on an Agilent (Santa Clara, CA, USA) 1200 Series HPLC system equipped with degasser, pump, auto sampler and column compartment, coupled with Corona charged aerosol detector (CAD) instrument (ESA, Chelmsford, MA, USA). Data processing was carried out with ChemStation B 04.02 software (Agilent). The N_2 pressure of the CAD was adjusted to 35 psi and response range was set to 100pA. Separations were performed on a Waters XBridge Amide column (4.6x250 mm; 2.5 μm). The mobile phase was consisted of water (A) and acetonitrile (B) with gradient elution of 75%-45% B at 0-30 min, 45-75% at 30-32 min and equilibrated with 75% B for 10 min. The column temperature was set at 30^0C. The flow rate was 1.0 mL/min; injection volume was 10 μL.

3D homology modeling of the endoinulinase from T. purpureogenus

The solved structure of homologous endoinulinase from *A. ficcum* (pdb 3RWK, in blue) was used as a template to create a 3D model of endo-inulinase from *T. purpuregenus* using the SWISS-MODEL server (Guex et al., 2009).

Example 1: Expression of WT inulinase from *T. purpureogenus*.

Directed enzyme evolution in *E. coli* cells enables high efficiency transformation, simple host cell handling and the use of a large array of molecular tools. However, most endoinulinases of fungal origin are not well expressed in *E. coli*.

Apart from effects such as cellular conditions, molecular chaperons and localization(Liu et al., 2017; Zelena et al., 2014), it has been shown that codon optimization improves the heterologous expression of enzymes and endoinulinases specificaly(He et al., 2014; Menzella, 2011). Therefore, a gene encoding the *T. purpureogenus* endoinulinase lacking its 25 amino acids signal peptide(Onodera et al., 1996) was codon-optimized, for expression in *E. coli* , using the OptimumGene™ algorithm and synthesized by GenScript®. The optimized gene is denoted herein as reTP (recombinant endoinulinase from *T. purpureogenus*). The optimized gene was cloned into a pMAL vector (NEB) downstream to the *malE* open reading frame (ORF), to drive cytoplasmic expression. Fusion of the endoinulinase to the maltose binding protein (MBP) resulting from the in-frame cloning with the *malE* gene served two purposes: increasing protein solubility and facilitating purification (**Figure 1**). The enzyme was then over-expressed in *E. coli*. Following purification (as described in the Material and Methods section), a functional biochemical assay was performed to verify the ability of the purified protein to hydrolyze inulin (using inulin with a DP ~10). A Michaelis-Menten plot was generated following a DNS assay **(Fig. 2C)** and the enzyme's kinetic profile served as a reference for the functionality of the recombinant enzyme. Our heterologous expression system yielded a functional fusion protein with properties similar to that of the endoinulinase previously purified from the fungus *T. purpureogenus*(Onodera and Shiomi, 1988) (annotated here as TPwt) (**Table 1**) indicating that the fusion protein (MBP) and the host organism had no significant effect on the enzymes kinetic properties.

The maximal activity was obtained by inducing protein expression at 30^0C for 40h, however, over 80% of the expressed protein ended up in the insoluble fraction (**Figure 3**).

Example 2. Library construction and directed evolution.

To improve the functional yield and the catalytic properties of reTP, a random genetic library was constructed using the GenemorphII kit (Agilent). The mutation rate was calibrated to incorporate ~2 nonsynonymous mutations per gene, thereby creating a library with a theoretical complexity of $6.5e^{10}$ (see the Material and Methods section for the calculation). Transformation of the initial library into *E. coli* cells resulted in ~20,000 individual colonies. For each round of screening, 600 randomly chosen colonies were grown and protein expression was induced in 96

deep-well plates. Cells lysates were then incubated with inulin, and assayed for activity using a DNS assay. In each round 10-15 clones exhibiting above 2 folds improvement in activity compared to reTP were selected. Improved variants were streaked on agar plates and expressed in 3 ml cultures in triplicates to validate their improvements, and to evaluate product range by analyzing the reaction products using TLC. Improved variants were therefore selected for higher expression levels, solubility, hydrolysis rates and product range. This procedure was repeated three times with the 3-5 of the best variants from each round serving as starting points for the next round of mutagenesis and screening. The acquired mutations in selected variants from each round are summarized in **Table 1**.

Example 3. Evolved endoinulinase variants exhibit increased solubility and activity.

Variants from each screening round (R1, R2 and R3) were expressed and their activity and soluble expression were analyzed and compared to reTP, by an inulin hydrolysis assay and SDS-PAGE, respectively. A clear increase in soluble protein expression was achieved, from <20% solubility in the reTP to approximately 80% in R3:5-12G, a variant isolated after the third round of screening (**Fig. 4A**). In addition, variants R2:1-8G and R3:5-12G demonstrated an up to 5-fold improvement in hydrolysis activity in cell lysates compared to reTP (**Fig. 4B**).

WT and improved variants were expressed in *E. coli* as described in materials and methods and the soluble protein fraction was separated after cell lysis. Equal amount of cell lysate was used to compere between the variants in an assay for inulin degradation. The activity of the evolved variants showed up to 5-fold improvement in functional expression over the WT clone (**Figure 4**C).

Example 4: Characterization of the evolved variants.

In an effort to identify the molecular bases leading for improved solubility and functional activity, the selected variants were subjected to various biochemical characterizations. To this end, the recombinant proteins were purified to near homogeneity, using an amylose affinity chromatography followed by gel filtration, as described in the methods section (**Fig 2A and B**). The inulin hydrolyzing activity of the purified enzymes was then measured. A specific activity of 413 U/mg protein was measured for reTP, which is approximately 5-fold higher than the activity previously

reported for the enzyme purified from *P. purpurogenum*(Onodera and Shiomi, 1988). The evolved variants displayed higher k_{cat} values, and variant R1:1-7B exhibited 15-fold increase in k_{cat} compared to that of the TPwt purified from *P. purpurogenum*(Onodera and Shiomi, 1988), and 2-fold higher than reTP, **Table 1**.

Example 5. Endoinulinase variants with altered pH optimum can be achieved using directed evolution

The pH optimum of most of the characterized inulinases is acidic, as in the case of the *A. awamori* inulinase (pH 4.5) (Arand and Golubev 2002) and the *A. ficcum* inulinase (pH 5.0)(Chen et al., 2012). A similar pH optimum was observed for reTP (**Fig. 6A**). However, inulin is degraded spontaneously at pH < 5 and at temperatures exceeding 60°C(Glibowski and Pikus, 2011). To reduce spontaneous uncontrolled inulin degradation during the enzymatic reaction, we aimed to increase the enzyme's optimal pH for activity. Hence, we set the pH during the screening process to 5.4, where less spontaneous hydrolysis occurs, and the reTP enzyme exhibit 80% of its maximal activity. As Figure 3a indicates, the pH optimum of the evolved variants shifted to 5.4 in response to the selection pressure. The pH stability of the variants was tested as well, and showed similar activity profiles to the optimized reTP (**Fig. 6B**). These results indicate that variants with specific properties can be easily selected by setting the appropriate screening conditions, leading the way for the selection of tailor-made enzymes for specific and favorable reaction conditions.

The optimal temperature of reTP and its evolved variants was 55°C, which is in agreement with those reported for other microbial inulinases(Xu et al., 2016) (**Fig. 7A**). In addition, both reTP and its variants demonstrated thermal stability, with > 80% residual activity when incubated at temperatures up to 50°C (**Fig. 7B**). This finding is in agreement with the thermal stability reported for the enzyme purified from *T. purpureogenus*(Onodera and Shiomi, 1988).

Example 6. Addition of Mg^{2+} ions is required to maintain high activity of variants from advanced rounds of evolution

The effect of different additives on enzyme's activity was tested by supplementing purified enzyme before the addition of inulin with the following: K^+, Ca^{2+}, Mg^{2+}, Al^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, EDTA, DTT at 1 mM concentration, and SDS or

Triton X-100 at 0.1% (*v/v*). The addition of EDTA decreased the activity of reTP, R2:1-8G and R3:5-12G by 70%, 82% and 50%, respectively (**Table 2**). In accordance, the addition of metal ions and Mg^{2+} in particular, increased the inulinase activity. In the presence of Mg^{2+}, the relative activity of reTP, R2:1-8G and R3:5-12G increased to 136%, 210% and 198%, respectively, as compared to the reactions without the additions of the metal ion (**Table 2**). These results suggest that the activity of the evolved variants became more dependent on the presence of metal ions than that of the reTP, especially after rounds #2 and #3. To further test this, we included Mg^{2+} in the growth medium used for protein expression and in the buffers used for protein purification, and compered the activity of the enzymes that were expressed and purified with and without the addition of Mg^{2+}. As seen in **Fig. 8**, the activity of reTP increased by 2-fold, of R2:1-8G by 4.5-fold and of R3:5-12G by 8-fold. Moreover, the specific activity of R3:5-12G in the presence of Mg^{2+} reached a value of 1380 U/mg, which is higher than that of most endoinulinases described in the literature. For example, *A. ficuum* endoinulinase expressed and purified from *E. coli* exhibited a specific activity of 75.22 U/mg(Wang et al., 2016), the *Bacilus smithii* T7 endoinulinase had a reported specific activity of 833 U/mg(Gao et al., 2009) and the *Xanthomonas campestris pv. phaseoli* KM 24 mutant endoinulinase showed a specific activity of 119 U/mg(Naidoo et al., 2015). Recently, expression of the *A. fumigatus* Cl1 endoinulinase in *E.coli* using high cell-density fermentation, yielded an enzyme with specific activity similar to that of our evolved variants (1590 U/mg)(Chen et al., 2015).

Example 7. Analysis of IOS distribution resulting from inulin hydrolysis using the evolved variants

TLC and HPLC were used to characterize the distribution of products resulting from inulin hydrolysis by the purified reTP and its evolved variants. The ability of the methods to detect the reaction product were validated using inulin and inulin type FOS ranging from DP2-6 (**Figs 5A-B**). As expected, both methods indicated that IOS were the predominant end products of the hydrolysis; HPLC analysis indicated that ~91% of the products are IOS (not shown). Upon the use of 2% (w/v) inulin as substrate in the reaction mixture, a mixture of IOS with a DP ranging between DP2 and DP6 was obtained after 1h incubation, while after 24h of incubation, the main product was mostly DP3 (**Fig. 5a**). When a higher inulin concentration was used

(6%), a broader product distribution was observed and even after incubation for 24h, the products ranged between DP2-8 (**Fig 5b**). TLC results indicated that only variant R3:5-12G was able to fully consume the high concentration (6%) of inulin (**Fig 5b**).

Example 8. Analysis of mutations accumulated during the evolution process

Understanding the function of mutations selected by directed enzyme evolution can be achieved, in some cases, by phylogenetic and structural analysis. As there is currently no available structure for the *T. purpuregenus* endoinulinase, we used the atomic structure of the homolog from *A. ficcum* endoinulinase (pdb 3RWK, 73% identity) to generate a 3D model using the Swiss-model server(Biasini et al., 2014), (**Fig 9**). The reconstructed model indicated that mutation Y128H, which is in close proximity to the active site, had a definite role in the modified enzyme catalytic activity, as it was acquired in the first round, and increased the k_{cat} of R1:1-7B (only mutation) by 2-fold compered to reTP. Since R1:1-7B did not exhibit increased functional expression levels and was least affected by the addition of metal ions (**Fig. 4B** and **8**), it seems that its selection in the screening process was purely due to its increased catalytic activity.

Table 1. *Kinetic parameters of TPwt, reTP and the evolved variants (purified from E.coli.)and their corresponding mutations.*

Endoinulinase	Mutations	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [s^{-1}/M^{-1}]	*SEQ ID NO. of encoding DNA*[b]
TPwt[a]		0.21	120	$0.57*10^6$	
reTP (MBP fused)		0.78±0.14	850±52	$1.15*10^6$	2
R1:1-7B (MBP fused)	Y128H	2.82±0.04	1797±97	$0.64*10^6$	3
R2:1-8G (MBP fused)	Y128H 344K T504M	2.40±0.04	792±45	$0.33*10^6$	4
R3:2-2E (MBP fused)	Y128H E344K Q350L T504M	NA	NA	NA	5
R3:5-12G (MBP fused)	Y128H A316T E344K T504M	3.12±0.38	685±31	$0.2*10^6$	6

[a] from(Onodera and Shiomi, 1988)
[b] sequences of reTP not including MBP

Table 2. Effect of metal ions and additives on the activity of reTP and its evolved variants.

Additives	**Concentration**	**reTP**	**R1:1-7B**	**R2:1-8G**	**R3:5-12G**
Control	1mM	100±19	100±6	100±8	100±7
K^+	1mM	98±15	109±6 [a]	111±3	91±9 [a]
Zn^{2+}	1mM	66±9 [a]	83±28	76±5 [a]	112±15
Ca^{2+}	1mM	116±6	147±9 [a]	126±1 [a]	130±24
Ni^{2+}	1mM	86±9	96±3	111±3	133±14
Cu^{2+}	1mM	50±6 [a]	2±0 [a]	3±1 [a]	6±2 [a]

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CLAIMS

1. A modified endoinulinase comprising modified wild-type *T. purpuregenus* endoinulinase, or a functional fragment thereof, in which an amino acid residue at one or more positions corresponding to position 128, 316, 344, 350 or 504 of wild-type *T. purpuregenus* endoinulinase is substituted, wherein:

(i) a tyrosine residue corresponding to Y128 is substituted with H, K or R; a glutamate residue corresponding to E344 is substituted with K, H or R; and a threonine residue corresponding to T504 is substituted with M, S or Y; and optionally an alanine residue corresponding to A316 is substituted with T, S, C or M;

(ii) a tyrosine residue corresponding to Y128 is substituted with H, K or R; a glutamate residue corresponding to E344 is substituted with K, H or R; a threonine residue corresponding to T504 is substituted with M, S or Y; and a glutamine residue corresponding to Q350 is substituted with L, G, A, V or I; or

(iii) a tyrosine residue corresponding to Y128 is substituted with H, K or R.

2. The modified endoinulinase or functional fragment thereof of claim 1, wherein:

(i) the tyrosine residue corresponding to Y128 is substituted with H (Y128H);

(ii) the glutamate residue corresponding to E344 is substituted with K (E344K);

(iii) the threonine residue corresponding to T504 is substituted with M (T504M);

(iv) the alanine residue corresponding to A316 is substituted with T (A316T); and

(v) the glutamine residue corresponding to Q350 is substituted with L (Q350L).

3. The modified endoinulinase or functional fragment thereof of claim 2 or 3, wherein the tyrosine residue corresponding to Y128 is substituted with H; the glutamate residue corresponding to E344 is substituted with K; the threonine residue

corresponding to T504 is substituted with M; and the alanine residue corresponding to A316 is substituted with T.

4. The modified endoinulinase or functional fragment thereof of any one of claims 1 to 3, which has an amino acid sequence, not including a signal peptide, which is at least 80% identical to the sequence of wild-type *T. purpuregenus* endoinulinase of SEQ ID NO: 3.

5. The modified endoinulinase or functional fragment thereof of any one of claims 1 to 3, in which no other modifications are made to the amino acid sequence of the wild-type *T. purpuregenus* endoinulinase of SEQ ID NO: 3.

6. An isolated nucleic acid molecule comprising a nucleic acid sequence encoding the modified endoinulinase, or a functional fragment thereof, of any one of claims 1 to 5.

7. The isolated nucleic acid molecule of claim 6, optimized for expression in *E. coli*.

8. The isolated nucleic acid molecule of claim 7 comprising the nucleic acid sequence as set forth in SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

9. An isolated nucleic acid molecule comprising a nucleic acid sequence codon-optimized for expression in *E. coli*, wherein said nucleic acid sequence is as set forth in SEQ ID NO: 2 encoding wild-type *T. purpuregenus* endoinulinase.

10. An expression vector comprising the isolated nucleic acid molecule of any one of claims 7 to 9 operably linked to a promoter.

11. A cell comprising the isolated nucleic acid molecule of any one of claims 9 to 9 or the expression vector of claim 10.

12. The cell of claim 11, selected from a bacterial, fungal, mammal or plant cell, preferably *E.coli*.

13. A method of producing a modified endoinulinase, or a functional fragment thereof, comprising:

(i) cultivating a cell of claim 11 or 12; and

(ii) separating said modified endoinulinase from said cell, thereby obtaining a modified endoinulinase.

14. The method of claim 13, wherein said cell is selected from a bacterial, fungal, mammal or plant cell, preferably *E.coli*.

15. The method of claim 13 or 14, wherein more than 50% of the activity of said modified endoinulinase, or functional fragment thereof, is in a soluble fraction, and its functional activity is about five-fold higher, as compared with unmodified endoinulinase.

16. The method of any one of claims 13 to 15, wherein said modified endoinulinase is *T. purpuregenus* endoinulinase, in which:

(i) Y128H;

(ii) Y128H, E344K and T504M;

(iii) Y128J, E344K, T504M and Q350L; or

(iv) Y128J, E344K, T504M and A316T.

17. The method of any one of claims 13 to 16, in which the modified endoinulinase or functional fragment thereof has an amino acid sequence which is at least 80% identical to the sequence of unmodified wild-type *T. purpuregenus* endoinulinase.

18. The method of any one of claims 13 to 16, in which no other modifications are made to the amino acid sequence.

19. A method for producing fructooligosaccharides comprising contacting inulin with a modified endoinulinase or a functional fragment thereof, of any one of claims 1 to 5.

Fig. 1

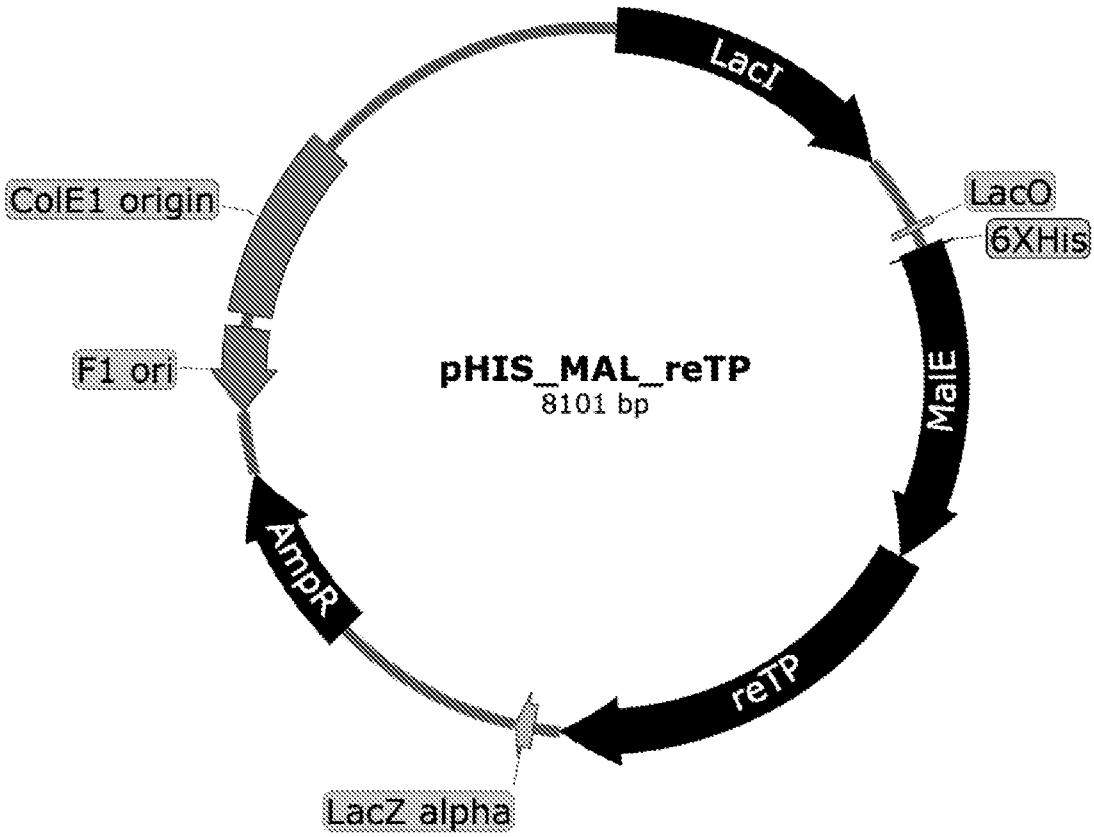

LacI
LacO
6XHis
MalE
reTP
LacZ alpha
AmpR
F1 ori
ColE1 origin
pHIS_MAL_reTP
8101 bp

Fig. 2A

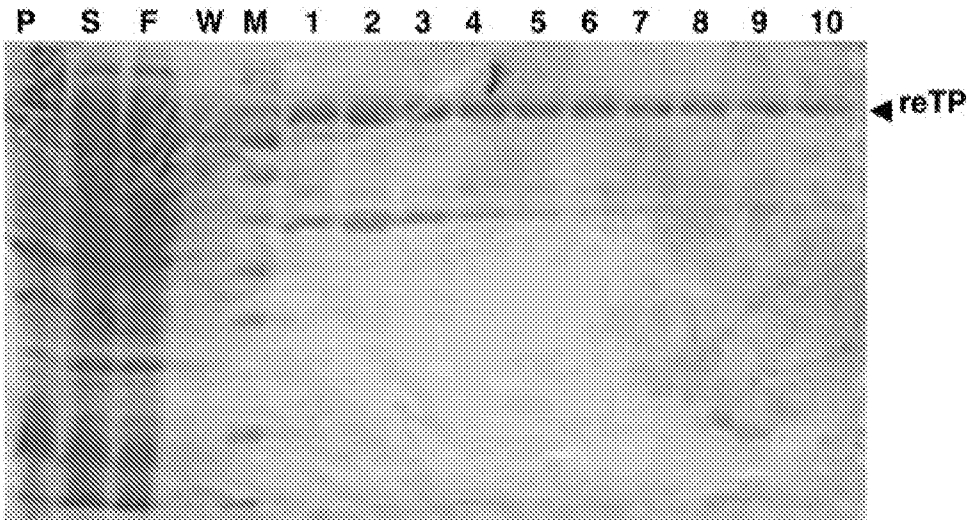

P S F W M 1 2 3 4 5 6 7 8 9 10
reTP

Fig. 2B

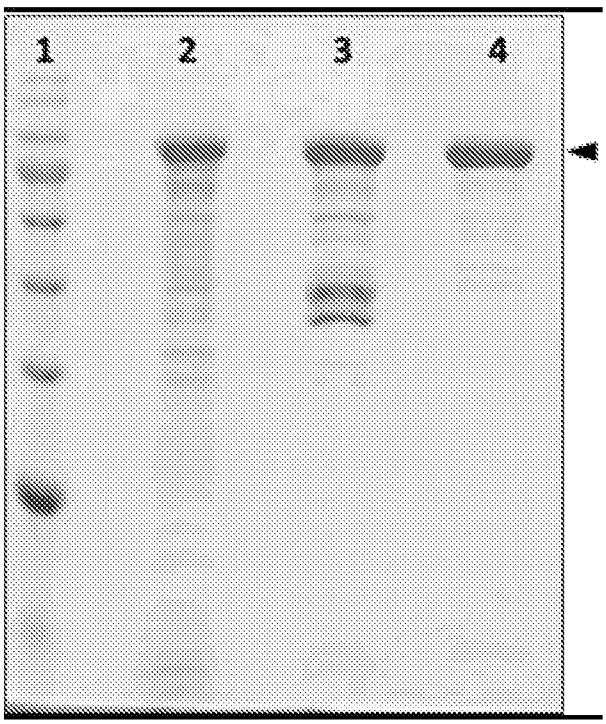


Fig. 2C

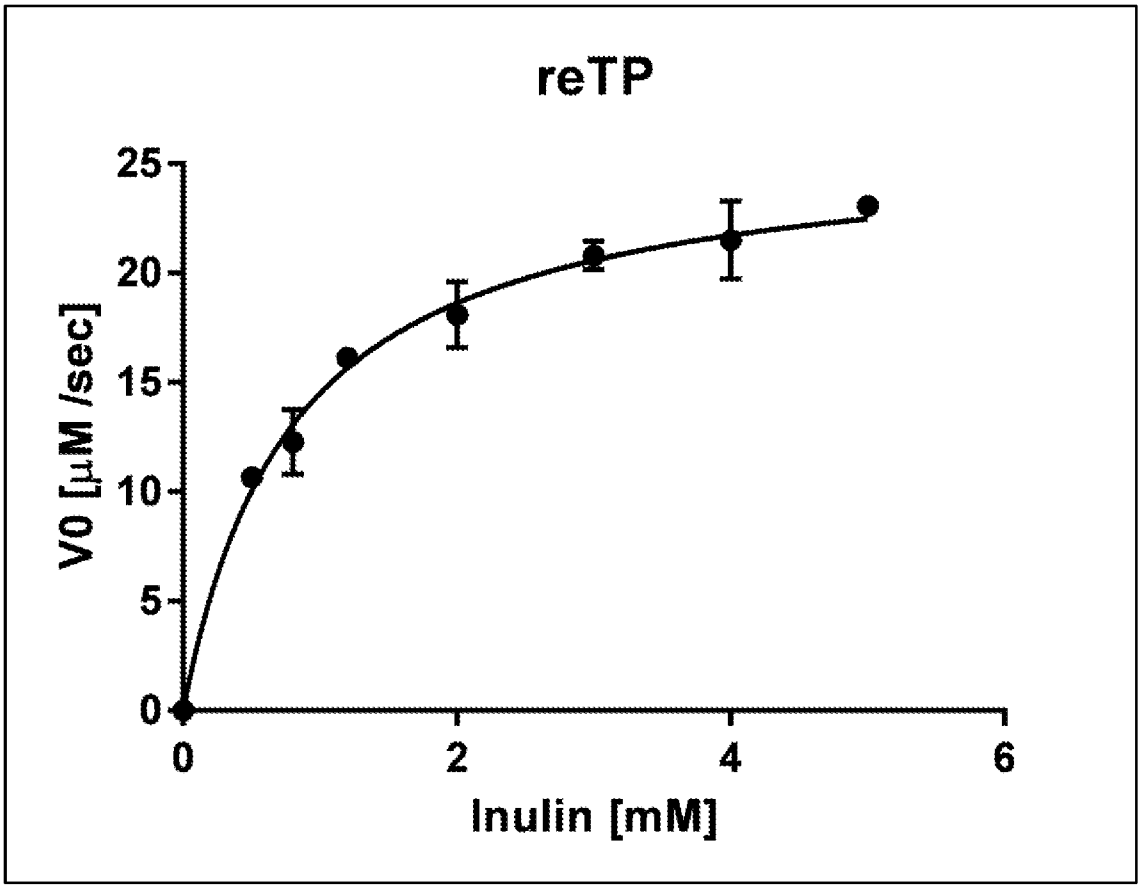


Fig. 3

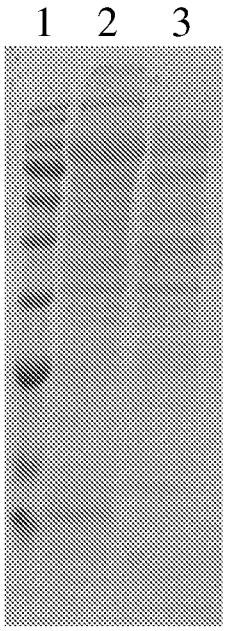

1 2 3

Fig. 4A

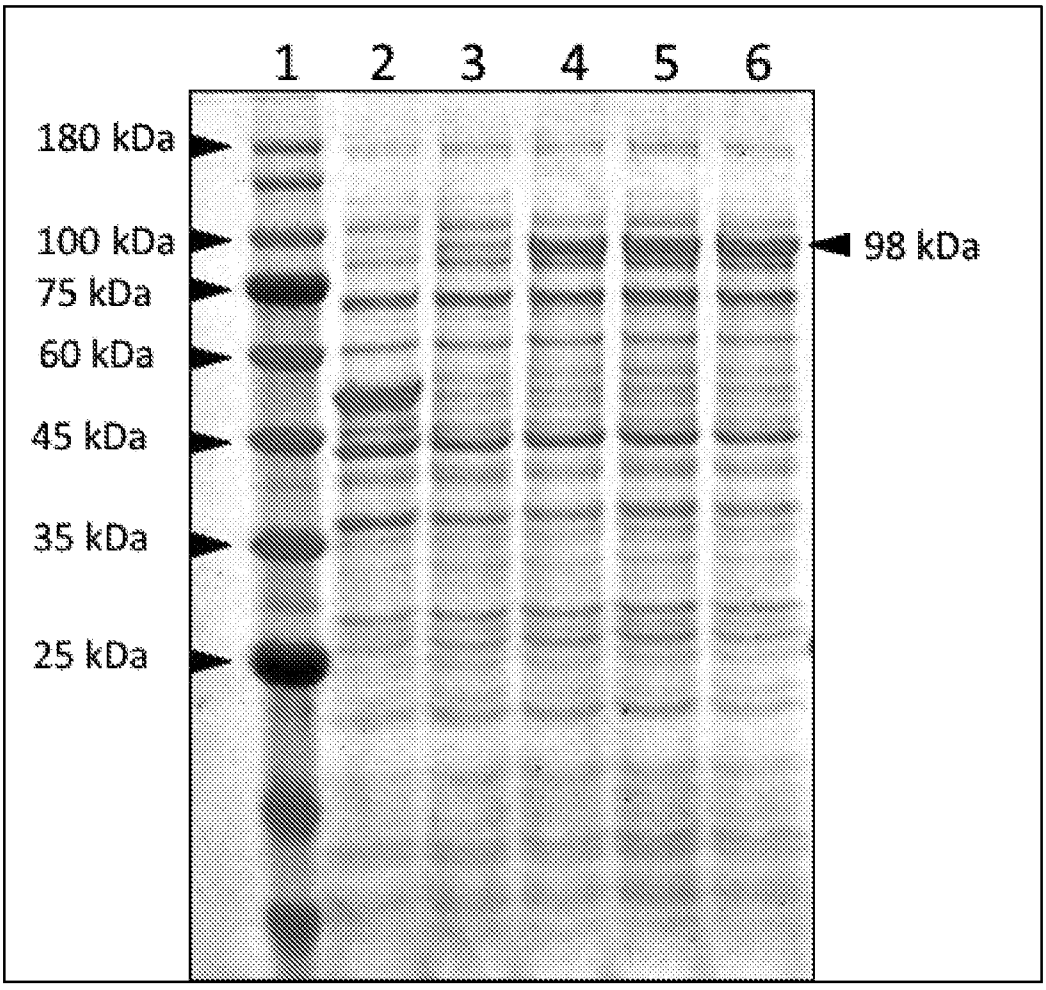

1 2 3 4 5 6
180 kDa
100 kDa
75 kDa
60 kDa
45 kDa
35 kDa
25 kDa
98 kDa

Fig. 4B

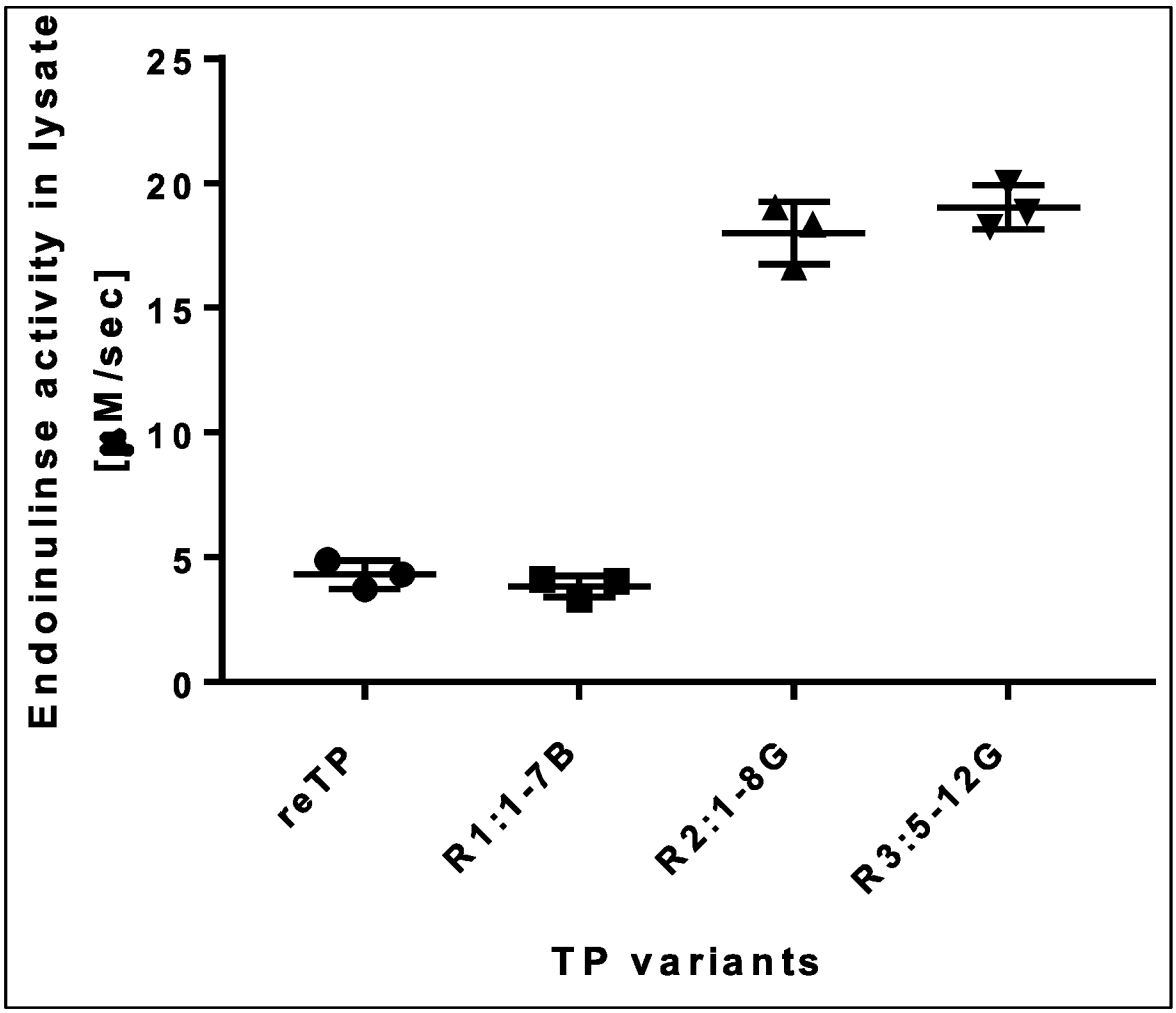

Endoinulinse activity in lysate
[μM/sec]
25
20
15
10
5
0
reTP
R1:1-7B
R2:1-8G
R3:5-12G
TP variants

Fig. 4C

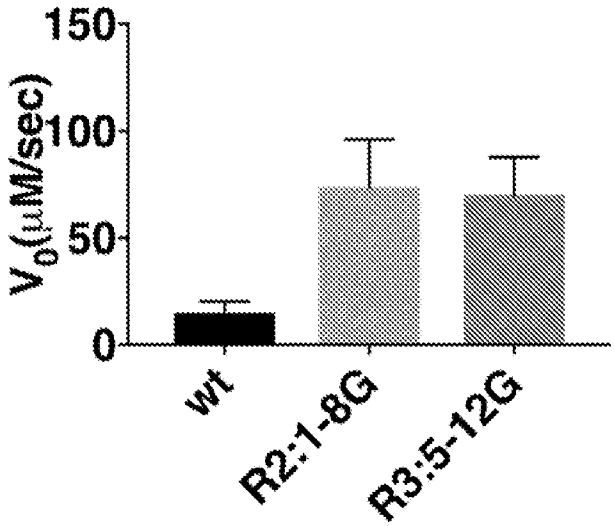

V_0(μM/sec)
150
100
50
0
wt
R2:1-8G
R3:5-12G

Fig. 5

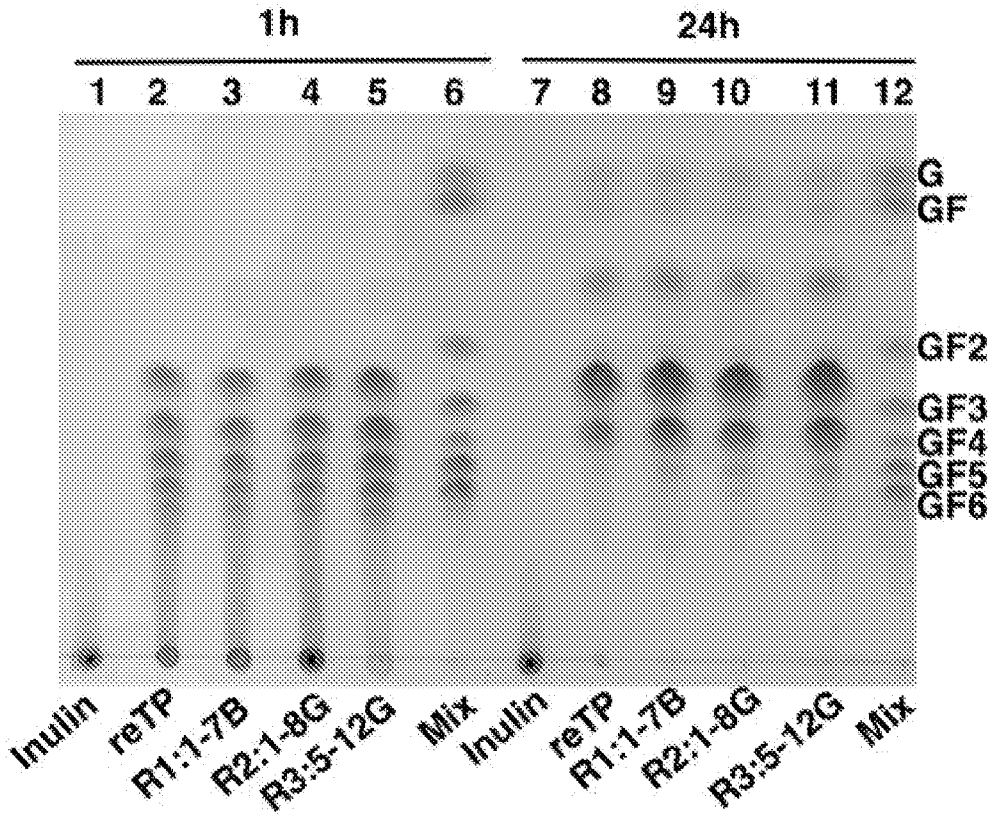

1h
24h
1 2 3 4 5 6 7 8 9 10 11 12
G
GF
GF2
GF3
GF4
GF5
GF6
Inulin
reTP
R1:1-7B
R2:1-8G
R3:5-12G
Mix
Inulin
reTP
R1:1-7B
R2:1-8G
R3:5-12G
Mix

Fig. 6A

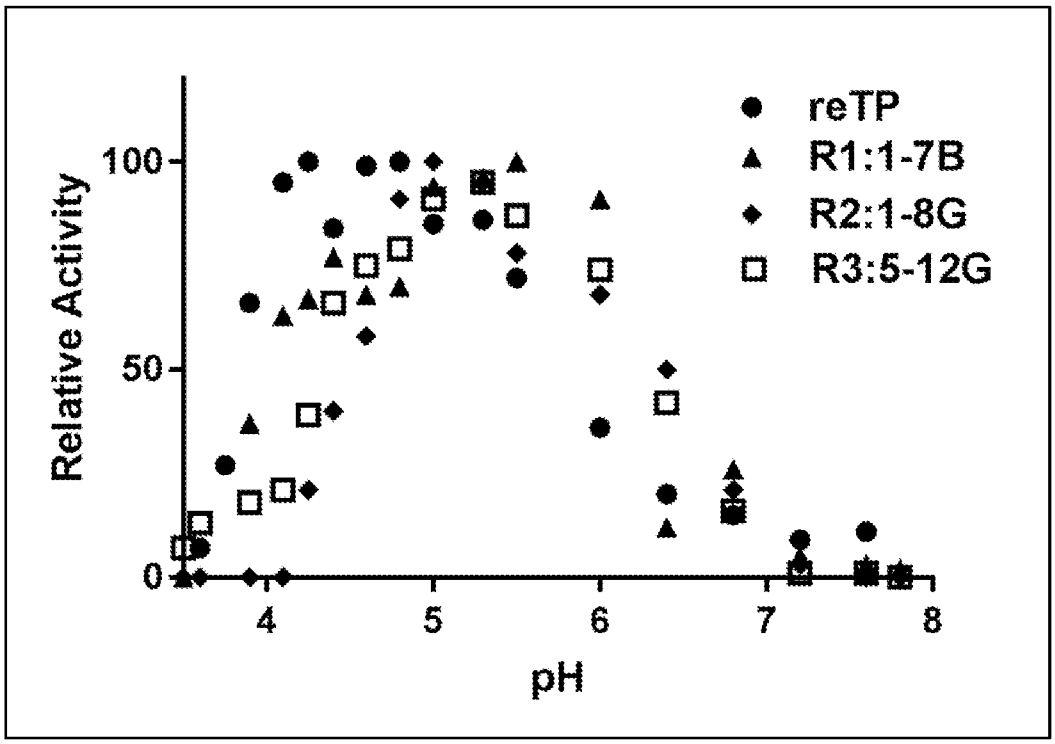

reTP
R1:1-7B
R2:1-8G
R3:5-12G
Relative Activity
100
50
0
4
5
6
7
8
pH

Fig. 6B

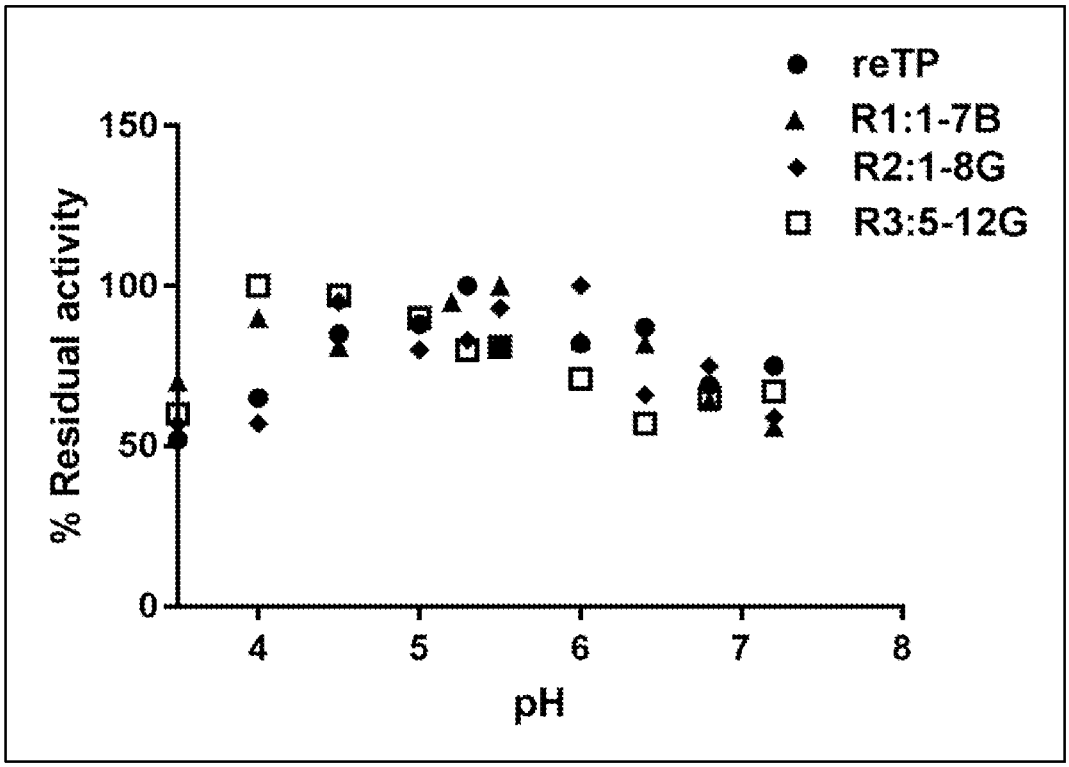

reTP
R1:1-7B
R2:1-8G
R3:5-12G
% Residual activity
150
100
50
0
4
5
6
7
8
pH

Fig. 7A

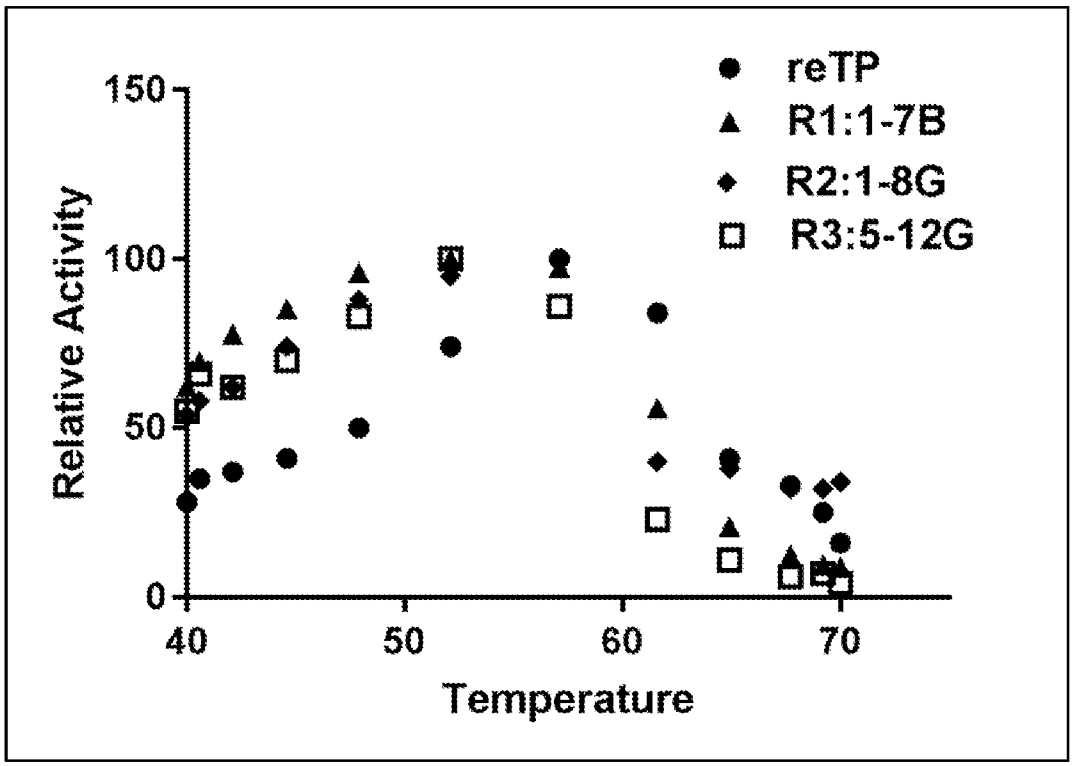

reTP
R1:1-7B
R2:1-8G
R3:5-12G
Relative Activity
150
100
50
0
40
50
60
70
Temperature

Fig. 7B

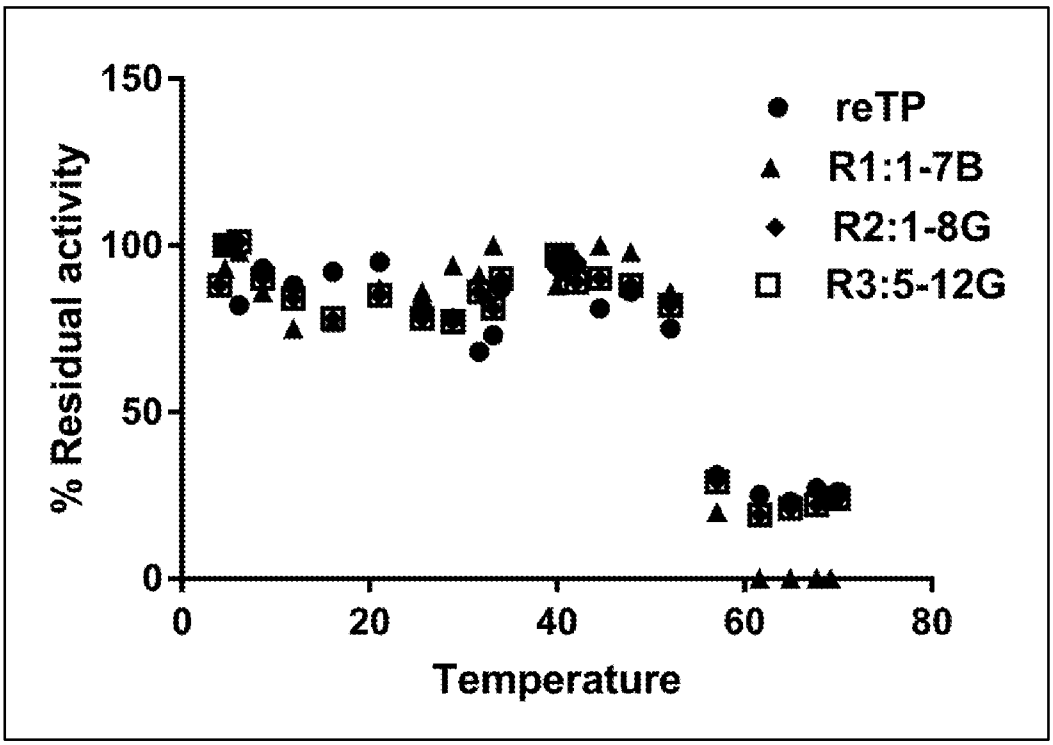

150
100
50
0
% Residual activity
0
20
40
60
80
Temperature
reTP
R1:1-7B
R2:1-8G
R3:5-12G

Fig. 8

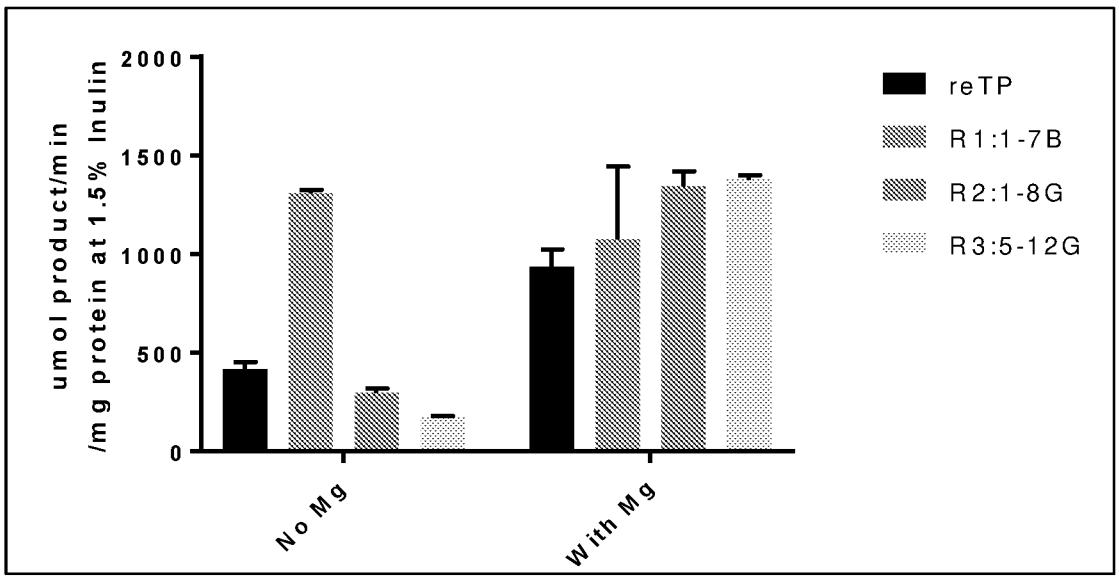
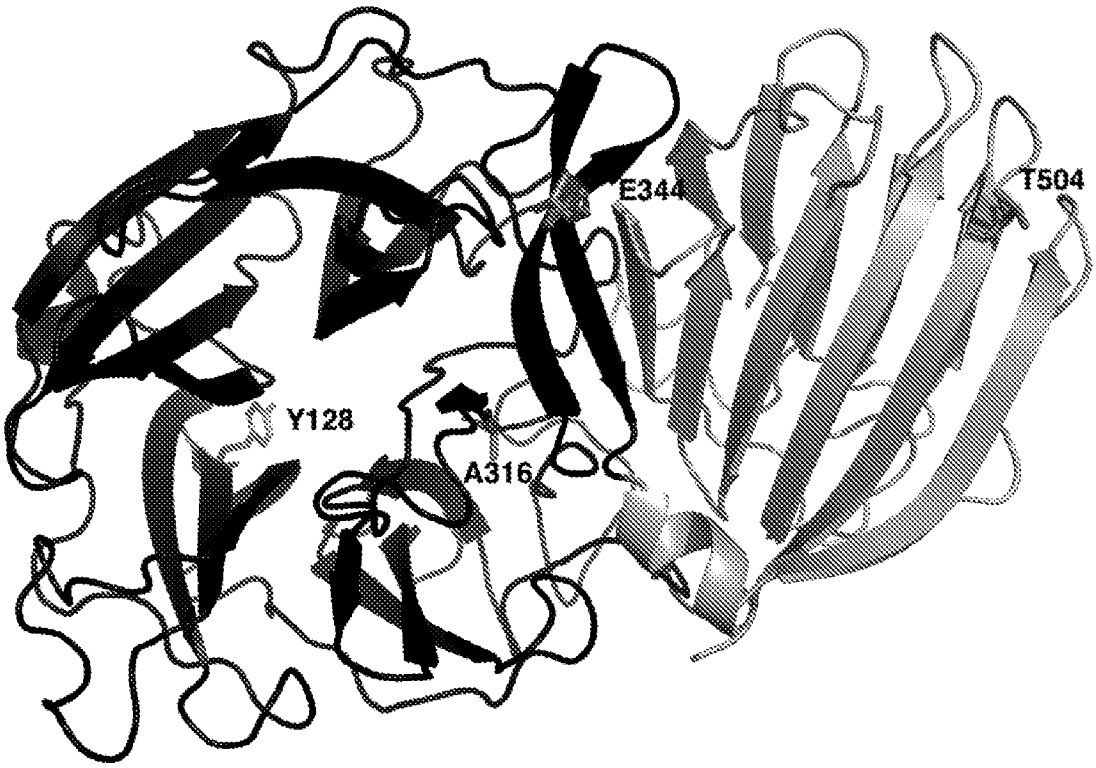

umol product/min /mg protein at 1.5% Inulin
2000
1500
1000
500
0
No Mg
With Mg
reTP
R1:1-7B
R2:1-8G
R3:5-12G

Fig. 9



INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2018/050872

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2018.01) C12N 9/24, C12N 15/01, C07H 3/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2018.01) C12N 9/24, C12N 15/01, C07H 3/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Databases consulted: PATENTSCOPE, THOMSON INNOVATION, Esp@cenet, Google Patents, Google Scholar
Search terms used: "Talaromyces purpureogenus" mutant mutation endoinulinase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Purification and Characterization of an Endoinulinase from Xanthomonas campestris pv. phaseoli KM 24 Mutant. Food technology and biotechnology, 53(2), 146. Naidoo, K., Kumar, A., Sharma, V., Permaul, K., & Singh, S. 23 Feb 2015 (2015/02/23) Whole Document	1-8,19
X	Molecular cloning and nucleotide sequences of cDNA and gene encoding endo-inulinase from Penicillium purpurogenum. Bioscience, biotechnology, and biochemistry, 60(11), 1780-1785. Onodera, S., Murakami, T., Ito, H., Mori, H., Matsui, H., Honma, M., ... & Shiomi, N. 08 Apr 1994 (1994/04/08) Figure 3	9-14,17,18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 Nov 2018	21 Nov 2018

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