

PENICILLIN-G ACYLASES

[0001] The present application claims priority to US Prov. Pat. Appln. Ser. No. 62/158,118, filed May 7, 2015, hereby incorporated by reference in its entirety for all purposes.

REFERENCE TO SEQUENCE LISTING, TABLE OR COMPUTER PROGRAM

[0002] The Sequence Listing concurrently submitted herewith under 37 C.F.R. §1.821 in a computer readable form (CRF) via EFS-Web as file name CX2-149WO1__ST25.txt is herein incorporated by reference. The electronic copy of the Sequence Listing was created on April 28, 2016, with a file size of 88 Kbytes.

FIELD OF THE INVENTION

[0003] The present invention provides engineered penicillin G acylase (PGA) enzymes, polynucleotides encoding the enzymes, compositions comprising the enzymes, and methods of using the engineered PGA enzymes.

BACKGROUND OF THE INVENTION

[0004] Penicillin G acylase (PGA) (penicillin amidase, EC 3.5.1.11) catalyzes the cleavage of the amide bond of penicillin G (benzylpenicillin) side chain. The enzyme is used commercially in the manufacture of 6-amino-penicillanic acid (6-APA) and phenyl-acetic acid (PAA). 6-APA is a key compound in the industrial production of semi-synthetic β -lactam antibiotics such as amoxicillin, ampicillin and cephalexin. The naturally occurring PGA enzyme shows instability in commercial processes, requiring immobilization on solid substrates for commercial applications. PGA has been covalently bonded to various supports and PGA immobilized systems have been reported as useful tools for the synthesis of pure optical isomers. Attachment to solid surfaces, however, leads to compromised enzyme properties, such as reduced activity and/or selectivity, and limitations to solute access. Moreover, although attachment to solid substrates allows capture of enzymes and reuse in additional processing cycles, the stability of the enzyme is such that such applications may be limited. The enzymatic catalysis by PGA of penicillin G to 6-APA is regiospecific (it does not cleave the lactam amide bond) and stereospecific. The production of 6-APA constitutes perhaps the largest utilization of enzymatic catalysis in the production of pharmaceuticals. The enzymatic activity of PGA, associated with the phenacetyl moiety, allows the stereospecific hydrolysis of a rich variety of phenacetyl derivatives of primary amines as well as alcohols.

SUMMARY OF THE INVENTION

[0005] The present invention provides engineered penicillin G acylase (PGA) enzymes, polynucleotides encoding the enzymes, compositions comprising the enzymes, and methods of using the engineered PGA enzymes.

[0006] The present invention provides engineered penicillin G acylases capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups from insulin to produce free insulin, wherein the penicillin G acylase is at least about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more identical to SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some embodiments, the present invention provides engineered penicillin G acylases capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups from insulin to produce free insulin, wherein the penicillin G acylase is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more identical to SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some additional embodiments, the present invention provides engineered penicillin G acylases capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups from insulin to produce free insulin, wherein the penicillin G acylase comprises SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some further embodiments, the penicillin G acylase comprises at least one mutation as provided in Table 5.1, Table 6.2, and/or Table 6.3.

[0007] The present invention also provides a penicillin G acylase encoded by a polynucleotide sequence having at least about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more sequence identity to a sequence selected from SEQ ID NOS:3, 5, 7, 9, and 11.

[0008] In some embodiments, the penicillin G acylase encoded by a polynucleotide sequence has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more sequence identity to a sequence selected from SEQ ID NOS:3, 5, 7, 9, and 11. In some embodiments, the penicillin G acylase encoded by a polynucleotide sequence selected from SEQ ID NOS:3, 5, 7, 9, and 11. The present invention also provides vectors comprising the polynucleotide sequences provided herein (e.g., SEQ ID NOS:3, 5, 7, 9, and/or 11). The present invention also provides host cells comprising the vectors provided herein (e.g., vectors comprising the polynucleotide sequences of SEQ ID NOS:3, 5, 7, 9, and/or 11).

[0009] The present invention also provides methods for producing free insulin, comprising: i) providing at least one engineered penicillin G acylase provided herein, and insulin comprising A1/B1/B29 tri-phenyl acetate protecting groups; and ii) exposing the engineered penicillin G acylase to the insulin comprising A1/B1/B29 tri-phenyl acetate protecting groups, under conditions such that the engineered penicillin G acylase removes the A1/B1/B29 tri-phenyl acetate protecting groups and free insulin is produced. In some embodiments of the methods, the penicillin G acylase is at least about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about

93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more identical to SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some embodiments of the methods, the penicillin G acylase is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more identical to SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some further embodiments of the methods, , the penicillin G acylase comprises SEQ ID NO:2, 4, 6, 8, 10, and/or 12. In some embodiments, the engineered penicillin G acylase produces more than 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more free insulin. The present invention also provides compositions comprising free insulin produced according to the method(s) provided herein.

[0009A] The invention also provides an engineered non-naturally occurring penicillin G acylase capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups from insulin to produce free insulin, wherein said penicillin G acylase comprises SEQ ID NO:12.

BRIEF DESCRIPTION OF THE FIGURES

[0010] Figure 1 provides a graph showing the substrate inhibition activity observed with Variant 1.

[0011] Figure 2 provides a graph showing the amount of free insulin produced using seven variant PGAs.

[0012] Figure 3 provides a graph showing % yield of free insulin produced using three variant PGAs.

[0013] Figure 4 provides a graph showing % yield of free insulin produced using three variant PGAs in the presence of DMSO in the reactions.

DESCRIPTION OF THE INVENTION

[0014] The present invention provides engineered penicillin G acylases (PGA) that are capable of cleaving penicillin to phenylacetic acid and 6-aminopenicillanic acid (6-APA), which is a key intermediate in the synthesis of a large variety of β -lactam antibiotics. In particular, the present invention provides engineered PGAs that are capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups to release free insulin.

[0015] Generally, naturally occurring PGAs are heterodimeric enzymes composed of an alpha subunit and a beta-subunit. Wild-type PGA is naturally synthesized as a pre-pro-PGA polypeptide, containing an N-terminal signal peptide that mediates translocation to the periplasm and a linker region connecting the C-terminus of the alpha subunit to the N-terminus of the beta subunit. Proteolytic processing leads to the mature heterodimeric enzyme. The intermolecular linker region can also function in promoting proper folding of the enzyme. The PGAs described herein are based on the PGA from *Kluyvera citrophila* in which various modifications have been introduced to generate improved enzymatic properties as described in detail below.

[0016] For the descriptions provided herein, the use of the singular includes the plural (and vice versa) unless specifically stated otherwise. For instance, the singular forms “a”, “an” and “the”

[0017] include plural referents unless the context clearly indicates otherwise. Similarly, “comprise,” “comprises,” “comprising” “include,” “includes,” and “including” are interchangeable and not intended to be limiting. It is to be further understood that where descriptions of various embodiments

use the term “comprising,” those skilled in the art would understand that in some specific instances, an embodiment can be alternatively described using language “consisting essentially of” or “consisting of.”

[0017] Both the foregoing general description, including the drawings, and the following detailed description are exemplary and explanatory only and are not restrictive of this disclosure. Moreover, the section headings used herein are for organizational purposes only and not to be construed as limiting the subject matter described.

Definitions

[0018] As used herein, the following terms are intended to have the following meanings.

[0019] In reference to the present disclosure, the technical and scientific terms used in the descriptions herein will have the meanings commonly understood by one of ordinary skill in the art, unless specifically defined otherwise. Accordingly, the following terms are intended to have the following meanings. All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference. Unless otherwise indicated, the practice of the present invention involves conventional techniques commonly used in molecular biology, fermentation, microbiology, and related fields, which are known to those of skill in the art. Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Indeed, it is intended that the present invention not be limited to the particular methodology, protocols, and reagents described herein, as these may vary, depending upon the context in which they are used. The headings provided herein are not limitations of the various aspects or embodiments of the present invention.

[0020] Nonetheless, in order to facilitate understanding of the present invention, a number of terms are defined below. Numeric ranges are inclusive of the numbers defining the range. Thus, every numerical range disclosed herein is intended to encompass every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein. It is also intended that every maximum (or minimum) numerical limitation disclosed herein includes every lower (or higher) numerical limitation, as if such lower (or higher) numerical limitations were expressly written herein.

[0021] As used herein, the term “comprising” and its cognates are used in their inclusive sense (*i.e.*, equivalent to the term “including” and its corresponding cognates).

[0022] As used herein and in the appended claims, the singular “a”, “an” and “the” include the plural reference unless the context clearly dictates otherwise. Thus, for example, reference to a “host cell” includes a plurality of such host cells.

[0023] Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation and amino acid sequences are written left to right in amino to carboxy orientation, respectively.

[0024] The headings provided herein are not limitations of the various aspects or embodiments of the invention that can be had by reference to the specification as a whole. Accordingly, the terms defined below are more fully defined by reference to the specification as a whole.

[0025] As used herein, the terms “protein,” “polypeptide,” and “peptide” are used interchangeably herein to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (*e.g.*, glycosylation, phosphorylation, lipidation, myristilation, ubiquitination, etc.). Included within this definition are D- and L-amino acids, and mixtures of D- and L-amino acids.

[0026] As used herein, “polynucleotide” and “nucleic acid” refer to two or more nucleosides that are covalently linked together. The polynucleotide may be wholly comprised ribonucleosides (*i.e.*, an RNA), wholly comprised of 2' deoxyribonucleotides (*i.e.*, a DNA) or mixtures of ribo- and 2' deoxyribonucleosides. While the nucleosides will typically be linked together via standard phosphodiester linkages, the polynucleotides may include one or more non-standard linkages. The polynucleotide may be single-stranded or double-stranded, or may include both single-stranded regions and double-stranded regions. Moreover, while a polynucleotide will typically be composed of the naturally occurring encoding nucleobases (*i.e.*, adenine, guanine, uracil, thymine, and cytosine), it may include one or more modified and/or synthetic nucleobases (*e.g.*, inosine, xanthine, hypoxanthine, etc.). Preferably, such modified or synthetic nucleobases will be encoding nucleobases.

[0027] As used herein, “hybridization stringency” relates to hybridization conditions, such as washing conditions, in the hybridization of nucleic acids. Generally, hybridization reactions are performed under conditions of lower stringency, followed by washes of varying but higher stringency. The term “moderately stringent hybridization” refers to conditions that permit target-DNA to bind a complementary nucleic acid that has about 60% identity, preferably about 75% identity, about 85% identity to the target DNA; with greater than about 90% identity to target-polynucleotide. Exemplary moderately stringent conditions are conditions equivalent to hybridization in 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42°C., followed by washing in 0.2×SSPE, 0.2% SDS, at 42°C. “High stringency hybridization” refers generally to conditions that are about 10°C or less from the thermal melting temperature T_m as determined under the solution condition for a defined polynucleotide sequence. In some embodiments, a high stringency condition refers to conditions that permit hybridization of only those nucleic acid sequences that form stable hybrids in 0.018M NaCl at 65°C. (*i.e.*, if a hybrid is not stable in 0.018M NaCl at 65°C, it will not be stable under high stringency conditions, as contemplated herein). High stringency conditions can be provided, for example, by hybridization in conditions equivalent to 50% formamide, 5× Denhart's solution, 5×SSPE, 0.2% SDS at 42°C, followed by washing in 0.1×SSPE, and 0.1% SDS at 65°C. Another high stringency

condition is hybridizing in conditions equivalent to hybridizing in 5X SSC containing 0.1% (w:v) SDS at 65°C and washing in 0.1x SSC containing 0.1% SDS at 65°C. Other high stringency hybridization conditions, as well as moderately stringent conditions, are known to those of skill in the art.

[0028] As used herein, “coding sequence” refers to that portion of a nucleic acid (*e.g.*, a gene) that encodes an amino acid sequence of a protein.

[0029] As used herein, “codon optimized” refers to changes in the codons of the polynucleotide encoding a protein to those preferentially used in a particular organism such that the encoded protein is efficiently expressed in the organism of interest. In some embodiments, the polynucleotides encoding the PGA enzymes may be codon optimized for optimal production from the host organism selected for expression. Although the genetic code is degenerate in that most amino acids are represented by several codons, called “synonyms” or “synonymous” codons, it is well known that codon usage by particular organisms is nonrandom and biased towards particular codon triplets. This codon usage bias may be higher in reference to a given gene, genes of common function or ancestral origin, highly expressed proteins versus low copy number proteins, and the aggregate protein coding regions of an organism's genome. In some embodiments, the polynucleotides encoding the PGAs enzymes may be codon optimized for optimal production from the host organism selected for expression.

[0030] As used herein, “preferred, optimal, high codon usage bias codons” refers interchangeably to codons that are used at higher frequency in the protein coding regions than other codons that code for the same amino acid. The preferred codons may be determined in relation to codon usage in a single gene, a set of genes of common function or origin, highly expressed genes, the codon frequency in the aggregate protein coding regions of the whole organism, codon frequency in the aggregate protein coding regions of related organisms, or combinations thereof. Codons whose frequency increases with the level of gene expression are typically optimal codons for expression. A variety of methods are known for determining the codon frequency (*e.g.*, codon usage, relative synonymous codon usage) and codon preference in specific organisms, including multivariate analysis, for example, using cluster analysis or correspondence analysis, and the effective number of codons used in a gene (See *e.g.*, GCG CodonPreference, Genetics Computer Group Wisconsin Package; CodonW, John Peden, University of Nottingham; McInerney, *Bioinform.*, 14:372-73 [1998]; Stenico et al., *Nucleic Acids Res.*, 22:437-46 [1994]; and Wright, *Gene* 87:23-29 [1990]). Codon usage tables are available for a growing list of organisms (See *e.g.*, Wada et al., *Nucleic Acids Res.*, 20:2111-2118 [1992]; Nakamura et al., *Nucl. Acids Res.*, 28:292 [2000]; Duret, et al., *supra*; Henaut and Danchin, “*Escherichia coli* and *Salmonella*,” Neidhardt, et al. (eds.), ASM Press, Washington D.C., [1996], p. 2047-2066. The data source for obtaining codon usage may rely on any available nucleotide sequence capable of coding for a protein. These data sets include nucleic acid sequences actually known to encode expressed proteins (*e.g.*, complete protein coding sequences-CDS), expressed sequence tags (ESTS),

or predicted coding regions of genomic sequences (See e.g., Uberbacher, Meth. Enzymol., 266:259-281 [1996]; Tiwari et al., Comput. Appl. Biosci., 13:263-270 [1997]).

[0031] As used herein, “control sequence” is defined herein to include all components, which are necessary or advantageous for the expression of a polynucleotide and/or polypeptide of the present disclosure. Each control sequence may be native or foreign to the polynucleotide of interest. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator.

[0032] As used herein, “operably linked” is defined herein as a configuration in which a control sequence is appropriately placed (*i.e.*, in a functional relationship) at a position relative to a polynucleotide of interest such that the control sequence directs or regulates the expression of the polynucleotide and/or polypeptide of interest.

[0033] As used herein, “promoter sequence” refers to a nucleic acid sequence that is recognized by a host cell for expression of a polynucleotide of interest, such as a coding sequence. The control sequence may comprise an appropriate promoter sequence. The promoter sequence contains transcriptional control sequences, which mediate the expression of a polynucleotide of interest. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

[0034] As used herein, “naturally occurring” or “wild-type” refers to the form found in nature. For example, a naturally occurring or wild-type polypeptide or polynucleotide sequence is a sequence present in an organism that can be isolated from a source in nature and which has not been intentionally modified by human manipulation.

[0035] As used herein, “non-naturally occurring,” “engineered,” and “recombinant” when used in the present disclosure with reference to (*e.g.*, a cell, nucleic acid, or polypeptide), refers to a material, or a material corresponding to the natural or native form of the material, that has been modified in a manner that would not otherwise exist in nature. In some embodiments the material is identical to naturally occurring material, but is produced or derived from synthetic materials and/or by manipulation using recombinant techniques. Non-limiting examples include, among others, recombinant cells expressing genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise expressed at a different level.

[0036] As used herein, “percentage of sequence identity,” “percent identity,” and “percent identical” refer to comparisons between polynucleotide sequences or polypeptide sequences, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) as compared to the reference sequence for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which either the identical nucleic acid base or amino acid residue occurs in both sequences or a nucleic acid base or

amino acid residue is aligned with a gap to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Determination of optimal alignment and percent sequence identity is performed using the BLAST and BLAST 2.0 algorithms (See e.g., Altschul et al., J. Mol. Biol. 215: 403-410 [1990]; and Altschul et al., Nucl. Acids Res. 3389-3402 [1977]). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information website.

[0037] Briefly, the BLAST analyses involve first identifying high scoring sequence pairs (HSPs) by identifying short words of length *W* in the query sequence, which either match or satisfy some positive-valued threshold score *T* when aligned with a word of the same length in a database sequence. *T* is referred to as, the neighborhood word score threshold (Altschul et al., *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters *M* (reward score for a pair of matching residues; always >0) and *N* (penalty score for mismatching residues; always <0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity *X* from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters *W*, *T*, and *X* determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (*W*) of 11, an expectation (*E*) of 10, *M*=5, *N*=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (*W*) of 3, an expectation (*E*) of 10, and the BLOSUM62 scoring matrix (See e.g., Henikoff and Henikoff, Proc Natl Acad Sci USA 89:10915 [1989]).

[0038] Numerous other algorithms are available and known in the art that function similarly to BLAST in providing percent identity for two sequences. Optimal alignment of sequences for comparison can be conducted using any suitable method known in the art (*e.g.*, by the local homology algorithm of Smith and Waterman, Adv. Appl. Math. 2:482 [1981]; by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443 [1970]; by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85:2444 [1988]; and/or by computerized implementations of these algorithms [GAP, BESTFIT, FASTA, and TFASTA in the GCG Wisconsin Software Package]), or by visual inspection, using methods commonly known in the art.

Additionally, determination of sequence alignment and percent sequence identity can employ the BESTFIT or GAP programs in the GCG Wisconsin Software package (Accelrys, Madison WI), using the default parameters provided.

[0039] As used herein, “substantial identity” refers to a polynucleotide or polypeptide sequence that has at least 80 percent sequence identity, at least 85 percent identity and 89 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 residue positions, frequently over a window of at least 30-50 residues, wherein the percentage of sequence identity is calculated by comparing the reference sequence to a sequence that includes deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. In specific embodiments applied to polypeptides, the term “substantial identity” means that two polypeptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 89 percent sequence identity, at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). In some preferred embodiments, residue positions that are not identical differ by conservative amino acid substitutions.

[0040] As used herein, “reference sequence” refers to a defined sequence to which another sequence is compared. A reference sequence may be a subset of a larger sequence, for example, a segment of a full-length gene or polypeptide sequence. Generally, a reference sequence is at least 20 nucleotide or amino acid residues in length, at least 25 residues in length, at least 50 residues in length, or the full length of the nucleic acid or polypeptide. Since two polynucleotides or polypeptides may each (1) comprise a sequence (*i.e.*, a portion of the complete sequence) that is similar between the two sequences, and (2) may further comprise a sequence that is divergent between the two sequences, sequence comparisons between two (or more) polynucleotides or polypeptide are typically performed by comparing sequences of the two polynucleotides over a comparison window to identify and compare local regions of sequence similarity. The term “reference sequence” is not intended to be limited to wild-type sequences, and can include engineered or altered sequences. For example, in some embodiments, a “reference sequence” can be a previously engineered or altered amino acid sequence.

[0041] As used herein, “comparison window” refers to a conceptual segment of at least about 20 contiguous nucleotide positions or amino acids residues wherein a sequence may be compared to a reference sequence of at least 20 contiguous nucleotides or amino acids and wherein the portion of the sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The comparison window can be longer than 20 contiguous residues, and includes, optionally 30, 40, 50, 100, or longer windows.

[0042] As used herein, “corresponding to,” “reference to,” and “relative to” when used in the context of the numbering of a given amino acid or polynucleotide sequence refers to the numbering of the residues of a specified reference sequence when the given amino acid or polynucleotide sequence is compared to the reference sequence. In other words, the residue number or residue position of a given polymer is designated with respect to the reference sequence rather than by the actual numerical

position of the residue within the given amino acid or polynucleotide sequence. For example, a given amino acid sequence, such as that of an engineered PGA, can be aligned to a reference sequence by introducing gaps to optimize residue matches between the two sequences. In these cases, although the gaps are present, the numbering of the residue in the given amino acid or polynucleotide sequence is made with respect to the reference sequence to which it has been aligned. As used herein, a reference to a residue position, such as "Xn" as further described below, is to be construed as referring to "a residue corresponding to", unless specifically denoted otherwise. Thus, for example, "X94" refers to any amino acid at position 94 in a polypeptide sequence.

[0043] As used herein, "improved enzyme property" refers to a PGA that exhibits an improvement in any enzyme property as compared to a reference PGA. For the engineered PGA polypeptides described herein, the comparison is generally made to the wild-type PGA enzyme, although in some embodiments, the reference PGA can be another improved engineered PGA. Enzyme properties for which improvement is desirable include, but are not limited to, enzymatic activity (which can be expressed in terms of percent conversion of the substrate at a specified reaction time using a specified amount of PGA), thermal stability, solvent stability, pH activity profile, cofactor requirements, refractoriness to inhibitors (*e.g.*, product inhibition), stereospecificity, and stereoselectivity (including enantioselectivity).

[0044] As used herein, "increased enzymatic activity" refers to an improved property of the engineered PGA polypeptides, which can be represented by an increase in specific activity (*e.g.*, product produced/time/weight protein) or an increase in percent conversion of the substrate to the product (*e.g.*, percent conversion of starting amount of substrate to product in a specified time period using a specified amount of PGA) as compared to the reference PGA enzyme. Exemplary methods to determine enzyme activity are provided in the Examples. Any property relating to enzyme activity may be affected, including the classical enzyme properties of K_m , V_{max} or k_{cat} , changes of which can lead to increased enzymatic activity. Improvements in enzyme activity can be from about 1.5 times the enzymatic activity of the corresponding wild-type PGA enzyme, to as much as 2 times, 5 times, 10 times, 20 times, 25 times, 50 times, 75 times, 100 times, or more enzymatic activity than the naturally occurring PGA or another engineered PGA from which the PGA polypeptides were derived. In specific embodiments, the engineered PGA enzyme exhibits improved enzymatic activity in the range of 1.5 to 50 times, 1.5 to 100 times greater than that of the parent PGA enzyme. It is understood by the skilled artisan that the activity of any enzyme is diffusion limited such that the catalytic turnover rate cannot exceed the diffusion rate of the substrate, including any required cofactors. The theoretical maximum of the diffusion limit, or k_{cat}/K_m , is generally about 10^8 to 10^9 ($M^{-1} s^{-1}$). Hence, any improvements in the enzyme activity of the PGA will have an upper limit related to the diffusion rate of the substrates acted on by the PGA enzyme. PGA activity can be measured by any one of standard assays used for measuring the release of phenylacetic acid upon cleavage of penicillin G, such as by titration (See *e.g.*, Simons and Gibson, *Biotechnol. Tech.*, 13:365-367 [1999]).

In some embodiments, the PGA activity can be measured by using 6-nitrophenylacetamido benzoic acid (NIPAB), which cleavage product 5-amino-2-nitro-benzoic acid is detectable spectrophotometrically ($\lambda_{\text{max}} = 405 \text{ nm}$). Comparisons of enzyme activities are made using a defined preparation of enzyme, a defined assay under a set condition, and one or more defined substrates, as further described in detail herein. Generally, when lysates are compared, the numbers of cells and the amount of protein assayed are determined as well as use of identical expression systems and identical host cells to minimize variations in amount of enzyme produced by the host cells and present in the lysates.

[0045] As used herein, “increased enzymatic activity” and “increased activity” refer to an improved property of an engineered enzyme, which can be represented by an increase in specific activity (*e.g.*, product produced/time/weight protein) or an increase in percent conversion of the substrate to the product (*e.g.*, percent conversion of starting amount of substrate to product in a specified time period using a specified amount of PGA) as compared to a reference enzyme as described herein. Any property relating to enzyme activity may be affected, including the classical enzyme properties of K_m , V_{max} or k_{cat} , changes of which can lead to increased enzymatic activity. In some embodiments, the PGA enzymes provided herein frees insulin by removing tri-phenyl acetate protecting groups from specific residues of insulin. Comparisons of enzyme activities are made using a defined preparation of enzyme, a defined assay under a set condition, and one or more defined substrates, as further described in detail herein. Generally, when enzymes in cell lysates are compared, the numbers of cells and the amount of protein assayed are determined as well as use of identical expression systems and identical host cells to minimize variations in amount of enzyme produced by the host cells and present in the lysates.

[0046] As used herein, “conversion” refers to the enzymatic transformation of a substrate to the corresponding product.

[0047] As used herein “percent conversion” refers to the percent of the substrate that is converted to the product within a period of time under specified conditions. Thus, for example, the “enzymatic activity” or “activity” of a PGA polypeptide can be expressed as “percent conversion” of the substrate to the product.

[0048] As used herein, “chemoselectivity” refers to the preferential formation in a chemical or enzymatic reaction of one product over another.

[0049] As used herein, “thermostable” and “thermal stable” are used interchangeably to refer to a polypeptide that is resistant to inactivation when exposed to a set of temperature conditions (*e.g.*, 40-80°C) for a period of time (*e.g.*, 0.5-24 hrs) compared to the untreated enzyme, thus retaining a certain level of residual activity (*e.g.*, more than 60% to 80%) after exposure to elevated temperatures.

[0050] As used herein, “solvent stable” refers to the ability of a polypeptide to maintain similar activity (*e.g.*, more than *e.g.*, 60% to 80%) after exposure to varying concentrations (*e.g.*, 5-99%) of solvent (*e.g.*, isopropyl alcohol, tetrahydrofuran, 2-methyltetrahydrofuran, acetone, toluene,

butylacetate, methyl tert-butylether, etc.) for a period of time (*e.g.*, 0.5-24 hrs) compared to the untreated enzyme.

[0051] As used herein, “pH stable” refers to a PGA polypeptide that maintains similar activity (*e.g.*, more than 60% to 80%) after exposure to high or low pH (*e.g.*, 4.5-6 or 8 to 12) for a period of time (*e.g.*, 0.5-24 hrs) compared to the untreated enzyme.

[0052] As used herein, “thermo- and solvent stable” refers to a PGA polypeptide that is both thermostable and solvent stable.

[0053] As used herein, “hydrophilic amino acid or residue” refers to an amino acid or residue having a side chain exhibiting a hydrophobicity of less than zero according to the normalized consensus hydrophobicity scale of Eisenberg et al., (Eisenberg et al., *J. Mol. Biol.*, 179:125-142 [1984]). Genetically encoded hydrophilic amino acids include L-Thr (T), L-Ser (S), L-His (H), L-Glu (E), L-Asn (N), L-Gln (Q), L-Asp (D), L-Lys (K) and L-Arg (R).

[0054] As used herein, “acidic amino acid or residue” refers to a hydrophilic amino acid or residue having a side chain exhibiting a pK value of less than about 6 when the amino acid is included in a peptide or polypeptide. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Genetically encoded acidic amino acids include L-Glu (E) and L-Asp (D).

[0055] As used herein, “basic amino acid or residue” refers to a hydrophilic amino acid or residue having a side chain exhibiting a pK value of greater than about 6 when the amino acid is included in a peptide or polypeptide. Basic amino acids typically have positively charged side chains at physiological pH due to association with hydronium ion. Genetically encoded basic amino acids include L-Arg (R) and L-Lys (K).

[0056] As used herein, “polar amino acid or residue” refers to a hydrophilic amino acid or residue having a side chain that is uncharged at physiological pH, but which has at least one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Genetically encoded polar amino acids include L-Asn (N), L-Gln (Q), L-Ser (S) and L-Thr (T).

[0057] As used herein, “hydrophobic amino acid or residue” refers to an amino acid or residue having a side chain exhibiting a hydrophobicity of greater than zero according to the normalized consensus hydrophobicity scale of Eisenberg et al., (Eisenberg et al., *J. Mol. Biol.*, 179:125-142 [1984]). Genetically encoded hydrophobic amino acids include L-Pro (P), L-Ile (I), L-Phe (F), L-Val (V), L-Leu (L), L-Trp (W), L-Met (M), L-Ala (A) and L-Tyr (Y).

[0058] As used herein, “aromatic amino acid or residue” refers to a hydrophilic or hydrophobic amino acid or residue having a side chain that includes at least one aromatic or heteroaromatic ring. Genetically encoded aromatic amino acids include L-Phe (F), L-Tyr (Y) and L-Trp (W). Although owing to the pKa of its heteroaromatic nitrogen atom L-His (H) it is sometimes classified as a basic residue, or as an aromatic residue as its side chain includes a heteroaromatic ring, herein histidine is classified as a hydrophilic residue or as a “constrained residue” (see below).

[0059] As used herein, “constrained amino acid or residue” refers to an amino acid or residue that has a constrained geometry. Herein, constrained residues include L-Pro (P) and L-His (H). Histidine has a constrained geometry because it has a relatively small imidazole ring. Proline has a constrained geometry because it also has a five membered ring.

[0060] As used herein, “non-polar amino acid or residue” refers to a hydrophobic amino acid or residue having a side chain that is uncharged at physiological pH and which has bonds in which the pair of electrons shared in common by two atoms is generally held equally by each of the two atoms (*i.e.*, the side chain is not polar). Genetically encoded non-polar amino acids include L-Gly (G), L-Leu (L), L-Val (V), L-Ile (I), L-Met (M) and L-Ala (A).

[0061] As used herein, “aliphatic amino acid or residue” refers to a hydrophobic amino acid or residue having an aliphatic hydrocarbon side chain. Genetically encoded aliphatic amino acids include L-Ala (A), L-Val (V), L-Leu (L) and L-Ile (I).

[0062] It is noted that cysteine (or “L-Cys” or “[C]”) is unusual in that it can form disulfide bridges with other L-Cys (C) amino acids or other sulfanyl- or sulfhydryl-containing amino acids. The “cysteine-like residues” include cysteine and other amino acids that contain sulfhydryl moieties that are available for formation of disulfide bridges. The ability of L-Cys (C) (and other amino acids with -SH containing side chains) to exist in a peptide in either the reduced free -SH or oxidized disulfide-bridged form affects whether L-Cys (C) contributes net hydrophobic or hydrophilic character to a peptide. While L-Cys (C) exhibits a hydrophobicity of 0.29 according to the normalized consensus scale of Eisenberg (Eisenberg et al., 1984, *supra*), it is to be understood that for purposes of the present disclosure L-Cys (C) is categorized into its own unique group.

[0063] As used herein, “small amino acid or residue” refers to an amino acid or residue having a side chain that is composed of a total three or fewer carbon and/or heteroatoms (excluding the α -carbon and hydrogens). The small amino acids or residues may be further categorized as aliphatic, non-polar, polar or acidic small amino acids or residues, in accordance with the above definitions. Genetically-encoded small amino acids include L-Ala (A), L-Val (V), L-Cys (C), L-Asn (N), L-Ser (S), L-Thr (T) and L-Asp (D).

[0064] As used herein, “hydroxyl-containing amino acid or residue” refers to an amino acid containing a hydroxyl (-OH) moiety. Genetically-encoded hydroxyl-containing amino acids include L-Ser (S) L-Thr (T) and L-Tyr (Y).

[0065] As used herein, “amino acid difference” and “residue difference” refer to a difference in the amino acid residue at a position of a polypeptide sequence relative to the amino acid residue at a corresponding position in a reference sequence. The positions of amino acid differences generally are referred to herein as “Xn,” where n refers to the corresponding position in the reference sequence upon which the residue difference is based. For example, a “residue difference at position X40 as compared to SEQ ID NO:2” refers to a difference of the amino acid residue at the polypeptide

position corresponding to position 40 of SEQ ID NO:2. Thus, if the reference polypeptide of SEQ ID NO:2 has a histidine at position 40, then a “residue difference at position X40 as compared to SEQ ID NO:2” refers to an amino acid substitution of any residue other than histidine at the position of the polypeptide corresponding to position 40 of SEQ ID NO:2. In most instances herein, the specific amino acid residue difference at a position is indicated as “XnY” where “Xn” specified the corresponding position as described above, and “Y” is the single letter identifier of the amino acid found in the engineered polypeptide (*i.e.*, the different residue than in the reference polypeptide). In some instances, the present disclosure also provides specific amino acid differences denoted by the conventional notation “AnB”, where A is the single letter identifier of the residue in the reference sequence, “n” is the number of the residue position in the reference sequence, and B is the single letter identifier of the residue substitution in the sequence of the engineered polypeptide. In some instances, a polypeptide of the present disclosure can include one or more amino acid residue differences relative to a reference sequence, which is indicated by a list of the specified positions where residue differences are present relative to the reference sequence. In some embodiments, where more than one amino acid can be used in a specific residue position of a polypeptide, the various amino acid residues that can be used are separated by a “/” (*e.g.*, X192A/G). The present disclosure includes engineered polypeptide sequences comprising one or more amino acid differences that include either/or both conservative and non-conservative amino acid substitutions. The amino acid sequences of the specific recombinant carbonic anhydrase polypeptides included in the Sequence Listing of the present disclosure include an initiating methionine (M) residue (*i.e.*, M represents residue position 1). The skilled artisan, however, understands that this initiating methionine residue can be removed by biological processing machinery, such as in a host cell or in vitro translation system, to generate a mature protein lacking the initiating methionine residue, but otherwise retaining the enzyme’s properties. Consequently, the term “amino acid residue difference relative to SEQ ID NO:2 at position Xn” as used herein may refer to position “Xn” or to the corresponding position (*e.g.*, position (X-1)n) in a reference sequence that has been processed so as to lack the starting methionine.

[0066] As used herein, the phrase “conservative amino acid substitutions” refers to the interchangeability of residues having similar side chains, and thus typically involves substitution of the amino acid in the polypeptide with amino acids within the same or similar defined class of amino acids. By way of example and not limitation, in some embodiments, an amino acid with an aliphatic side chain is substituted with another aliphatic amino acid (*e.g.*, alanine, valine, leucine, and isoleucine); an amino acid with a hydroxyl side chain is substituted with another amino acid with a hydroxyl side chain (*e.g.*, serine and threonine); an amino acids having aromatic side chains is substituted with another amino acid having an aromatic side chain (*e.g.*, phenylalanine, tyrosine, tryptophan, and histidine); an amino acid with a basic side chain is substituted with another amino acid with a basis side chain (*e.g.*, lysine and arginine); an amino acid with an acidic side chain is substituted with another amino acid with an acidic side chain (*e.g.*, aspartic acid or glutamic acid);

and/or a hydrophobic or hydrophilic amino acid is replaced with another hydrophobic or hydrophilic amino acid, respectively. Exemplary conservative substitutions are provided in Table 1.

Table 1. Exemplary Conservative Amino Acid Substitutions	
Residue	Potential Conservative Substitutions
A, L, V, I	Other aliphatic (A, L, V, I) Other non-polar (A, L, V, I, G, M)
G, M	Other non-polar (A, L, V, I, G, M)
D, E	Other acidic (D, E)
K, R	Other basic (K, R)
N, Q, S, T	Other polar
H, Y, W, F	Other aromatic (H, Y, W, F)
C, P	Non-polar

[0067] As used herein, the phrase “non-conservative substitution” refers to substitution of an amino acid in the polypeptide with an amino acid with significantly differing side chain properties. Non-conservative substitutions may use amino acids between, rather than within, the defined groups and affects (a) the structure of the peptide backbone in the area of the substitution (e.g., proline for glycine) (b) the charge or hydrophobicity, or (c) the bulk of the side chain. By way of example and not limitation, an exemplary non-conservative substitution can be an acidic amino acid substituted with a basic or aliphatic amino acid; an aromatic amino acid substituted with a small amino acid; and a hydrophilic amino acid substituted with a hydrophobic amino acid.

[0068] As used herein, “deletion” refers to modification of the polypeptide by removal of one or more amino acids from the reference polypeptide. Deletions can comprise removal of 1 or more amino acids, 2 or more amino acids, 5 or more amino acids, 10 or more amino acids, 15 or more amino acids, or 20 or more amino acids, up to 10% of the total number of amino acids, or up to 20% of the total number of amino acids making up the polypeptide while retaining enzymatic activity and/or retaining the improved properties of an engineered enzyme. Deletions can be directed to the internal portions and/or terminal portions of the polypeptide. In various embodiments, the deletion can comprise a continuous segment or can be discontinuous.

[0069] As used herein, “insertion” refers to modification of the polypeptide by addition of one or more amino acids to the reference polypeptide. In some embodiments, the improved engineered PGA enzymes comprise insertions of one or more amino acids to the naturally occurring PGA polypeptide as well as insertions of one or more amino acids to engineered PGA polypeptides. Insertions can be in the internal portions of the polypeptide, or to the carboxy or amino terminus. Insertions as used herein

include fusion proteins as is known in the art. The insertion can be a contiguous segment of amino acids or separated by one or more of the amino acids in the naturally occurring polypeptide.

[0070] The term "amino acid substitution set" or "substitution set" refers to a group of amino acid substitutions in a polypeptide sequence, as compared to a reference sequence. A substitution set can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more amino acid substitutions. In some embodiments, a substitution set refers to the set of amino acid substitutions that is present in any of the variant PGAs listed in the Tables provided in the Examples.

[0071] As used herein, "fragment" refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is identical to the corresponding positions in the sequence. Fragments can typically have about 80%, about 90%, about 95%, about 98%, or about 99% of the full-length PGA polypeptide, for example the polypeptide of SEQ ID NO:2. In some embodiments, the fragment is "biologically active" (i.e., it exhibits the same enzymatic activity as the full-length sequence).

[0072] As used herein, "isolated polypeptide" refers to a polypeptide that is substantially separated from other contaminants that naturally accompany it, *e.g.*, protein, lipids, and polynucleotides. The term embraces polypeptides which have been removed or purified from their naturally-occurring environment or expression system (*e.g.*, host cell or in vitro synthesis). The improved PGA enzymes may be present within a cell, present in the cellular medium, or prepared in various forms, such as lysates or isolated preparations. As such, in some embodiments, the engineered PGA polypeptides of the present disclosure can be an isolated polypeptide.

[0073] As used herein, "substantially pure polypeptide" refers to a composition in which the polypeptide species is the predominant species present (*i.e.*, on a molar or weight basis it is more abundant than any other individual macromolecular species in the composition), and is generally a substantially purified composition when the object species comprises at least about 50 percent of the macromolecular species present by mole or % weight. Generally, a substantially pure engineered PGA polypeptide composition comprises about 60% or more, about 70% or more, about 80% or more, about 90% or more, about 91% or more, about 92% or more, about 93% or more, about 94% or more, about 95% or more, about 96% or more, about 97% or more, about 98% or more, or about 99% of all macromolecular species by mole or % weight present in the composition. Solvent species, small molecules (<500 Daltons), and elemental ion species are not considered macromolecular species. In some embodiments, the isolated improved PGA polypeptide is a substantially pure polypeptide composition.

[0074] As used herein, when used in reference to a nucleic acid or polypeptide, the term "heterologous" refers to a sequence that is not normally expressed and secreted by an organism (*e.g.*, a wild-type organism). In some embodiments, the term encompasses a sequence that comprises two or more subsequences which are not found in the same relationship to each other as normally found in nature, or is recombinantly engineered so that its level of expression, or physical relationship to other

nucleic acids or other molecules in a cell, or structure, is not normally found in nature. For instance, a heterologous nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged in a manner not found in nature (*e.g.*, a nucleic acid open reading frame (ORF) of the invention operatively linked to a promoter sequence inserted into an expression cassette, such as a vector). In some embodiments, “heterologous polynucleotide” refers to any polynucleotide that is introduced into a host cell by laboratory techniques, and includes polynucleotides that are removed from a host cell, subjected to laboratory manipulation, and then reintroduced into a host cell.

[0075] As used herein, “suitable reaction conditions” refer to those conditions in the biocatalytic reaction solution (*e.g.*, ranges of enzyme loading, substrate loading, cofactor loading, temperature, pH, buffers, co-solvents, etc.) under which a PGA polypeptide of the present disclosure is capable of releasing free insulin by removing tri-phenyl acetate protecting groups. Exemplary “suitable reaction conditions” are provided in the present disclosure and illustrated by the Examples.

[0076] As used herein, “loading,” such as in “compound loading,” “enzyme loading,” or “cofactor loading” refers to the concentration or amount of a component in a reaction mixture at the start of the reaction.

[0077] As used herein, “substrate” in the context of a biocatalyst mediated process refers to the compound or molecule acted on by the biocatalyst.

[0078] As used herein “product” in the context of a biocatalyst mediated process refers to the compound or molecule resulting from the action of the biocatalyst.

[0079] As used herein, “equilibration” as used herein refers to the process resulting in a steady state concentration of chemical species in a chemical or enzymatic reaction (*e.g.*, interconversion of two species A and B), including interconversion of stereoisomers, as determined by the forward rate constant and the reverse rate constant of the chemical or enzymatic reaction.

[0080] As used herein “acylase” and “acyltransferases” are used interchangeably to refer to enzymes that are capable of transferring an acyl group from a donor to an acceptor to form esters or amides. The acylase mediated reverse reaction results in hydrolysis of the ester or amide.

[0081] As used herein, “penicillin G” and “benzylpenicillin” refer to the antibiotic also known as (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (C₁₆H₁₈N₂O₄S). It is primarily effective against Gram-positive organisms, although some Gram-negative organisms are also susceptible to it.

[0082] As used herein, “penicillin G acylase” and “PGA” are used interchangeably to refer to an enzyme having the capability of mediating cleavage of penicillin G (benzylpenicillin) to phenylacetic acid (PHA) and 6-aminopenicillanic acid (6-APA). In some embodiments, PGA activity can be based on cleavage of model substrates, for instance the cleavage of 6-nitro-3-(phenylacetamide)benzoic acid to phenylacetic acid and 5-amino-2-nitro-benzoic acid. PGAs are also capable of carrying out the reverse reaction of transferring an acyl group of an acyl donor to an acyl acceptor. PGAs as used herein include naturally occurring (wild type) PGAs as well as non-naturally occurring PGA enzymes

comprising one or more engineered polypeptides generated by human manipulation. The wild-type PGA gene is a heterodimer consisting of alpha subunit (23.8 KDa) and beta subunit (62.2KDa) linked by a spacer region of 54 amino acids. Due to the presence of the spacer region, an auto-processing step is required to form the active protein.

[0083] As used herein, “acyl donor” refers to that portion of the acylase substrate which donates the acyl group to an acyl acceptor to form esters or amides.

[0084] As used herein, “acyl acceptor” refers to that portion of the acylase substrate which accepts the acyl group of the acyl donor to form esters or amides.

[0085] As used herein, “ α -chain sequence” refers to an amino acid sequence that corresponds to (e.g., has at least 85% identity to) the residues at positions 27 to 235 of SEQ ID NO: 2. As used herein, a single chain polypeptide can comprise an “ α -chain sequence” and additional sequence(s).

[0086] As used herein, “ β -chain sequence” refers to an amino acid sequence that corresponds to (e.g., has at least 85% identity to) residues at positions 290 to 846 of SEQ ID NO:2. As used herein, a single chain polypeptide can comprise an “ β -chain sequence” and additional sequence(s).

[0087] As used herein, “derived from” when used in the context of engineered PGA enzymes, identifies the originating PGA enzyme, and/or the gene encoding such PGA enzyme, upon which the engineering was based. For example, the engineered PGA enzyme of SEQ ID NO: 60 was obtained by artificially evolving, over multiple generations the gene encoding the *K. citrophila* PGA alpha-chain and beta-chain sequences of SEQ ID NO:2. Thus, this engineered PGA enzyme is “derived from” the naturally occurring or wild-type PGA of SEQ ID NO: 2.

[0088] As used herein, “insulin” refers to the polypeptide hormone produced by the beta-cells of the pancreas in normal individuals. Insulin is necessary for regulating carbohydrate metabolism, by reducing blood glucose levels. Systematic deficiency of insulin results in diabetes. Insulin is comprised of 51 amino acids and has a molecular weight of approximately 5800 daltons. Insulin is comprised of two peptide chains (designated “A” and “B”), containing one intrasubunit and two intersubunit disulfide bonds. The A chain is composed of 21 amino acids and the B chain is composed of 30 amino acids. The two chains form a highly ordered structure, with several alpha-helical regions in both the A and B chains. Isolated chains are inactive. In solution, insulin is either a monomer, dimer, or hexamer. It is hexameric in the highly concentrated preparations used for subcutaneous injection, but becomes monomeric as it is diluted in body fluids. The definition is intended to encompass proinsulin and any purified isolated polypeptide having part or all of the primary structural conformation and at least one of the biological properties of naturally-occurring insulin. It is further intended to encompass natural and synthetically-derived insulin, including glycoforms, as well as analogs (e.g., polypeptides having deletions, insertions, and/or substitutions).

[0089] Insulin contains three nucleophilic amines that can potentially react with a phenylacetate-donor and be deprotected by PGA. These residues include a Lys on the B-chain at position 29 (B29) and two N-terminal free amines, Gly on the A-chain at position 1 (A1) and Phe on the B-chain at

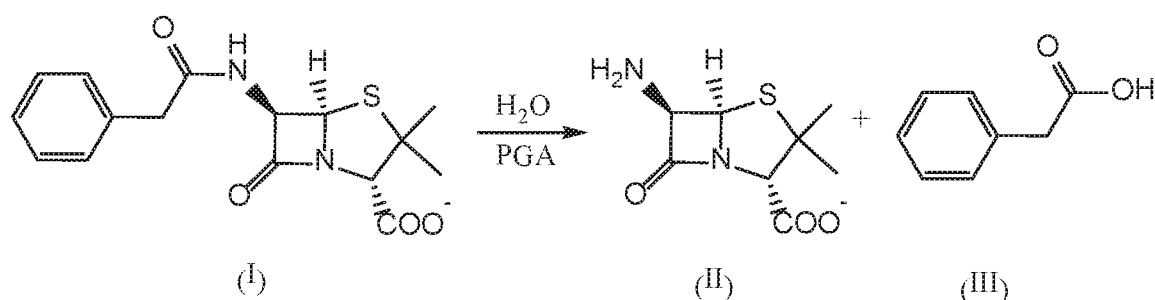
position 1 (B1). The present invention provides tri-protected insulin (phenyl acetate chemically attached to A1, B1, B29 residues on human insulin). PGA has previously been reported to catalyze hydrolysis of N-phenylacetate-protected peptides and insulin with exclusive selectivity for the phenylacetate amide bond, leaving the rest of the peptide bonds of the protein intact (Brtnik et al., Coll. Czech. Chem. Commun., 46 (8), 1983-1989 [1981]; and Wang et al. Biopolym. 25 (Suppl.), S109-S114 [1986]).

[0090] As used herein, “tri-phenyl acetate protecting group,” refers to an insulin molecule that has the three primary amines at the B1, B29 and A1 positions that are protected with a phenyl acyl group, as described herein.

Penicillin G Acylases

[0091] Penicillin acylase was first described from *Penicillium chrysogenum* Wisc. Q176 by Sakaguchi and Murao (Sakaguchi and Murao, J. Agr.Chem. Soc. Jpn., 23:411 [1950]). Penicillin G acylase is a hydrolytic enzyme that acts on the side chains of penicillin G, cephalosporin G, and related antibiotics to produce the β -lactam antibiotic intermediates 6-amino penicillanic acid and 7-amino des-acetoxy cephalosporanic acid, with phenyl acetic acid as a common by-product. These antibiotic intermediates are among the potential building blocks of semi-synthetic antibiotics, such as ampicillin, amoxicillin, cloxacillin, cephalixin, and cefatoxime.

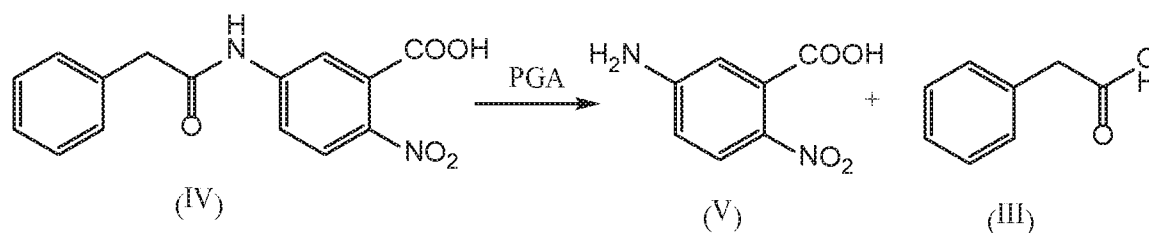
[0092] As indicated above, penicillin G acylases (PGA) are characterized by the ability to catalyze the hydrolytic cleavage of penicillin G, with a conjugate base of structural formula (I), to 6-amino penicillanic acid, with a conjugate base of structural formula (II), and phenylacetic acid of structural formula (III), as shown in Scheme 1:



Scheme 1

[0093] While not being bound by theory, substrate specificity appears associated with recognition of the hydrophobic phenyl group while a nucleophile, which in some PGAs is a serine residue at the N-terminus of the beta-chain acts as the acceptor of beta-lactam and a variety of other groups, such as beta-amino acids. PGAs can also be characterized by the ability to cleave a model substrates

analogous to penicillin G, for instance cleavage of 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB) of structural formula (IV), as shown in Scheme 2:



Scheme 2

to phenylacetic acid of structural formula (III) and 5-amino-2-nitro-benzoic acid of structural formula (V) (See e.g., Alkema et al., *Anal. Biochem.*, 275:47-53 [1999]). Because the 5-amino-2-nitro-benzoic acid is chromogenic, the substrate of formula (IV) provides a convenient way of measuring PGA activity. In addition to the foregoing reactions, PGAs can also be used in the kinetic resolution of DL-tert leucine for the preparation of optically pure tert leucine (See e.g., Liu et al., *Prep. Biochem. Biotechnol.*, 36:235-41 [2006]).

[0094] The PGAs of the present disclosure are based on the enzyme obtained from the organism *Kluyvera citrophila* (*K. citrophila*). As with PGAs from other organisms, the PGA of *K. citrophila* is a heterodimeric enzyme comprised of an alpha-subunit and a beta-subunit that is generated by proteolytic processing of a pre-pro-PGA polypeptide. Removal of a signal peptide and a spacer peptide produces the mature heterodimer (See e.g., Barbero et al., *Gene* 49:69-80 [1986]). The amino acid sequence of the naturally occurring pre-pro-PGA polypeptide of *K. citrophila* is publicly available (See e.g., Genbank accession No. P07941, [gi:129551]) and is provided herein as SEQ ID NO:2. The alpha-chain sequence of the naturally occurring *K. citrophila* PGA corresponds to residues 27 to 235 of SEQ ID NO:2. The beta-chain sequence of the naturally occurring *K. citrophila* PGA corresponds to residues 290 to 846 of SEQ ID NO:2. Residues 1 to 26 of SEQ ID NO:2 correspond to the signal peptide and residues 236-289 of SEQ ID NO:2 correspond to the linking propeptide, both of which are removed to generate the naturally occurring mature PGA enzyme which is a heterodimer comprising an α -chain subunit and a β -chain subunit.

[0095] In some embodiments, the present invention provides engineered PGA polypeptides with amino acid sequences that have at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to SEQ ID NOS:2, 4, 6, 8, 10 and/or 12.

[0096] The present invention provides insulin-specific deacylation biocatalysts suitable for laboratory scale preparative use. Directed evolution was used to develop efficient acylase variants capable of completely removing the A1/B1/B29-tri-phenyl acetate protecting groups on insulin and generate >99% free insulin. Following only two rounds of evolution, variants that generate more than 99% free insulin in less than 6 hrs. at 0.8g/L enzyme loading were produced. The final best variant

PGA_005 was ~8-fold improved over the initial backbone. The PGA variants provided herein are capable of accepting a wide range of acyl groups, exhibit increased solvent stability, and improved thermostability, as compared to the wild-type PGA. The variant PGAs provided herein lack the spacer region. Thus, the auto-processing step is not required in order to produce active enzymes.

[0097] The present invention also provides polynucleotides encoding the engineered PGA polypeptides. In some embodiments, the polynucleotides are operatively linked to one or more heterologous regulatory sequences that control gene expression, to create a recombinant polynucleotide capable of expressing the polypeptide. Expression constructs containing a heterologous polynucleotide encoding the engineered PGA polypeptides can be introduced into appropriate host cells to express the corresponding PGA polypeptide.

[0098] Because of the knowledge of the codons corresponding to the various amino acids, availability of a protein sequence provides a description of all the polynucleotides capable of encoding the subject. The degeneracy of the genetic code, where the same amino acids are encoded by alternative or synonymous codons allows an extremely large number of nucleic acids to be made, all of which encode the improved PGA enzymes disclosed herein. Thus, having identified a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the protein. In this regard, the present disclosure specifically contemplates each and every possible variation of polynucleotides that could be made by selecting combinations based on the possible codon choices, and all such variations are to be considered specifically disclosed for any polypeptide disclosed herein, including the amino acid sequences presented in the Tables in Examples 5 and 6.

[0099] In various embodiments, the codons are preferably selected to fit the host cell in which the protein is being produced. For example, preferred codons used in bacteria are used to express the gene in bacteria; preferred codons used in yeast are used for expression in yeast; and preferred codons used in mammals are used for expression in mammalian cells.

[0100] In certain embodiments, all codons need not be replaced to optimize the codon usage of the PGA polypeptides since the natural sequence will comprise preferred codons and because use of preferred codons may not be required for all amino acid residues. Consequently, codon optimized polynucleotides encoding the PGA enzymes may contain preferred codons at about 40%, 50%, 60%, 70%, 80%, or greater than 90% of codon positions of the full length coding region.

[0101] In some embodiments, the polynucleotide comprises a nucleotide sequence encoding a PGA polypeptide with an amino acid sequence that has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to the alpha-chain and/or beta-chain any of the reference engineered PGA polypeptides described herein. Accordingly, in some embodiments, the polynucleotide encodes an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to a

reference alpha- and beta-chain sequences based on SEQ ID NO:2. In some embodiments, the polynucleotide encodes an alpha- and/or beta-chain amino acid sequence of SEQ ID NO:2.

[0102] In some embodiments, the polynucleotide comprises a nucleotide sequence encoding a PGA polypeptide with an amino acid sequence that has at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more sequence identity to SEQ ID NO:4, 6, 8, 10, and/or 12. Accordingly, in some embodiments, the polynucleotide encodes an amino acid sequence that is at least about 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:1, 3, 5, 7, 9, and/or 11.

[0103] In some embodiments, an isolated polynucleotide encoding an improved PGA polypeptide was manipulated in a variety of ways to provide for improved activity and/or expression of the polypeptide. Manipulation of the isolated polynucleotide prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotides and nucleic acid sequences utilizing recombinant DNA methods are well known in the art.

[0104] For example, mutagenesis and directed evolution methods can be readily applied to polynucleotides to generate variant libraries that can be expressed, screened, and assayed.

Mutagenesis and directed evolution methods are well known in the art (*See e.g.*, US Patent Nos.

5,605,793, 5,830,721, 6,132,970, 6,420,175, 6,277,638, 6,365,408, 6,602,986, 7,288,375, 6,287,861, 6,297,053, 6,576,467, 6,444,468, 5,811,238, 6,117,679, 6,165,793, 6,180,406, 6,291,242, 6,995,017, 6,395,547, 6,506,602, 6,519,065, 6,506,603, 6,413,774, 6,573,098, 6,323,030, 6,344,356, 6,372,497, 7,868,138, 5,834,252, 5,928,905, 6,489,146, 6,096,548, 6,387,702, 6,391,552, 6,358,742, 6,482,647, 6,335,160, 6,653,072, 6,355,484, 6,03,344, 6,319,713, 6,613,514, 6,455,253, 6,579,678, 6,586,182, 6,406,855, 6,946,296, 7,534,564, 7,776,598, 5,837,458, 6,391,640, 6,309,883, 7,105,297, 7,795,030, 6,326,204, 6,251,674, 6,716,631, 6,528,311, 6,287,862, 6,335,198, 6,352,859, 6,379,964, 7,148,054, 7,629,170, 7,620,500, 6,365,377, 6,358,740, 6,406,910, 6,413,745, 6,436,675, 6,961,664, 7,430,477, 7,873,499, 7,702,464, 7,783,428, 7,747,391, 7,747,393, 7,751,986, 6,376,246, 6,426,224, 6,423,542, 6,479,652, 6,319,714, 6,521,453, 6,368,861, 7,421,347, 7,058,515, 7,024,312, 7,620,502, 7,853,410, 7,957,912, 7,904,249, and all related non-US counterparts; Ling *et al.*, Anal. Biochem., 254(2):157-78 [1997]; Dale *et al.*, Meth. Mol. Biol., 57:369-74 [1996]; Smith, Ann. Rev. Genet., 19:423-462 [1985]; Botstein *et al.*, Science, 229:1193-1201 [1985]; Carter, Biochem. J., 237:1-7 [1986]; Kramer *et al.*, Cell, 38:879-887 [1984]; Wells *et al.*, Gene, 34:315-323 [1985]; Minshull *et al.*, Curr. Op. Chem. Biol., 3:284-290 [1999]; Christians *et al.*, Nat. Biotechnol., 17:259-264 [1999]; Cramer *et al.*, Nature, 391:288-291 [1998]; Cramer *et al.*, Nat. Biotechnol., 15:436-438 [1997]; Zhang *et al.*, Proc. Nat. Acad. Sci. U.S.A., 94:4504-4509 [1997]; Cramer *et al.*, Nat. Biotechnol., 14:315-319 [1996]; Stemmer, Nature, 370:389-391 [1994]; Stemmer, Proc. Nat. Acad. Sci. USA, 91:10747-10751 [1994]; WO 95/22625; WO 97/0078; WO 97/35966; WO 98/27230; WO 00/42651; WO 01/75767; and WO 2009/152336, all of which are incorporated herein by reference).

[0105] In some embodiments, the variant PGA acylases of the present invention further comprise additional sequences that do not alter the encoded activity of the enzyme. For example, in some embodiments, the variant PGA acylases are linked to an epitope tag or to another sequence useful in purification.

[0106] In some embodiments, the variant PGA acylase polypeptides of the present invention are secreted from the host cell in which they are expressed (e.g., a yeast or filamentous fungal host cell) and are expressed as a pre-protein including a signal peptide (i.e., an amino acid sequence linked to the amino terminus of a polypeptide and which directs the encoded polypeptide into the cell secretory pathway).

[0107] In some embodiments, the signal peptide is an endogenous *K. citrophila* PGA acylase signal peptide. In some other embodiments, signal peptides from other *K. citrophila* secreted proteins are used. In some embodiments, other signal peptides find use, depending on the host cell and other factors. Effective signal peptide coding regions for filamentous fungal host cells include, but are not limited to, the signal peptide coding regions obtained from *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, *Humicola lanuginosa* lipase, and *T. reesei* cellobiohydrolase II. Signal peptide coding regions for bacterial host cells include, but are not limited to the signal peptide coding regions obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* β -lactamase, *Bacillus stearothermophilus* neutral proteases (nprT, nprS, nprM), and *Bacillus subtilis* prsA. In some additional embodiments, other signal peptides find use in the present invention (See e.g., Simonen and Palva, Microbiol. Rev., 57: 109-137 [1993], incorporated herein by reference). Additional useful signal peptides for yeast host cells include those from the genes for *Saccharomyces cerevisiae* alpha-factor, *Saccharomyces cerevisiae* SUC2 invertase (See e.g., Taussig and Carlson, Nucl. Acids Res., 11:1943-54 [1983]; SwissProt Accession No. P00724; and Romanos *et al.*, Yeast 8:423-488 [1992]). In some embodiments, variants of these signal peptides and other signal peptides find use. Indeed, it is not intended that the present invention be limited to any specific signal peptide, as any suitable signal peptide known in the art finds use in the present invention.

[0108] In some embodiments, the present invention provides polynucleotides encoding variant PGA acylase polypeptides, and/or biologically active fragments thereof, as described herein. In some embodiments, the polynucleotide is operably linked to one or more heterologous regulatory or control sequences that control gene expression to create a recombinant polynucleotide capable of expressing the polypeptide. In some embodiments, expression constructs containing a heterologous polynucleotide encoding a variant PGA acylase is introduced into appropriate host cells to express the variant PGA acylase.

[0109] Those of ordinary skill in the art understand that due to the degeneracy of the genetic code, a multitude of nucleotide sequences encoding variant PGA acylase polypeptides of the present

invention exist. For example, the codons AGA, AGG, CGA, CGC, CGG, and CGU all encode the amino acid arginine. Thus, at every position in the nucleic acids of the invention where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described above without altering the encoded polypeptide. It is understood that “U” in an RNA sequence corresponds to “T” in a DNA sequence. The invention contemplates and provides each and every possible variation of nucleic acid sequence encoding a polypeptide of the invention that could be made by selecting combinations based on possible codon choices.

[0110] As indicated above, DNA sequence encoding a PGA may also be designed for high codon usage bias codons (codons that are used at higher frequency in the protein coding regions than other codons that code for the same amino acid). The preferred codons may be determined in relation to codon usage in a single gene, a set of genes of common function or origin, highly expressed genes, the codon frequency in the aggregate protein coding regions of the whole organism, codon frequency in the aggregate protein coding regions of related organisms, or combinations thereof. A codon whose frequency increases with the level of gene expression is typically an optimal codon for expression. In particular, a DNA sequence can be optimized for expression in a particular host organism. A variety of methods are well-known in the art for determining the codon frequency (*e.g.*, codon usage, relative synonymous codon usage) and codon preference in specific organisms, including multivariate analysis (*e.g.*, using cluster analysis or correspondence analysis,) and the effective number of codons used in a gene. The data source for obtaining codon usage may rely on any available nucleotide sequence capable of coding for a protein. These data sets include nucleic acid sequences actually known to encode expressed proteins (*e.g.*, complete protein coding sequences-CDS), expressed sequence tags (ESTs), or predicted coding regions of genomic sequences, as is well-known in the art. Polynucleotides encoding variant PGAs can be prepared using any suitable methods known in the art. Typically, oligonucleotides are individually synthesized, then joined (*e.g.*, by enzymatic or chemical ligation methods, or polymerase-mediated methods) to form essentially any desired continuous sequence. In some embodiments, polynucleotides of the present invention are prepared by chemical synthesis using, any suitable methods known in the art, including but not limited to automated synthetic methods. For example, in the phosphoramidite method, oligonucleotides are synthesized (*e.g.*, in an automatic DNA synthesizer), purified, annealed, ligated and cloned in appropriate vectors. In some embodiments, double stranded DNA fragments are then obtained either by synthesizing the complementary strand and annealing the strands together under appropriate conditions, or by adding the complementary strand using DNA polymerase with an appropriate primer sequence. There are numerous general and standard texts that provide methods useful in the present invention are well known to those skilled in the art.

[0111] The engineered PGAs can be obtained by subjecting the polynucleotide encoding the naturally occurring PGA to mutagenesis and/or directed evolution methods, as discussed above. Mutagenesis may be performed in accordance with any of the techniques known in the art, including

random and site-specific mutagenesis. Directed evolution can be performed with any of the techniques known in the art to screen for improved variants including shuffling. Other directed evolution procedures that find use include, but are not limited to staggered extension process (StEP), in vitro recombination, mutagenic PCR, cassette mutagenesis, splicing by overlap extension (SOEing), ProSAR™ directed evolution methods, etc., as well as any other suitable methods.

[0112] The clones obtained following mutagenesis treatment are screened for engineered PGAs having a desired improved enzyme property. Measuring enzyme activity from the expression libraries can be performed using the standard biochemistry technique of monitoring the rate of product formation. Where an improved enzyme property desired is thermal stability, enzyme activity may be measured after subjecting the enzyme preparations to a defined temperature and measuring the amount of enzyme activity remaining after heat treatments. Clones containing a polynucleotide encoding a PGA are then isolated, sequenced to identify the nucleotide sequence changes (if any), and used to express the enzyme in a host cell.

[0113] When the sequence of the engineered polypeptide is known, the polynucleotides encoding the enzyme can be prepared by standard solid-phase methods, according to known synthetic methods. In some embodiments, fragments of up to about 100 bases can be individually synthesized, then joined (*e.g.*, by enzymatic or chemical ligation methods, or polymerase mediated methods) to form any desired continuous sequence. For example, polynucleotides and oligonucleotides of the invention can be prepared by chemical synthesis (*e.g.*, using the classical phosphoramidite method described by Beaucage et al., *Tet. Lett.*, 22:1859-69 [1981], or the method described by Matthes et al., *EMBO J.*, 3:801-05 [1984], as it is typically practiced in automated synthetic methods). According to the phosphoramidite method, oligonucleotides are synthesized (*e.g.*, in an automatic DNA synthesizer), purified, annealed, ligated and cloned in appropriate vectors. In addition, essentially any nucleic acid can be obtained from any of a variety of commercial sources (*e.g.*, The Midland Certified Reagent Company, Midland, TX, The Great American Gene Company, Ramona, CA, ExpressGen Inc. Chicago, IL, Operon Technologies Inc., Alameda, CA, and many others).

[0114] The present invention also provides recombinant constructs comprising a sequence encoding at least one variant PGA, as provided herein. In some embodiments, the present invention provides an expression vector comprising a variant PGA polynucleotide operably linked to a heterologous promoter. In some embodiments, expression vectors of the present invention are used to transform appropriate host cells to permit the host cells to express the variant PGA protein. Methods for recombinant expression of proteins in fungi and other organisms are well known in the art, and a number of expression vectors are available or can be constructed using routine methods. In some embodiments, nucleic acid constructs of the present invention comprise a vector, such as, a plasmid, a cosmid, a phage, a virus, a bacterial artificial chromosome (BAC), a yeast artificial chromosome (YAC), and the like, into which a nucleic acid sequence of the invention has been inserted. In some embodiments, polynucleotides of the present invention are incorporated into any one of a variety of

expression vectors suitable for expressing variant PGA polypeptide(s). Suitable vectors include, but are not limited to chromosomal, nonchromosomal and synthetic DNA sequences (*e.g.*, derivatives of SV40), as well as bacterial plasmids, phage DNA, baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, pseudorabies, adenovirus, adeno-associated virus, retroviruses, and many others. Any suitable vector that transduces genetic material into a cell, and, if replication is desired, which is replicable and viable in the relevant host finds use in the present invention. In some embodiments, the construct further comprises regulatory sequences, including but not limited to a promoter, operably linked to the protein encoding sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art. Indeed, in some embodiments, in order to obtain high levels of expression in a particular host it is often useful to express the variant PGAs of the present invention under the control of a heterologous promoter. In some embodiments, a promoter sequence is operably linked to the 5' region of the variant PGA coding sequence using any suitable method known in the art. Examples of useful promoters for expression of variant PGAs include, but are not limited to promoters from fungi. In some embodiments, a promoter sequence that drives expression of a gene other than a PGA gene in a fungal strain finds use. As a non-limiting example, a fungal promoter from a gene encoding an endoglucanase may be used. In some embodiments, a promoter sequence that drives the expression of a PGA gene in a fungal strain other than the fungal strain from which the PGAs were derived finds use. Examples of other suitable promoters useful for directing the transcription of the nucleotide constructs of the present invention in a filamentous fungal host cell include, but are not limited to promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (glaA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, and *Fusarium oxysporum* trypsin-like protease (*See e.g.*, WO 96/00787, incorporated herein by reference), as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase), promoters such as *cbh1*, *cbh2*, *egl1*, *egl2*, *pepA*, *hfb1*, *hfb2*, *xyn1*, *amy*, and *glaA* (*See e.g.*, Nunberg *et al.*, Mol. Cell Biol., 4:2306 -2315 [1984]; Boel *et al.*, EMBO J., 3:1581-85 [1984]; and European Patent Appln. 137280, all of which are incorporated herein by reference), and mutant, truncated, and hybrid promoters thereof.

[0115] In yeast host cells, useful promoters include, but are not limited to those from the genes for *Saccharomyces cerevisiae* enolase (*eno-1*), *Saccharomyces cerevisiae* galactokinase (*gal1*), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (*ADH2/GAP*), and *S. cerevisiae* 3-phosphoglycerate kinase. Additional useful promoters useful for yeast host cells are known in the art (*See e.g.*, Romanos *et al.*, Yeast 8:423-488 [1992], incorporated herein by reference). In addition, promoters associated with chitinase production in fungi find use in

the present invention (See e.g., Blaiseau and Lafay, Gene 120243-248 [1992]; and Limon *et al.*, Curr. Genet., 28:478-83 [1995], both of which are incorporated herein by reference).

[0116] For bacterial host cells, suitable promoters for directing transcription of the nucleic acid constructs of the present disclosure, include but are not limited to the promoters obtained from the *E. coli* lac operon, *E. coli* trp operon, bacteriophage λ , *Streptomyces coelicolor* agarase gene (dagA), *Bacillus subtilis* levansucrase gene (sacB), *Bacillus licheniformis* alpha-amylase gene (amyL), *Bacillus stearothermophilus* maltogenic amylase gene (amyM), *Bacillus amyloliquefaciens* alpha-amylase gene (amyQ), *Bacillus licheniformis* penicillinase gene (penP), *Bacillus subtilis* xylA and xylB genes, and prokaryotic beta-lactamase gene (See e.g., Villa-Kamaroff *et al.*, Proc. Natl. Acad. Sci. USA 75: 3727-3731 [1978]), as well as the *tac* promoter (See e.g., DeBoer *et al.*, Proc. Natl. Acad. Sci. USA 80: 21-25 [1983]).

[0117] In some embodiments, cloned variant PGAs of the present invention also have a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator that is functional in the host cell of choice finds use in the present invention. Exemplary transcription terminators for filamentous fungal host cells include, but are not limited to those obtained from the genes for *Aspergillus oryzae* TKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease (See also, US Patent No. 7,399,627, incorporated herein by reference). In some embodiments, exemplary terminators for yeast host cells include those obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1), and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are well-known to those skilled in the art (See e.g., Romanos *et al.*, Yeast 8:423-88 [1992]).

[0118] In some embodiments, a suitable leader sequence is part of a cloned variant PGA sequence, which is a nontranslated region of an mRNA that is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice finds use in the present invention. Exemplary leaders for filamentous fungal host cells include, but are not limited to those obtained from the genes for *Aspergillus oryzae* TKA amylase and *Aspergillus nidulans* triose phosphate isomerase. Suitable leaders for yeast host cells include, but are not limited to those obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

[0119] In some embodiments, the sequences of the present invention also comprise a polyadenylation sequence, which is a sequence operably linked to the 3' terminus of the nucleic acid sequence and

which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice finds use in the present invention. Exemplary polyadenylation sequences for filamentous fungal host cells include, but are not limited to those obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase. Useful polyadenylation sequences for yeast host cells are known in the art (See e.g., Guo and Sherman, Mol. Cell. Biol., 15:5983-5990 [1995]).

[0120] In some embodiments, the control sequence comprises a signal peptide coding region encoding an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region that is foreign to the coding sequence. The foreign signal peptide coding region may be required where the coding sequence does not naturally contain a signal peptide coding region.

[0121] Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the polypeptide. However, any signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used in the present invention.

[0122] Effective signal peptide coding regions for bacterial host cells include, but are not limited to the signal peptide coding regions obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* neutral proteases (nprT, nprS, nprM), and *Bacillus subtilis* prsA. Further signal peptides are known in the art (See e.g., Simonen and Palva, Microbiol. Rev., 57: 109-137 [1993]).

[0123] Effective signal peptide coding regions for filamentous fungal host cells include, but are not limited to the signal peptide coding regions obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, and *Humicola lamuginosa* lipase.

[0124] Useful signal peptides for yeast host cells include, but are not limited to genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding regions are known in the art (See e.g., Romanos et al., [1992], *supra*).

[0125] In some embodiments, the control sequence comprises a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature active PGA polypeptide by

catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the genes for *Bacillus subtilis* alkaline protease (aprE), *Bacillus subtilis* neutral protease (nprT), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* lactase (See e.g., WO 95/33836).

[0126] Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

[0127] In some embodiments, regulatory sequences are also used to allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. In prokaryotic host cells, suitable regulatory sequences include, but are not limited to the lac, tac, and trp operator systems. In yeast host cells, suitable regulatory systems include, as examples, the ADH2 system or GAL1 system. In filamentous fungi, suitable regulatory sequences include the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter.

[0128] Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene, which is amplified in the presence of methotrexate, and the metallothionein genes, which are amplified with heavy metals. In these cases, the nucleic acid sequence encoding the PGA polypeptide of the present invention would be operably linked with the regulatory sequence.

[0129] Thus, in additional embodiments, the present invention provides recombinant expression vectors comprising a polynucleotide encoding an engineered PGA polypeptide or a variant thereof, and one or more expression regulating regions such as a promoter and a terminator, a replication origin, etc., depending on the type of hosts into which they are to be introduced. In some embodiments, the various nucleic acid and control sequences described above are joined together to produce a recombinant expression vector that may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the polypeptide at such sites. Alternatively, in some embodiments, the nucleic acid sequences are expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

[0130] The recombinant expression vector comprises any suitable vector (e.g., a plasmid or virus), that can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the polynucleotide sequence. The choice of the vector typically depends on the compatibility of the vector with the host cell into which the vector is to be introduced. In some embodiments, the vectors are linear or closed circular plasmids.

[0131] In some embodiments, the expression vector is an autonomously replicating vector (i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome). In some embodiments, the vector contains any means for assuring self-replication. Alternatively, in some other embodiments, upon being introduced into the host cell, the vector is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, in additional embodiments, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon find use.

[0132] In some embodiments, the expression vector of the present invention contains one or more selectable markers, which permit easy selection of transformed cells. A “selectable marker” is a gene, the product of which provides for biocide or viral resistance, resistance to antimicrobials or heavy metals, prototrophy to auxotrophs, and the like. Any suitable selectable markers for use in a filamentous fungal host cell find use in the present invention, including, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Additional markers useful in host cells such as *Aspergillus*, include but are not limited to the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae*, and the *bar* gene of *Streptomyces hygroscopicus*. Suitable markers for yeast host cells include, but are not limited to *ADE2*, *HIS3*, *LEU2*, *LYS2*, *MET3*, *TRP1*, and *URA3*. Examples of bacterial selectable markers include, but are not limited to the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers, which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol, and or tetracycline resistance.

[0133] In some embodiments, the expression vectors of the present invention contain an element(s) that permits integration of the vector into the host cell's genome or autonomous replication of the vector in the cell independent of the genome. In some embodiments involving integration into the host cell genome, the vectors rely on the nucleic acid sequence encoding the polypeptide or any other element of the vector for integration of the vector into the genome by homologous or nonhomologous recombination.

[0134] In some alternative embodiments, the expression vectors contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the host cell. The additional nucleic acid sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements preferably contain a sufficient number of nucleotides, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous

with the target sequence in the genome of the host cell. Furthermore, the integrational elements may be non-encoding or encoding nucleic acid sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

[0135] For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of bacterial origins of replication are P15A ori or the origins of replication of plasmids pBR322, pUC19, pACYCI77 (which plasmid has the P15A ori), or pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, or pAM β 1 permitting replication in *Bacillus*. Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6. The origin of replication may be one having a mutation which makes it's functioning temperature-sensitive in the host cell (See e.g., Ehrlich, Proc. Natl. Acad. Sci. USA 75:1433 [1978]).

[0136] In some embodiments, more than one copy of a nucleic acid sequence of the present invention is inserted into the host cell to increase production of the gene product. An increase in the copy number of the nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the nucleic acid sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the nucleic acid sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

[0137] Many of the expression vectors for use in the present invention are commercially available. Suitable commercial expression vectors include, but are not limited to the p3xFLAGTM expression vectors (Sigma-Aldrich Chemicals), which include a CMV promoter and hGH polyadenylation site for expression in mammalian host cells and a pBR322 origin of replication and ampicillin resistance markers for amplification in *E. coli*. Other suitable expression vectors include, but are not limited to pBluescriptII SK(-) and pBK-CMV (Stratagene), and plasmids derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pREP4, pCEP4 (Invitrogen) or pPoly (See e.g., Lathe et al., Gene 57:193-201 [1987]).

[0138] Thus, in some embodiments, a vector comprising a sequence encoding at least one variant PGA is transformed into a host cell in order to allow propagation of the vector and expression of the variant PGA(s). In some embodiments, the variant PGAs are post-translationally modified to remove the signal peptide and in some cases may be cleaved after secretion. In some embodiments, the transformed host cell described above is cultured in a suitable nutrient medium under conditions permitting the expression of the variant PGA(s). Any suitable medium useful for culturing the host cells finds use in the present invention, including, but not limited to minimal or complex media containing appropriate supplements. In some embodiments, host cells are grown in HTP media.

Suitable media are available from various commercial suppliers or may be prepared according to published recipes (e.g., in catalogues of the American Type Culture Collection).

[0139] In another aspect, the present invention provides host cells comprising a polynucleotide encoding an improved PGA polypeptide provided herein, the polynucleotide being operatively linked to one or more control sequences for expression of the PGA enzyme in the host cell. Host cells for use in expressing the PGA polypeptides encoded by the expression vectors of the present invention are well known in the art and include but are not limited to, bacterial cells, such as *E. coli*, *Bacillus megaterium*, *Lactobacillus kefir*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells (e.g., *Saccharomyces cerevisiae* or *Pichia pastoris* (ATCC Accession No. 201178)); insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, BHK, 293, and Bowes melanoma cells; and plant cells. Appropriate culture media and growth conditions for the above-described host cells are well known in the art.

[0140] Polynucleotides for expression of the PGA may be introduced into cells by various methods known in the art. Techniques include among others, electroporation, biolistic particle bombardment, liposome mediated transfection, calcium chloride transfection, and protoplast fusion. Various methods for introducing polynucleotides into cells are known to those skilled in the art.

[0141] In some embodiments, the host cell is a eukaryotic cell. Suitable eukaryotic host cells include, but are not limited to, fungal cells, algal cells, insect cells, and plant cells. Suitable fungal host cells include, but are not limited to, Ascomycota, Basidiomycota, Deuteromycota, Zygomycota, Fungi imperfecti. In some embodiments, the fungal host cells are yeast cells and filamentous fungal cells. The filamentous fungal host cells of the present invention include all filamentous forms of the subdivision Eumycotina and Oomycota. Filamentous fungi are characterized by a vegetative mycelium with a cell wall composed of chitin, cellulose and other complex polysaccharides. The filamentous fungal host cells of the present invention are morphologically distinct from yeast.

[0142] In some embodiments of the present invention, the filamentous fungal host cells are of any suitable genus and species, including, but not limited to *Achlya*, *Acremonium*, *Aspergillus*, *Aureobasidium*, *Bjerkandera*, *Ceriporiopsis*, *Cephalosporium*, *Chrysosporium*, *Cochliobolus*, *Corynascus*, *Cryphonectria*, *Cryptococcus*, *Coprinus*, *Coriolus*, *Diplodia*, *Endothia*, *Fusarium*, *Gibberella*, *Gliocladium*, *Hemicella*, *Hypocrea*, *Myceliophthora*, *Mucor*, *Neurospora*, *Penicillium*, *Podospora*, *Phlebia*, *Piromyces*, *Pyricularia*, *Rhizomucor*, *Rhizopus*, *Schizophyllum*, *Scytalidium*, *Sporotrichum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Trametes*, *Tolypocladium*, *Trichoderma*, *Verticillium*, and/or *Volvariella*, and/or teleomorphs, or anamorphs, and synonyms, basionyms, or taxonomic equivalents thereof.

[0143] In some embodiments of the present invention, the host cell is a yeast cell, including but not limited to cells of *Candida*, *Hansenula*, *Saccharomyces*, *Schizosaccharomyces*, *Pichia*, *Kluyveromyces*, or *Yarrowia* species. In some embodiments of the present invention, the yeast cell is *Hansenula polymorpha*, *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, *Saccharomyces*

diastaticus, *Saccharomyces norbensis*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia kodamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia quercuum*, *Pichia pijperi*, *Pichia stipitis*, *Pichia methanolica*, *Pichia angusta*, *Kluyveromyces lactis*, *Candida albicans*, or *Yarrowia lipolytica*.

[0144] In some embodiments of the invention, the host cell is an algal cell such as *Chlamydomonas* (e.g., *C. reinhardtii*) and *Phormidium* (P. sp. ATCC29409).

[0145] In some other embodiments, the host cell is a prokaryotic cell. Suitable prokaryotic cells include, but are not limited to Gram-positive, Gram-negative and Gram-variable bacterial cells. Any suitable bacterial organism finds use in the present invention, including but not limited to *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Acinetobacter*, *Acidothrmus*, *Arthrobacter*, *Azobacter*, *Bacillus*, *Bifidobacterium*, *Brevibacterium*, *Butyrivibrio*, *Buchnera*, *Campestris*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Chromatium*, *Coprococcus*, *Escherichia*, *Enterococcus*, *Enterobacter*, *Erwinia*, *Fusobacterium*, *Faecalibacterium*, *Francisella*, *Flavobacterium*, *Geobacillus*, *Haemophilus*, *Helicobacter*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Ilyobacter*, *Micrococcus*, *Microbacterium*, *Mesorhizobium*, *Methylobacterium*, *Methylobacterium*, *Mycobacterium*, *Neisseria*, *Pantoea*, *Pseudomonas*, *Prochlorococcus*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodopseudomonas*, *Roseburia*, *Rhodospirillum*, *Rhodococcus*, *Scenedesmus*, *Streptomyces*, *Streptococcus*, *Synecoccus*, *Saccharomonospora*, *Staphylococcus*, *Serratia*, *Salmonella*, *Shigella*, *Thermoanaerobacterium*, *Tropheryma*, *Tularensis*, *Temecula*, *Thermosynechococcus*, *Thermococcus*, *Ureaplasma*, *Xanthomonas*, *Xylella*, *Yersinia* and *Zymomonas*. In some embodiments, the host cell is a species of *Agrobacterium*, *Acinetobacter*, *Azobacter*, *Bacillus*, *Bifidobacterium*, *Buchnera*, *Geobacillus*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Enterococcus*, *Erwinia*, *Flavobacterium*, *Lactobacillus*, *Lactococcus*, *Pantoea*, *Pseudomonas*, *Staphylococcus*, *Salmonella*, *Streptococcus*, *Streptomyces*, or *Zymomonas*. In some embodiments, the bacterial host strain is non-pathogenic to humans. In some embodiments the bacterial host strain is an industrial strain. Numerous bacterial industrial strains are known and suitable in the present invention. In some embodiments of the present invention, the bacterial host cell is an *Agrobacterium* species (e.g., *A. radiobacter*, *A. rhizogenes*, and *A. rubi*). In some embodiments of the present invention, the bacterial host cell is an *Arthrobacter* species (e.g., *A. aureus*, *A. citreus*, *A. globiformis*, *A. hydrocarboglutamicus*, *A. mysorens*, *A. nicotianae*, *A. paraffineus*, *A. protophomiae*, *A. roseoparaffinus*, *A. sulfureus*, and *A. ureafaciens*). In some embodiments of the present invention, the bacterial host cell is a *Bacillus* species (e.g., *B. thuringensis*, *B. anthracis*, *B. megaterium*, *B. subtilis*, *B. lentus*, *B. circulans*, *B. pumilus*, *B. lautus*, *B. coagulans*, *B. brevis*, *B. firmus*, *B. alkaophilus*, *B. licheniformis*, *B. clausii*, *B. stearothermophilus*, *B. halodurans*, and *B. amyloliquefaciens*). In some embodiments, the host cell is an industrial *Bacillus* strain including but not limited to *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. megaterium*, *B. clausii*, *B. stearothermophilus*, or *B. amyloliquefaciens*. In some embodiments, the *Bacillus* host cells are *B.*

subtilis, *B. licheniformis*, *B. megaterium*, *B. stearothermophilus*, and/or *B. amyloliquefaciens*. In some embodiments, the bacterial host cell is a *Clostridium* species (e.g., *C. acetobutylicum*, *C. tetani* E88, *C. lituseburense*, *C. saccharobutylicum*, *C. perfringens*, and *C. beijerinckii*). In some embodiments, the bacterial host cell is a *Corynebacterium* species (e.g., *C. glutamicum* and *C. acetoacidophilum*). In some embodiments the bacterial host cell is an *Escherichia* species (e.g., *E. coli*). In some embodiments, the bacterial host cell is an *Erwinia* species (e.g., *E. uredovora*, *E. carotovora*, *E. ananas*, *E. herbicola*, *E. punctata*, and *E. terreus*). In some embodiments, the bacterial host cell is a *Pantoea* species (e.g., *P. citrea*, and *P. agglomerans*). In some embodiments the bacterial host cell is a *Pseudomonas* species (e.g., *P. putida*, *P. aeruginosa*, *P. mevalonii*, and *P. sp.* D-01 10). In some embodiments, the bacterial host cell is a *Streptococcus* species (e.g., *S. equisimiles*, *S. pyogenes*, and *S. uberis*). In some embodiments, the bacterial host cell is a *Streptomyces* species (e.g., *S. ambifaciens*, *S. achromogenes*, *S. avermitilis*, *S. coelicolor*, *S. aureofaciens*, *S. aureus*, *S. fungicidicus*, *S. griseus*, and *S. lividans*). In some embodiments, the bacterial host cell is a *Zymomonas* species (e.g., *Z. mobilis*, and *Z. lipolytica*).

[0146] An exemplary host cell is *Escherichia coli* W3110. The expression vector was created by operatively linking a polynucleotide encoding an improved PGA into the plasmid pCK110900 operatively linked to the *lac* promoter under control of the *lacI* repressor. The expression vector also contained the P15a origin of replication and the chloramphenicol resistance gene. Cells containing the subject polynucleotide in *Escherichia coli* W3110 were isolated by subjecting the cells to chloramphenicol selection.

[0147] Many prokaryotic and eukaryotic strains that find use in the present invention are readily available to the public from a number of culture collections such as American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

[0148] In some embodiments, host cells are genetically modified to have characteristics that improve protein secretion, protein stability and/or other properties desirable for expression and/or secretion of a protein. Genetic modification can be achieved by genetic engineering techniques and/or classical microbiological techniques (e.g., chemical or UV mutagenesis and subsequent selection). Indeed, in some embodiments, combinations of recombinant modification and classical selection techniques are used to produce the host cells. Using recombinant technology, nucleic acid molecules can be introduced, deleted, inhibited or modified, in a manner that results in increased yields of PGA variant(s) within the host cell and/or in the culture medium. For example, knockout of *Alp1* function results in a cell that is protease deficient, and knockout of *pyr5* function results in a cell with a pyrimidine deficient phenotype. In one genetic engineering approach, homologous recombination is used to induce targeted gene modifications by specifically targeting a gene *in vivo* to suppress expression of the encoded protein. In alternative approaches, siRNA, antisense and/or ribozyme

technology find use in inhibiting gene expression. A variety of methods are known in the art for reducing expression of protein in cells, including, but not limited to deletion of all or part of the gene encoding the protein and site-specific mutagenesis to disrupt expression or activity of the gene product. (See e.g., Chaveroche et al., Nucl. Acids Res., 28:22 e97 [2000]; Cho et al., Molec. Plant Microbe Interact., 19:7-15 [2006]; Maruyama and Kitamoto, Biotechnol Lett., 30:1811-1817 [2008]; Takahashi et al., Mol. Gen. Genom., 272: 344-352 [2004]; and You et al., Arch. Microbiol., 191:615-622 [2009], all of which are incorporated by reference herein). Random mutagenesis, followed by screening for desired mutations also finds use (See e.g., Combier et al., FEMS Microbiol. Lett., 220:141-8 [2003]; and Firon et al., Eukary. Cell 2:247-55 [2003], both of which are incorporated by reference).

[0149] Introduction of a vector or DNA construct into a host cell can be accomplished using any suitable method known in the art, including but not limited to calcium phosphate transfection, DEAE-Dextran mediated transfection, PEG-mediated transformation, electroporation, or other common techniques known in the art.

[0150] In some embodiments, the engineered host cells (*i.e.*, “recombinant host cells”) of the present invention are cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants, or amplifying the PGA polynucleotide. Culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and are well-known to those skilled in the art. As noted, many standard references and texts are available for the culture and production of many cells, including cells of bacterial, plant, animal (especially mammalian) and archeobacterial origin.

[0151] In some embodiments, cells expressing the variant PGA polypeptides of the invention are grown under batch or continuous fermentations conditions. Classical “batch fermentation” is a closed system, wherein the compositions of the medium is set at the beginning of the fermentation and is not subject to artificial alternations during the fermentation. A variation of the batch system is a “fed-batch fermentation” which also finds use in the present invention. In this variation, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is likely to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Batch and fed-batch fermentations are common and well known in the art. “Continuous fermentation” is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth. Continuous fermentation systems strive to maintain steady state growth conditions. Methods for modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

[0152] In some embodiments of the present invention, cell-free transcription/translation systems find use in producing variant PGA(s). Several systems are commercially available and the methods are well-known to those skilled in the art.

[0153] The present invention provides methods of making variant PGA polypeptides or biologically active fragments thereof. In some embodiments, the method comprises: providing a host cell transformed with a polynucleotide encoding an amino acid sequence that comprises at least about 70% (or at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99%) sequence identity to SEQ ID NO:2 and comprising at least one mutation as provided herein; culturing the transformed host cell in a culture medium under conditions in which the host cell expresses the encoded variant PGA polypeptide; and optionally recovering or isolating the expressed variant PGA polypeptide, and/or recovering or isolating the culture medium containing the expressed variant PGA polypeptide. In some embodiments, the methods further provide optionally lysing the transformed host cells after expressing the encoded PGA polypeptide and optionally recovering and/or isolating the expressed variant PGA polypeptide from the cell lysate. The present invention further provides methods of making a variant PGA polypeptide comprising cultivating a host cell transformed with a variant PGA polypeptide under conditions suitable for the production of the variant PGA polypeptide and recovering the variant PGA polypeptide. Typically, recovery or isolation of the PGA polypeptide is from the host cell culture medium, the host cell or both, using protein recovery techniques that are well known in the art, including those described herein. In some embodiments, host cells are harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including, but not limited to freeze-thaw cycling, sonication, mechanical disruption, and/or use of cell lysing agents, as well as many other suitable methods well known to those skilled in the art.

[0154] Engineered PGA enzymes expressed in a host cell can be recovered from the cells and/or the culture medium using any one or more of the well known techniques for protein purification, including, among others, lysozyme treatment, sonication, filtration, salting-out, ultra-centrifugation, and chromatography. Suitable solutions for lysing and the high efficiency extraction of proteins from bacteria, such as *E. coli*, are commercially available under the trade name CellLytic B™ (Sigma-Aldrich). Thus, in some embodiments, the resulting polypeptide is recovered/isolated and optionally purified by any of a number of methods known in the art. For example, in some embodiments, the polypeptide is isolated from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, chromatography (*e.g.*, ion exchange, affinity, hydrophobic interaction, chromatofocusing, and size exclusion), or precipitation. In some embodiments, protein refolding steps are used, as desired, in completing the configuration of the mature protein. In addition, in some embodiments, high performance liquid chromatography

(HPLC) is employed in the final purification steps. For example, in some embodiments, methods known in the art, find use in the present invention (See e.g., Parry et al., *Biochem. J.*, 353:117 [2001]; and Hong et al., *Appl. Microbiol. Biotechnol.*, 73:1331 [2007], both of which are incorporated herein by reference). Indeed, any suitable purification methods known in the art find use in the present invention.

[0155] Chromatographic techniques for isolation of the PGA polypeptide include, but are not limited to reverse phase chromatography high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, and affinity chromatography. Conditions for purifying a particular enzyme will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity, molecular weight, molecular shape, etc., are known to those skilled in the art.

[0156] In some embodiments, affinity techniques find use in isolating the improved PGA enzymes. For affinity chromatography purification, any antibody which specifically binds the PGA polypeptide may be used. For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats, etc., may be immunized by injection with the PGA. The PGA polypeptide may be attached to a suitable carrier, such as BSA, by means of a side chain functional group or linkers attached to a side chain functional group. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (*Bacillus Calmette Guerin*) and *Corynebacterium parvum*.

[0157] In some embodiments, the PGA variants are prepared and used in the form of cells expressing the enzymes, as crude extracts, or as isolated or purified preparations. In some embodiments, the PGA variants are prepared as lyophilisates, in powder form (e.g., acetone powders), or prepared as enzyme solutions. In some embodiments, the PGA variants are in the form of substantially pure preparations.

[0158] In some embodiments, the PGA polypeptides are attached to any suitable solid substrate. Solid substrates include but are not limited to a solid phase, surface, and/or membrane. Solid supports include, but are not limited to organic polymers such as polystyrene, polyethylene, polypropylene, polyfluoroethylene, polyethyleneoxy, and polyacrylamide, as well as co-polymers and grafts thereof. A solid support can also be inorganic, such as glass, silica, controlled pore glass (CPG), reverse phase silica or metal, such as gold or platinum. The configuration of the substrate can be in the form of beads, spheres, particles, granules, a gel, a membrane or a surface. Surfaces can be planar, substantially planar, or non-planar. Solid supports can be porous or non-porous, and can have swelling or non-swelling characteristics. A solid support can be configured in the form of a well, depression, or other container, vessel, feature, or location. A plurality of supports can be configured

on an array at various locations, addressable for robotic delivery of reagents, or by detection methods and/or instruments.

[0159] In some embodiments, immunological methods are used to purify PGA variants. In one approach, antibody raised against a variant PGA polypeptide (*e.g.*, against a polypeptide comprising any of SEQ ID NOS:2, 4, 6, 8, 10, or 12, and/or an immunogenic fragment thereof) using conventional methods is immobilized on beads, mixed with cell culture media under conditions in which the variant PGA is bound, and precipitated. In a related approach, immunochromatography finds use.

[0160] In some embodiments, the variant PGAs are expressed as a fusion protein including a non-enzyme portion. In some embodiments, the variant PGA sequence is fused to a purification facilitating domain. As used herein, the term "purification facilitating domain" refers to a domain that mediates purification of the polypeptide to which it is fused. Suitable purification domains include, but are not limited to metal chelating peptides, histidine-tryptophan modules that allow purification on immobilized metals, a sequence which binds glutathione (*e.g.*, GST), a hemagglutinin (HA) tag (corresponding to an epitope derived from the influenza hemagglutinin protein; See *e.g.*, Wilson *et al.*, *Cell* 37:767 [1984]), maltose binding protein sequences, the FLAG epitope utilized in the FLAGS extension/affinity purification system (*e.g.*, the system available from Immunex Corp), and the like. One expression vector contemplated for use in the compositions and methods described herein provides for expression of a fusion protein comprising a polypeptide of the invention fused to a polyhistidine region separated by an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography; See *e.g.*, Porath *et al.*, *Prot. Exp. Purif.*, 3:263-281 [1992]) while the enterokinase cleavage site provides a means for separating the variant PGA polypeptide from the fusion protein. pGEX vectors (Promega) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to ligand-agarose beads (*e.g.*, glutathione-agarose in the case of GST-fusions) followed by elution in the presence of free ligand.

EXPERIMENTAL

[0161] Various features and embodiments of the disclosure are illustrated in the following representative examples, which are intended to be illustrative, and not limiting.

[0162] In the experimental disclosure below, the following abbreviations apply: ppm (parts per million); M (molar); mM (millimolar), μ M and μ M (micromolar); nM (nanomolar); mol (moles); gm and g (gram); mg (milligrams); μ g and μ g (micrograms); L and l (liter); ml and mL (milliliter); cm (centimeters); mm (millimeters); μ m and μ m (micrometers); sec. (seconds); min(s) (minute(s)); h(s) and hr(s) (hour(s)); U (units); MW (molecular weight); rpm (rotations per minute); °C (degrees Centigrade); RT (room temperature); CDS (coding sequence); DNA (deoxyribonucleic acid); RNA

(ribonucleic acid); TB (Terrific Broth; 12 g/L bacto-tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 65 mM potassium phosphate, pH 7.0, 1 mM MgSO₄); CAM (chloramphenicol); PMBS (polymyxin B sulfate); IPTG (isopropyl thiogalactoside); TFA (trifluoroacetic acid); HPLC (high performance liquid chromatography); FIOPC (fold improvement over positive control); HTP (high throughput); LB (Luria broth); Codexis (Codexis, Inc., Redwood City, CA); Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO); Millipore (Millipore, Corp., Billerica MA); Difco (Difco Laboratories, BD Diagnostic Systems, Detroit, MI); Daicel (Daicel, West Chester, PA); Genetix (Genetix USA, Inc., Beaverton, OR); Molecular Devices (Molecular Devices, LLC, Sunnyvale, CA); Applied Biosystems (Applied Biosystems, part of Life Technologies, Corp., Grand Island, NY), Agilent (Agilent Technologies, Inc., Santa Clara, CA); Thermo Scientific (part of Thermo Fisher Scientific, Waltham, MA); (Infors; Infors-HT, Bottmingen/Basel, Switzerland); Corning (Corning, Inc., Palo Alto, CA); and Bio-Rad (Bio-Rad Laboratories, Hercules, CA); Microfluidics (Microfluidics Corp., Newton, MA, United States of America).

[0163] The following sequences find use in the present invention.

SEQ ID NO:1 (PGA WT polynucleotide sequence)

ATGAAAAATAGAAATCGTATGATCGTGAACGGTATTGTGACTTCCCTGATCTGTTGTTCT
AGCCTGTCAGCGCTGGCGGCAAGCCCGCCAACCGAGGTTAAGATCGTTCGCGATGAATA
CGGCATGCCGCATATTTACGCCGATGATACCTATCGACTGTTTTACGGCTATGGCTACGT
GGTGGCGCAGGATCGCCTGTTCCAGATGGAAATGGCGCGCCGCAGTACTCAGGGGACCG
TCTCCGAGGTGCTGGGCAAAGCATTTCGTCAGTTTTTGATAAAGATATTCGCCAGAACTACT
GGCCGGATTCTATTTCGCGCGCAGATAGCTTCCCTCTCCGCTGAGGATAAATCCATTCTGC
AGGGCTATGCCGATGGCATGAATGCGTGGATCGATAAAGTGAACGCCAGCCCCGATAAG
CTGTTACCCCAGCAGTTCTCCACCTTTGGTTTTAAACCCAAGCATTGGGAACCGTTTGATG
TGGCGATGATTTTTGTCGGCACCATGGCGAACCGGTTTTCTGACAGCACCAGCGAAATTG
ATAACCTGGCGCTGCTGACGGCGCTAAAAGATAAATACGGCAAGCAGCAGGGCATGGCG
GTCTTTAACCAGCTGAAATGGCTGGTTAATCCTTCCGCGCCAACCACCATTCGCGCGCGG
GAAAGCGCCTATCCGCTGAAGTTTGATCTGCAAAACACGCAACGGCGGCGCTGCTGCC
GCGCTACGACCAGCCGGCACCGATGCTCGACCGCCCGGCAAAAGGGACCGATGGCGCGC
TGCTGGCGCTGACCGCCGATCAGAACCGGGAACTATCGCCGCGCAGTTCGCGCAAAGC
GGCGCTAACGGCCTGGCTGGCTACCCGACCACTAGCAATATGTGGGTGATTGGCAAAAA
CAAAGCCCAGGATGCGAAGGCCATTATGGTCAATGGGCCGCAGTTTGGTTGGTATGCGC
CGGCGTACACCTACGGTATCGGCCTGCACGGCGCGGGCTATGACGTCACCGGCAATACG
CCGTTTGCCTATCCGGGCCTCGTTTTTGGTCACAACGGCACCATTTTCATGGGGATCCACC
GCCGGTTTTGGTGATGATGTTCGATATCTTTGCCGAAAACTTTCGCGCCGAGAAGCCGGGC
TATTACCAGCATAACGGCGAGTGGGTGAAGATGTTGAGCCGCAAGGAGACTATTGCGGT

CAAAGACGGCCAGCCGGAGACCTTTACCGTTTGGCGCACGCTGCACGGCAACGTCATTA
AAACCGATACTGCGACGCAGACCGCCTATGCCAAAGCGCGCGCCTGGGATGGCAAAGAG
GTGGCGTCCCTGCTGGCGTGGACGCACCAGATGAAGGCCAAAACTGGCCGGAGTGGAC
GCAGCAGGCGGCCAAACAGGCGCTGACCATTAAGTGGTACTACGCCGATGTGAACGGCA
ATATCGGCTATGTGCATACCGGCGCCTATCCGGATCGCCAGCCCGGCCACGACCCGCGTT
TGCCGGTTCCCGGCACTGGAAAAATGGGACTGGAAAGGGTTGCTGTGCTTTGATTTGAATC
CGAAAGTGTATAACCCGCAGTCGGGCTATATCGCCAACTGGAACAACCTCGCCGCAAAAA
GACTACCCGGCCTCTGATCTGTTCGCGTTCTGTGGGGCGGTGCGGATCGAGTTACTGAG
ATCGACACGATCCTCGATAAGCAACCGCGCTTCACCGCCGATCAGGCGTGGGATGTGAT
CCGCCAAACCAGCCGTCGGGATCTCAACCTGCGGTTGTTCTTACCGGCGCTGAAGGACGC
CACCGCGAACCTGGCGGAAAACGATCCGCGCCGCCAACTGGTGGATAAACTGGCGAGCT
GGGACGGTGAAAACCTTGTC AACGATGACGGAAAAACCTATCAGCAACCGGGATCGGCG
ATTCTTAACGCCTGGCTGACCAGCATGCTCAAGCGCACGGTGGTTGCCGCGGTCCCAGCG
CCGTTTGGCAAGTGGTACAGCGCCAGTGGCTATGAAACCACCCAGGACGGGGCCAACCGG
CTCGCTGAACATCAGCGTGGGGGCGAAAAATCCTCTACGAAGCTCTGCAGGGTGATAAGT
CGCCAATCCCGCAGGCGGTGATCTGTTTGGCGGGAAACCGCAGCAGGAAGTGATACTG
GCGGCGCTGGACGACGCTTGGCAGACGCTGTCAAAACGCTACGGTAACGACGTCACCGG
CTGGA AAAACCCCTGCCATGGCGCTTACCTTCCGGGCCAATAACTTCTTCGGCGTGCCGCA
GGCGGCAGCAAAAAGAGGCGCGTCATCAGGCGGAGTACCAGAACCGCGGTACGGAAAAAC
GACATGATTGTCTTCTCACCGACGTCGGGTAAACCGCCCGGTTCTTGCCTGGGATGTGGTG
GCGCCGGGGCAAAGCGGTTTTATCGCGCCGGATGGCAAAGCCGATAAGCACTATGACGA
TCAGCTGAAAATGTACGAGAGCTTTGGCCGTAAATCGCTGTGGTTAACGCCTCAGGACGT
TGACGAGCACAAAGAGTCTCAGGAAGTGCTGCAGGTACAGCGCTAA (SEQ ID NO:1)

SEQ ID NO:2 (PGA WT polypeptide sequence)

MKNRNRMI VNGIVTSLICSSLSALAA SPPT EVKIVRDEYGMPHIYADDTYRLFYGYGYVVA
QDRLFQMEMARRSTQGT VSEVLGKAFVSFDKDIRQNYWPDSIRAQIASLSAEDKSILQGYAD
GMNAWIDKVN ASPDKLLPQQFSTFGFKPKHWEPFDVAMIFVGTMANRFS DSTSEIDNLALLT
ALKDKY GKQQGMAVFNQLKWL VNPSAPT TIAARESAYPLKFDLQNTQTAALLPRYDQPAP
MLDRPAKGT DGALLALTADQNRE TIAAQFAQSGANGLAGYPTTSNMWVIGKNKAQDAKAI
MVNGPQFGWYAPAYTYGIGLHGAGYDVTGNTPFAYPGLVFGHNGTISWGSTAGFGDDVDIF
AEKLSAEKPGYYQHNGEWVKMLSRKETIAVKDGGPETFTVWRTLHGNVIKTD TATQTAYA
KARAWDGKEVASLLAWTHQMKAKNWPEWTQQA AKQALTINWYYADVNGNIGYVHTGAY
PDRQPGHDPRLPVP GTGKWDWKGLLSFDLNPKVYNPQSGYIANWNNSPQKDYPASDLFAFL
WGGADRVTEIDTILDKQPRFTADQAWDVIRQTSRRDLNLRLFLPALKDATANLAENDPRRQL
VDKLASWDGENLVNDDGKTYQQPGSAILNAWL TSM LKRTVVA AVPA PFGKWYSASGYETT
QDGPTGSLNISVGAKILYEALQGDKSPIPQAVDLFGGKPPQQEVILAALDDAWQTL SKRYGND

VTGWKTPAMALTFRANNFFGVPPQAAAKEARHQAHEYQNRGTENDMIVFSPTSGNRPVLAWD
VVAPGQSGFIAPDGKADKHYDDQLKMYESFGRKSLWLTPQDVDEHKESQEV LQVQR (SEQ
ID NO:2)

SEQ ID NO:3 (PGA Variant 1 polynucleotide sequence)

AGCAATATGTGGGTGATTGGCAAAAACAAAGCCCAGGATGCGAAGGCCATTATGGTCAA
TGGGCCGCAGTTTGGTTGGTATGTGCCGGCGTACACCTACGGTATCGGCCTGCACGGCGC
GGGCTATGACGTCACCGGCAATACGCCGTTTGCCTATCCGGGGCCTCGTTTTTGGTCACAA
CGGCACCATTTTCATGGGGATCCACCGCCGGTGGTGGTATGATGTTCGATATCTTTGCCGA
AAAACTTTCCGCCGAGAAGCCGGGCTATTACCAGCATAACGGCGAGTGGGTGAAGATGT
TGAGCCGCAAGGAGACTATTGCGGTCAAAGACGGCCAGCCGGAGACCTTTACCGTTTGG
CGCACGCTGCACGGCAACGTCATTAAAACCGATACTGCGACGCAGACCGCCTATGCCAA
AGCGCGCGCCTGGGATGGCAAAGAGGTGGCGTCCCTGCTGGCGTGACGCACCAGATGA
AGGCCAAAAACTGGCCGGAGTGGACGCAGCAGGCGGCCAAACAGGCGCTGACCATTAA
CTGGTACTACGCCGATGTGAACGGCAATATCGGCTATGTGCATACCGGCGCCTATCCGGA
TCGCCAGCCCCGGCCACGACCCGCGTTTGGCCGTTCCCGGCACTGGAAAATGGGACTGGA
AAGGGTTGCTGTCTGTTTGATTTGAATCCGAAAGTGTATAACCCGCAGTCGGGCTATATCG
CCAACTGGAACAACCTCGCCGAAAAAGACTACCCGGCCTCTGATCTGTTTCGCGTTCCCTGT
GGGGCGGTGCGGATCGAGTTACTGAGATCGACACGATCCTCGATAAGCAACCGCGCTTC
ACCGCCGATCAGGCGTGGGATGTGATCCGCCAAACCAGCCGTCGGGATCTCAACCTGCG
GTTGTTCTTACCGGCGCTGAAGGACGCCACCGCGAACCTGGCGGAAAACGATCCGCGCC
GCCAACTGGTGGATAAACTGGCGAGCTGGGACGGCGAAAACCTTGTC AACGATGACGGA
AAAACCTATCAGCAACCGGGATCGGCGATTCTTAACGCCTGGCTGACCAGCATGCTCAA
GCGCACGGTGGTTGCCGCGGTCCCAGCGCCGTTTGGTAAGTGGTACAGCGCCAGTGGCT
ATGAAACCACCCAGGACGGGCCAACCGGCTCGCTGAACATCAGCGTGGGGGGCGAAAATC
CTCTACGAAGCTCTGCAGGGTGATAAGTCGCCAATCCCGCAGGCGGTTCGATCTGTTTGGC
GGGAAACCGCAGCAGGAAGTAATACTGGCGGCGCTGGACGACGCTTGGCAGACGCTGTC
AAAACGCTACGGTAACGACGTCACCGGCTGGAAAACCCCTGCCATGGCGCTTACCTTCC
GGGCCAATAACTTCTTCGGCGTGCCGCAGGCGGCAGCAAAAGAGGCGCGTCATCAGGCG
GAGTACCAGAACCGCGGTACGGAAAACGACATGATTGTCTTCTACCGACGTCGGGTAA
CCGCCCCGGTTCTTGCTGGGATGTGGTGGCGCCGGGGCAAAGCGGTTTTATCGCGCCGGA
TGGCAAAGCCGATAAGCACTATGACGATCAGCTGAAAATGTACGAGAGCTTTGGCCGTA
AATCGCTGTGGTTAACGCCTCAGGACGTTGACGAGCACCAAGAGTCTCAGGAAGTGCTG
CAGGTACAGTTGGATCAGACCGAGGTAAAGATCGTTCGCGATGAATACGGCATGCCGCA
TATTTACGCCGATGATACCTATCGACTGTTTTACGGCTATGGCTACGTGGTGGCGCAGGA
TCGCCTGTTCCAGATGGAAAATGGCGCGCCGCAGTACTCAGGGGACCGTCTCCGAGGTGCT
GGGCAAAGCATTTCGTCAGTTTTGATAAAGATATTCGCCAGAACTACTGGCCGGATTCTAT

TCGCGCGCAGATAGCTTCCCTCTCCGCTGAGGATAAATCCATTCTGCAGGGCTATGCCGA
 TGGCATGAATGCGTGGATCGATAAAGTGAACGCCAGCCCCGATAAGCTGTTACCCCAGC
 AGTTCTCCACCTTTGGTTTTAAACCCAAGCATTGGGAACCGTTTGATGTGGCGATGATTTT
 TGTCGGCACCATTGGCGAACCGTTTCTCTGACAGCACCAGCGAAATTGATAACCTGGCGCT
 GCTGACGGCGCTAAAAGACAAATACGGCAAGCAGCAGGGCATGGCGGTCTTTAACCAGC
 TGAAATGGCTGGTTAATCCTTCCGCGCCAACCACCATTTGCGGCGCGGGAAAGCGCCTATC
 CGCTGAAGTTTGATCTGCAAAACACGCAAACGGCGTAA (SEQ ID NO:3)

SEQ ID NO:4 (PGA Variant 1 polypeptide sequence)

SNMWVIGKNKAQDAKAIMVNGPQFGWYVPAITYGIGLHGAGYDVTGNTPFAYPGLVFGH
 NGTISWGSTAGGGDDVDIFAELKSAEKPGYYQHNGEWVKMLSRKETIAVKDGQPETFTVWR
 TLHGNNVIKTDATQTAYAKARAWDGKEVASLLAWTHQMKAKNWPEWTQQAQKQALTN
 WYYADVNGNIGYVHTGAYPDRQPGHDPRLPVPGTGKWDWKGLLSFDLNPVKVYNPQSGYIA
 NWNNSPQKDYPASDLFAFLWGGADRVTEIDTILDKQPRFTADQAWDVIRQTSRRDLNLRFL
 PALKDATANLAENDPRRQLVDKLASWDGENLVNDDGKTYQQPGSAILNAWLTSMLKRTVV
 AAVPAPFGKWYSASGYETTQDGPTGSLNISVGAKILYEALQGDKSPIQAVDLFGGKPPQQEV
 LAALDDAWQTLISKRYGNDVTGWKTPAMALTFRANFFGVPQAAAKEARHQAQYQNRGTE
 NDMIVFSPTSGNRPVLAWDVVPAGQSGFIAPDGKADKHYDDQLKMYESFGRKSLWLTPQDV
 DEHQESQEVLLQVQLDQTEVKIVRDEYGMPIHYADDTYRLFYGYGYVVAQDRLFQMEMARR
 STQGTVSEVLGKAFVSFDKDIRQNYWPDSIRAQIASLSAEDKSILQGYADGMNAWIDKVNAS
 PDKLLPQQFSTFGFKPKHWEPFDVAMIFVGTMANRFSSTSEIDNLALLTALKDKYQKQGM
 AVFNQLKWLNVNPSAPTTIAARESAYPLKFDLQNTQTA (SEQ ID NO:4)

SEQ ID NO:5 (PGA Variant 6 polynucleotide sequence)

AGCAATATGTGGGTGATTGGCAAAAACAAAGCCAGGATGCGAAGGCCATTATGGTCAA
 TGGGCCGCAGTTTGGTTGGTATGTGCCGGCGTATACCTACGGTATCGGCCTGCACGGCGC
 GGGCTATGACGTCACCGGCAATACGCCGTTTGCCTATCCGGGCCTCGTTTTTGGTCAAA
 CGGCACCATTTTCATGGGGATCCACCGCCGGTGGTGGTGATGATGTCGATATCTTTGCCGA
 AAAACTTTCCGCCGAGAAGCCGGGCTATTACCAGCATAACGGCGAGTGGGTGAAGATGT
 TGAGCCGCAAGGAGACTATTGCGGTCAAAGACGGCCAGCCGGAGACCTTTACCGTTTGG
 CGCACGCTGCACGGCAACGTCATTAAAACCGATACTGCGACGCAGACCGCCTATGCCAA
 AGCGCGCGCCTGGGATGGCAAAGAGGTGGCGTCCCTGCTGGCGTGGACGCACCAGATGA
 AGGCCAAAAACTGGCCGGAGTGGACGCAGCAGGCGGCCAAACAGGCGCTGACCATCAA
 CTGGTACTACGCCGATGTGAACGGCAATATCGGCTATGTGCATACCGGCGCCTATCCGGA
 TCGCCAGCCCGGCCACGACCCGCGTTTGCCGGTTCCCGGCACTGGAAAATGGGACTGGA
 AAGGGTTGCTGTCTGTTTGAATCCGAAAGTGTATAACCCGCAGTCGGGCTATATCG
 CCAACTGGAACAACCTCGCCGCAAAAAGACTACCCGGCCTCTGATCTGTTCGCGTTCCCTGT

GGGGCGGTGCGGATCGAGCGACTGAGATCGACACGATCCTCGATAAGCAACCGCGCTTC
ACCGCCGATCAGGCGTGGGATGTGATCCGCCAAACCAGCCGTCGGGATCTCAACCTGCG
GTTGTTCTTACCGGCGCTGAAGGACGCCACCGCCAACCTGGCGGAAAACGATCCGCGCC
GCCAACTGGTGGATAAACTGGCGAGCTGGGACGGCGAAAACCTTGTC AACGATGACGGA
AAAACCTATCAGCAACCGGGATCGGCGATTCTTAACGCCTGGCTGACCAGCATGCTCAA
GCGCACGGTGGTTGCCGCGGTCCCAGCGCCGTTTGGTAAGTGGTACAGCGCCAGTGGCT
ATGAAACCACCCAGGACGGGGCCAACCGGCTCGCTGAACATCAGCGTGGGGGGCGAAAATC
CTCTACGAAGCTCTGCAGGGTGATAAGTCGCCAATCCCGCAGGCGGTGATCTGTTTGGC
GGGAAACCGCAGCAGGAAGTAATACTGGCGGGCGCTGGACGACGCTTGGCAGACGCTGTC
AAAACGCTACGGTAACGACGTCACCGGCTGGAAAACCCCTGCCATGGCGCTTACCTTCC
GGGCCAATAACTTCTTCGGCGTGCCGCAGGCGGCAGCAAAAGAGGCGCGTCATCAGGCG
GAGTACCAGAACCGCGGTACGGAAAACAACATGATTGTCTTCTACCGACGTCGGGTAA
CCGCCCGGTTCTTGCCTGGGATGTGGTGGCGCCGGGGCAAAGCGGTTTTATCGCGCCGGA
TGGCAAAGCCGATAAGCACTATGACGATCAGCTGAAAATGTACGAGAGCTTTGGCCGTA
AATCGCTGTGGTTAACGCCTCAGGACGTTGACGAGCACAAAGAGTCTCAGGAAGTGCTG
CAGGTACAGTTGGATCAGACCGAGGTAAAGATCGTTCGCGATGAATACGGCATGCCGCA
TATTTACGCCGATGATACCTATCGACTGTTTTACGGCTATGGCTACGTGGTGGCGCAGGA
TCGCTGTTCCAGATGGAAATGGCGCGCCGCGAGTACTCAGGGGACCGTCTCCGAGGTGCT
GGGCAAAGCATTCGTCAGTTTTGATAAAGATATTCGCCAGAACTACTGGCCGGATTCTAT
TCGCGCGCAGATAGCTTCCCTCTCCGCTGAGGATAAAATCCATTCTGCAGGGCTATGCCGA
TGGCATGAATGCGTGGATCGATAAAGTGAACGCCAGCCCCGATAAGCTGTTACCCCAGC
AGTTCTCCACCTTTGGTTTTAAACCCAAGCATTGGGAACCGTTTGATGTGGCGATGATTTT
TGTCGGCACCATGGCGAACCGTTTTTCTGACAGCACCGCGAAATTGATAACCTGGCGCT
GCTGACGGCGCTAAAAGACAAATACGGCAAGCAGCAGGGCATGGCGGTCTTTAACCAGC
TGAAATGGCTGGTTAATCCTTCCGCGCCAACCACCATTCGCGCGCGGGAAAGCGCCTATC
CGCTGAAGTTTGATCTGCAAAACACGCAAACGGCGTAA (SEQ ID NO:5)

SEQ ID NO:6 (PGA Variant 6 polypeptide sequence)

SNMWVIGKNKAQDAKAIMVNGPQFGWYVPAYTYGIGLHGAGYDVTGNTPFAYPGLVFGH
NGTISWGSTAGGGDDVDIFAELKSAEKPYYQHNGEWVKMLSRKETIAVKDQGQPETFTVWR
TLHGNNVIKTDATQTAYAKARAWDGKEVASLLAWTHQMKAKNWPEWTQQAQKQALTN
WYYADVNGNIGYVHTGAYPDRQPGHDPRLPVPGTGKWDWKGLLSFDLNPVYNPQSGYIA
NWNNSPQKDYPASDLFAFLWGGADRATEIDTILDKQPRFTADQAWDVIRQTSRRDLNLRFL
PALKDATANLAENDPRRQLVDKLSWDGENLVNDDGKTYQQPGSAILNAWLTSMLKRTVV
AAVPAPFGKWYSASGYETTQDGPTGSLNISVGAKILYEALQGDKSPIQAVDLFGGKPQQEVI
LAALDDAWQTLISKRYGNDVTGWKTPAMALTFRANFFGVPAQAAKEARHQAQYQNRGTE
NNMIVFSPTSGNRPVLAWDVVPAGQSGFIAPDGKADKHYDDQLKMYESFGRKSLWLTPQDV

DEHKESQEVQLQVQLDQTEVKIVRDEYGMPHIYADDTYRLFYGYGYVVAQDRLFQMEMARR
STQGTVSEVLGKAFVSFDKDIRQNYWPDSIRAQIASLSAEDKSILQGYADGMNAWIDKVNAS
PDKLLPQQFSTFGFKPKHWEPFDVAMIFVGTMANRFSSTSEIDNLALLTALKDKYGKQQGM
AVFNQLKWLNVNPSAPTTIAARESAYPLKFDLQNTQTA (SEQ ID NO:6)

SEQ ID NO:7 (PGA Variant 53 polynucleotide sequence)

AGCAATATGTGGGTGATTGGCAAAAACAAAGCCCAGGATGCGAAGGCCATTATGGTCAA
TGGGCCGCAGTTTGGTTGGTTTAATCCGGCGTACACCTACGGTATCGGCCTGCACGGCGC
GGGCTATGACGTCACCGGCAATACGCCGTTTGCCTATCCGGGGCCTCCTGTTTGGTCACAA
CGGCACCATTTTCATGGGGATCCACCGCCGGTGGTGGTGATGATGTCGATATCTTTGCCGA
AAAACTTTCCGCCGAGAAGCCGGGCTATTACCAGCATAACGGCGAGTGGGTGAAGATGT
TGAGCCGCAAGGAGACTATTGCGGTCAAAGACGGCCAGCCGGAGACCTTTACCGTTTGG
CGCACGCTGCACGGCAACGTCATTAAAACCGATACTGCGACGCAGACCGCCTATGCCAA
AGCGCGCGCCTGGGATGGCAAAGAGGTGGCGTCCCTGCTGGCGTGGACGCACCAGATGA
AGGCCAAAAACTGGCCGGAGTGGACGCAGCAGGCGGCCAAACAGGCGCTGACCATTAA
CTGGTACTACGCCGATGTGAACGGCAATATCGGCTATGTGCATACCGGCGCCTATCCGGA
TCGCCAGCCCCGGCCACGACCCGCGTTTGCCGGTTCCCGGCACTGGAAAATGGGACTGGA
AAGGGTTGCTGTGCTTTGATTTGAATCCGAAAGTGTATAACCCGCAGTCGGGCTATATCG
CCAACTGGAACAACCTCGCCGCAAAAAGACTACCCGGCCTCTGATCTGTTCGCGTTCCTGT
GGGGCGGTGCGGATCGAGTTACTGAGATCGACACGATCCTCGATAAGCAACCGCGCTTC
ACCGCCGATCAGGCGTGGGATGTGATCCGCCAAACCAGCCGTCGGGATCTCAACCTGCG
GTTGTTCTTACCGGCGCTGAAGGACGCCACCGCGAACCTGGCGGAAAACGATCCGCGCC
GCCAACTGGTGGATAAACTGGCGAGCTGGGACGGCGAAAACCTTGTC AACGATGACGGA
AAAACCTATCAGCAACCGGGATCGGCGATTCTTAACGCCTGGCTGACCAGCATGCTCAA
GCGCACGGTGGTTGCCGCGGTCCCAGCGCCGTTTGGTAAGTGGTACAGCGCCAGTGGCT
ATGAAACCACCCAGGACGGGCCAACCGGCTCGCTGAACATCAGCGTGGGGGGCGAAAATC
CTCTACGAAGCTCTGCAGGGTGATAAGTCGCCAATCCCGCAGGCGGTGATCTGTTTGGC
GGGAAACCGCAGCAGGAAGTAATACTGGCGGCGCTGGACGACGCTTGGCAGACGCTGTC
AAAACGCTACGGTAACGACGTCACCGGCTGGAAAACCCCTGCCATGGCGCTTACCTTCC
GGGCCAATAACTTCTTCGGCGTGCCGCAGGCGGCAGCAAAAGAGGCGCGTCATCAGGCG
GAGTACCAGAACCGCGGTACGGAAAACGACATGATTGTCTTCTACCGACGTCGGGTAA
CCGCCCGGTTCTTGCTGGGATGTGGTGGCGCCGGGGCAAAGCGGTTTTATCGCGCCGGA
TGGCAAAGCCGATAAGCACTATGACGATCAGCTGAAAATGTACGAGAGCTTTGGCCGTA
AATCGCTGTGGTTAACGCCTCAGGACGTTGACGAGACCAAGAGTCTCAGGAAGTGCTG
CAGGTACAGTTGGATCAGACCGAGGTAAAGATCGTTCGCGATGAATACGGCATGCCGCA
TATTTACGCCGATGATACCTATCGACTGTTTTACGGCTATGGCTACGTGGTGGCGCAGGA
TCGCCTGTTCCAGATGGAAAATGGCGCGCCGCACTACTCAGGGGACCGTCTCCGAGGTGCT

GGGCAAAGCTTTCGTTTCTTTTGATAAAGATATTCGCCAGAACTACTGGCCGGATTCTAT
TCGCGCGCAGATAGCTTCCCTCTCCGCTGAGGATAAATCCATTCTGCAGGGCTATGCCGA
TGGCATGAATGCGTGGATCGATAAAGTGAACGCCAGCCCCGATAAGCTGTTACCCCAGC
AGTTCTCCACCTTTGGTTTTAAACCCAAGCATTGGGAACCGTTTGATGTGGCGATGATTTT
TGTCGGCACCATGGCGAACCGTTTCTCTGACAGCACCAGCGAAATTGATAACCTGGCGCT
GCTGACGGCGCTAAAAGACAAATACGGCAAGCAGCAGGGCATGGCGGTCTTTAACCAGC
TGAAATGGCTGGTTAATCCTTCCGCGCCAACCACCATTTGCGGCGCGGGAAAGCGCCTATC
CGCTGAAGTTTGATCTGCAAAACACGCAAACGGCGTAA (SEQ ID NO:7)

SEQ ID NO:8 (PGA Variant 53 polypeptide sequence)

SNMWVIGKNKAQDAKAIMVNGPQFGWFNPAYTYGIGLHGAGYDVTGNTPFAYPGLLFGHN
GTISWGSTAGGGDDVDIFAELSAEKPGYYQHNGEWVKMLSRKETIAVKDGQPETFTVWRT
LHGNVIKTDATQTAYAKARAWDGKEVASLLAWTHQMKAKNWPEWTQQAQALTNW
YYADVNGNIGYVHTGAYPDRQPGHDPRLPVPGTGKWDWKGLLSFDLNPKVYNPQSGYIAN
WNNSPQKDYPASDLFAFLWGGADRVTEIDTILDKQPRFTADQAWDVIRQTSRRDLNLRLFLP
ALKDATANLAENDPRRQLVDKLASWDGENLVNDDGKTYQQPGSAILNAWLTSMLKRTVVA
AVPAPFGKWYSASGYETTQDGPTGSLNISVGAKILYEALQGDKSPIQAVDLFGGKPPQEVIL
AALDDAWQTLISKRYGNDVTGWKTPAMALTFRANNFFGVPQAAAKEARHQAQYQNRGTEN
DMIVFSPTSGNRPVLAWDVVAPGQSGFIAPDGKADKHYDDQLKMYESFGRKSLWLTPQDVD
EHQESQEVLLQVQLDQTEVKIVRDEYGMPHIYADDTYRLFYGYGYVVAQDRLFQMEMARRS
TQGTVSEVLGKAFVSFDKDIRQNYWPDSIRAQIASLSAEDKSILQGYADGMNAWIDKVNASP
DKLLPQQFSTFGFKPKHWEFPDVAMIFVGTMANRFSSTSEIDNLALLTALKDKYQKQQGM
AVFNQLKWLNVNPSAPTTIAARESAYPLKFDLQNTQTA (SEQ ID NO:8)

SEQ ID NO:9 (PGA Variant 261 polynucleotide sequence)

AGCAATATGTGGGTGATTGGCAAAAACAAAGCCCAGGATGCGAAGGCCATTATGGTCAA
TGGGCCGCAGTTTGGTTGGTATAATCCGGCGTATACCTACGGTATCGGCCCTGCACGGCGC
GGGCTATGACGTCACCGGCAATACGCCGTTTGCCTATCCGGGCCTCCTTTTTGGTCACAA
CGGCACCATTTTCATGGGGATCCACCGCCGGTGCCGGTGATGTCTGTCGATATCTTTGCCGA
AAAACTTTCCGCCGAGAAGCCGGGCTATTACCAGCATAACGGCGAGTGGGTGAAGATGT
TGAGCCGCAAGGAGACTATTGCGGTCAAAGACGGCCAGCCGGAGACCTTTACCGTTTGG
CGCACGCTGCACGGCAACGTCATTAAAACCGATACTGCGACGCAGACCGCCTATGCCAA
AGCGCGCGCCTGGGATGGCAAAGAGGTGGCGTCCCTGCTGGCGTGGACGCACCAGATGA
AGGCCAAAAACTGGCCGGAGTGGACGCAGCAGGCGGCCAAACAGGCGCTGACCATCAA
CTGGTACTACGCCGATGTGAACGGCAATATCGGCTATGTGCATACCGGCGCCTATCCGGA
TCGCCAGCCCGGCCACGACCCGCGTTTGCCGGTTCCCGGCACTGGAAAATGGGACTGGA
AAGGGTTGCTGTCTGTTGATTTGAATCCGAAAGTGTATAACCCGCAGTCGGGCTATATCG

CCAACTGGAACAACCTCGCCGCAAAAAGACTACCCGGCCTCTGATCTGTTTCGCGTTCCTGT
GGGGCGGTGCGGATCGAGCGACTGAGATCGACACGATCCTCGATAAGCAACCGCGCTTC
ACCGCCGATCAGGCGTGGGATGTGATCCGCCAAACCAGCCGTCGGGATCTCAACCTGCG
GTTGTTCTTACC GGCGCTGAAGGACGCCACCGCCAACCTGGCGGAAAACGATCCGCGCC
GCCAACTGGTGGATAAACTGGCGAGCTGGGACGGCGAAAACCTTGTCAACGATGACGGA
AAAACCTATCAGCAACCGGGATCGGCGATTCTTAACGCCTGGCTGACCAGCATGCTCAA
GCGCACGGTGGTTGCCGCGGTCCCAGCGCCGTTTGGTAAGTGGTACAGCGCCAGTGGCT
ATGAAACCACCCAGGACGGGCCAACCGGCTCGCTGAACATCAGCGTGGGGGCGAAAATC
CTCTACGAAGCTCTGCAGGGTGATAAGTCGCCAATCCCGCAGGCGGTTCGATCTGTTTGGC
GGGAAACCGCAGCAGGAAGTAATACTGGCGGCGCTGGACGACGCTTGGCAGACGCTGTC
AAAACGCTACGGTAACGACGTCACCGGCTGGAAAACCCCTGCCATGGCGCTTACCTTCC
GGGCCAATAACTTCTTCGGCGTGCCGACAGGCGGCAGCAAAAGAGGCGCGTCATCAGGCG
GAGTACCAGAACCGCGGTACGGAAAACAACATGATTGTCTTCTACCGACGTCGGGTAA
CCGCCCCGGTTCTTGCCTGGGATGTGGTGGCGCCGGGGCAAAGCGGTTTTATCGCGCCGGA
TGGCAAAGCCGATAAGCACTATGACGATCAGCTGAAAATGTACGAGAGCTTTGGCCGTA
AATCGCTGTGGTTAACGCCTCAGGACGTTGACGAGCACAAAGAGTCTCAGGAAGTGCTG
CAGGTACAGTTGGATCAGACCGAGGTAAAGATCGTTCGCGATGAATACGGCATGCCGCA
TATTTACGCCGATGATACCTATCGACTGTTTTACGGCTATGGCTACGTGGTGGCGCAGGA
TCGCTGTTCAGATGGAAATGGCGCGCCGACGTACTCAGGGGACCGTCTCCGAGGTGCT
GGGCAAAGCATTTCGTTTCATTTGATAAAGATATTCGCCAGAACTACTGGCCGGATTCTAT
TCGCGCGCAGATAGCTTCCCTCTCCGCTGAGGATAAAATCCATTCTGCAGGGCTATGCCGA
TGGCATGAATGCGTGGATCGATAAAGTGAACGCCAGCCCCGATAAGCTGTTACCCACAGC
AGTTCTCCACCTTTGGTTTTAAACCCAAGCATTGGGAACCGTTTGATGTGGCGATGATTTT
TGTCGGCACCATGGCGAACCGTTTTTCTGACAGCACCGCGAAATTGATAACCTGGCGCT
GCTGACGGCGCTAAAAGACAAATACGGCAAGCAGCAGGGCATGGCGGTCTTTAACCAGC
TGAAATGGCTGGTTAATCCTTCCGCGCCAACCACCATTTGCGGCGCGGGAAAGCGCCTATC
CGCTGAAGTTTGATCTGCAAAACACGCAAACGGCGTAA (SEQ ID NO:9)

SEQ ID NO:10 (PGA Variant 261 polypeptide sequence)

SNMWVIGKNKAQDAKAIMVNGPQFGWYNPAYTYGIGLHGAGYDVTGNTPFAYPGLLFGHN
GTISWGSTAGAGDVVDIFAEKLSAEKPGYYQHNGEWVKMLSRKETIAVKDGGPETFTVWRT
LHGNVIKTDATQTAYAKARAWDGKEVASLLAWTHQMKAKNWPEWTQQAQKQALTINW
YYADVNGNIGYVHTGAYPDRQPGHDPRLPVPGTGKWDWKGLLSFDLNPKVYNPQSGYIAN
WNNSPQKDYPASDLFAFLWGGADRATEIDTILDKQPRFTADQAWDVIRQTSRRDLNLRFLP
ALKDATANLAENDPRRQLVDKLASWDGENLVNDDGKTYQQPGSAILNAWLTSMLKRTVVA
AVPAPFGKWYSASGYETTQDGPTGSLNISVGAKILYEALQGDKSPIPQAVDLFGGKPPQEVIL
AALDDAWQTLISKRYGNDVTGWKTPAMALTFRANNFFGVPQAAAKEARHQAQYQNRGTEN

NMIVFSPTSGNRPVLAWDVVAPGQSGFIAPDGKADKHYDDQLKMYESFGRKSLWLTPQDVD
EHKESQEVQLVQLDQTEVKIVRDEYGMPHIYADDTYRLFYGYGYVVAQDRLFQMEMARRS
TQGTVSEVLGKAFVSFDKDIRQNYWPDSIRAQIASLSAEDKSILQGYADGMNAWIDKVNASP
DKLLPQQFSTFGFKPKHWEFPDVAMIFVGTMANRFS DSTSEIDNLALLTALKDKY GKQQGM
AVFNQLKWLNVNPSAPTTIAARESAYPLKFDLQNTQTA (SEQ ID NO:10)

SEQ ID NO:11 (PGA Variant 258 polynucleotide sequence)

AGCAATATGTGGGTGATTGGCAAAAACAAAGCCCAGGATGCGAAGGCCATTATGGTCAA
TGGGCCCGCAGTTTGGTTGGTATAATCCGGCGTATACCTACGGTATCGGCCCTGCACGGCGC
GGGCTATGACGTCACCGGCAATACGCCGTTTGCCTATCCGGGGCCTCCTTTTTGGTCACAA
CGGCACCATTTTCATGGGGATCCACCGCCGGTGCCGGTGATAGCGTCGATATCTTTGCCGA
AAAACTTTCCGCCGAGAAGCCGGGCTATTACCAGCATAACGGCGAGTGGGTGAAGATGT
TGAGCCGCAAGGAGACTATTGCGGTCAAAGACGGCCAGCCGGAGACCTTTACCGTTTGG
CGCACGCTGCACGGCAACGTCATTAAAACCGATACTGCGACGCAGACCGCCTATGCCAA
AGCGCGCGCCTGGGATGGCAAAGAGGTGGCGTCCCTGCTGGCGTGACGCACCAGATGA
AGGCCAAAAACTGGCCGGAGTGGACGCAGCAGGCGGCCAAACAGGCGCTGACCATCAA
CTGGTACTACGCCGATGTGAACGGCAATATCGGCTATGTGCATACCGGCGCCTATCCGGA
TCGCCAGCCCGGCCACGACCCGCGTTTGCCGGTTCCCGGCACTGGAAAATGGGACTGGA
AAGGGTTGCTGTCTGTTTGATTTGAATCCGAAAGTGTATAACCCGCAGTCGGGCTATATCG
CCAACTGGAACAACCTCGCCGCAAAAAGACTACCCGGCCTCTGATCTGTTTCGCGTTCCCTGT
GGGGCGGTGCGGATCGAGCGACTGAGATCGACACGATCCTCGATAAGCAACCGCGCTTC
ACCGCCGATCAGGCGTGGGATGTGATCCGCCAAACCAGCCGTCGGGATCTCAACCTGCG
GTTGTTCTTACCGGCGCTGAAGGACGCCACCGCCAACCTGGCGGAAAACGATCCGCGCC
GCCAACTGGTGGATAAACTGGCGAGCTGGGACGGCGAAAACCTTGTC AACGATGACGGA
AAAACCTATCAGCAACCGGGATCGGCGATTCTTAACGCCTGGCTGACCAGCATGCTCAA
GCGCACGGTGGTTGCCGCGGTCCCAGCGCCGTTTGGTAAGTGGTACAGCGCCAGTGGCT
ATGAAACCACCCAGGACGGGCCAACCGGCTCGCTGAACATCAGCGTGGGGGGCGAAAATC
CTCTACGAAGCTCTGCAGGGTGATAAGTCGCCAATCCCGCAGGCGGTGATCTGTTTGGC
GGGAAACCGCAGCAGGAAGTAATACTGGCGGCGCTGGACGACGCTTGGCAGACGCTGTC
AAAACGCTACGGTAACGACGTCACCGGCTGGAAAACCCCTGCCATGGCGCTTACCTTCC
GGGCCAATAACTTCTTCGGCGTGCCG CAGGCGGCAGCAAAAGAGGCGCGTCATCAGGCG
GAGTACCAGAACCGCGGTACGGAAAACAACATGATTGTCTTCTACCGACGTCGGGTAA
CCGCCCCGTTCTTGCTGGGATGTGGTGGCGCCGGGGCAAAGCGGTTTTATCGCGCCGGA
TGGCAAAGCCGATAAGCACTATGACGATCAGCTGAAAATGTACGAGAGCTTTGGCCGTA
AATCGCTGTGGTTAACGCCTCAGGACGTTGACGAGCACAAAGAGTCTCAGGAAGTGCTG
CAGGTACAGTTGGATCAGACCGAGGTAAAGATCGTTCGCGATGAATACGGCATGCCGCA
TATTTACGCCGATGATACCTATCGACTGTTTTACGGCTATGGCTACGTGGTGGCGCAGGA

TCGCCTGTTCCAGATGGAAATGGCGCGCCGCGAGTACTCAGGGGACCGTCTCCGAGGTGCT
 GGGCAAAGCATTTCGTTAAGTTTGATAAAGATATTCGCCAGAACTACTGGCCGGATTCTAT
 TCGCGCGCAGATAGCTTCCCTCTCCGCTGAGGATAAAATCCATTCTGCAGGGCTATGCCGA
 TGGCATGAATGCGTGGATCGATAAAGTGAACGCCAGCCCCGATAAGCTGTTACCCACAGC
 AGTTCTCCACCTTTTGGTTTTAAACCCAAGCATTGGGAACCGTTTGATGTGGCGATGATTTT
 TGTCGGCACCATGGCGAACCGTTTTTCTGACAGCACCAGCGAAATTGATAACCTGGCGCT
 GCTGACGGCGCTAAAAGACAAATACGGCAAGCAGCAGGGCATGGCGGTCTTTAACCAGC
 TGAAATGGCTGGTTAATCCTTCCGCGCCAACCACCATTGCGGCGCGGGAAAGCGCCTATC
 CGCTGAAGTTTGATCTGCAAAACACGCAAACGGCGTAA (SEQ ID NO:11)

SEQ ID NO:12 (PGA Variant 258 polypeptide sequence)

SNMWVIGKNKAQDAKAIMVNGPQFGWYNPAYTYGIGLHGAGYDVTGNTPFAYPGLLFGHN
 GTISWGSTAGAGDSVDIFAEKLSAEKPGYYQHNGEWVKMLSRKETIAVKDGPETFTVWRT
 LHGNVIKTDATQTAYAKARAWDGKEVASLLAWTHQMKAKNWPEWTQQAQKQALTINW
 YYADVNGNIGYVHTGAYPDRQPGHDPRLPVPGTGKWDWKGLLSFDLNPKVYNPQSGYIAN
 WNNSPQKDYPASDLFAFLWGGADRATEIDTILDKQPRFTADQAWDVIRQTSRRDLNLRLFLP
 ALKDATANLAENDPRRQLVDKLASWDGENLVNDDGKTYQQPGSAILNAWLTSMLKRTVVA
 AVPAFPGKWYSASGYETTQDGPTGSLNISVGAKILYEALQGDKSPIQAVDLFGGKPPQEVIL
 AALDDAWQTLISKRYGNDVTGWKTPAMALTFRANNFFGVPQAAAKEARHQAQYQNRGTEN
 NMIVFSPTSGNRPVLAWDVVAPGQSGFIAPDGKADKHYDDQLKMYESFGRKSLWLTPQDQD
 EHKESEVLQVQLDQTEVKIVRDEYGMPIHYADDTYRLFYGYGYVVAQDRLFQMEMARRS
 TQGTVSEVLGKAFVKFDKDIRQNYWPDSIRAQIASLSAEDKSILQGYADGMNAWIDKVNASP
 DKLLPQQFSTFGFKPKHWEFPDVAMIFVGTMANRFSDDSTSEIDNLALLTALKDKYQKQGM
 AVFNQLKWLNVNPSAPTTIAARESAYPLKFDLQNTQTA (SEQ ID NO:12)

EXAMPLE 1

***E. coli* Expression Hosts Containing Recombinant PGA Genes**

[0164] The initial PGA enzymes used to produce the variants of the present invention were obtained from the Codex[®] Acylase Panel (Codexis). The PGA panel plate comprises a collection of engineered PGA polypeptides that have improved properties, as compared to the wild-type *Khuyvera citrophila* PGA. The wild type PGA gene is a heterodimer consisting of alpha subunit (23.8 KDa) and beta subunit (62.2KDa) linked by 54aa spacer region. Due to the presence of spacer region, an autoprocessing step is required to form the active protein. The wild-type gene was modified to eliminate the spacer region thus eliminating the auto processing step. The Codex[®] Acylase Panel (Codexis) contains PGA variants that lack the spacer region (See e.g., US Pat. Appln. Publn. 2010/0143968 A1). The PGA-encoding genes were cloned into the expression vector pCK110900

(See, FIG. 3 of US Pat. Appln. Publn. No. 2006/0195947) operatively linked to the lac promoter under control of the lacI repressor. The expression vector also contains the P15a origin of replication and the chloramphenicol resistance gene. The resulting plasmids were transformed into *E. coli* W3110, using standard methods known in the art. The transformants were isolated by subjecting the cells to chloramphenicol selection, as known in the art (See e.g., US Pat. No. 8,383,346 and WO2010/144103).

EXAMPLE 2

Preparation of HTP PGA-Containing Wet Cell Pellets

[0165] *E. coli* cells containing recombinant PGA-encoding genes from monoclonal colonies were inoculated into 180µl LB containing 1% glucose and 30 µg/mL chloramphenicol in the wells of 96 well shallow well microtiter plates. The cultures were grown overnight at 30°C., 200 rpm and 85% humidity. Then, 10µl of each of the cell cultures were transferred into the wells of 96 well deep well plates containing 390 µL TB and 30 µg/mL CAM. The deep-well plates were incubated for 3 hours (OD600 0.6-0.8) at 30°C, 250 rpm and 85% humidity. The cell cultures were then induced by IPTG to a final concentration of 1 mM and incubated overnight under the same conditions as originally used. The cells were then pelleted using centrifugation at 4000 rpm for 10 min. The supernatants were discarded and the pellets frozen at -80°C prior to lysis.

EXAMPLE 3

Preparation and Analysis of HTP PGA-Containing Cell Lysates

[0166] First, 250µl lysis buffer containing 20 mM Tris-HCl buffer, pH 7.5, 1 mg/mL lysozyme, and 0.5 mg/mL PMBS was added to the cell paste in each well produced as described in Example 2. The cells were lysed at room temperature for 2 hours with shaking on a bench top shaker. The plate was then centrifuged for 15 min at 4000 rpm and 4° C. The clear supernatants used in biocatalytic reactions to determine their activity levels.

[0167] The activity of the PGA variants was evaluated based on the efficiency of the variant in removing the three phenyl acetate groups chemically attached to the A1 (glycine), B1 (phenylalanine), and B29 (Lysine) residues of insulin. HTP reactions were carried out in 96 well deep well plates. First, 0.3 ml of the reaction mixture contained 0.1M Tris-HCl, pH 8.0, 5g/L tri-protected insulin and HTP lysate between 25-125µl (depending on the linear curve). The HTP plates were incubated in Thermotron® shakers (3 mm throw, model #AJ185, Infors at 30°C, 300rpm), for 6 or 22 hrs. The reactions were quenched with 300µl acetonitrile and mixed for 3 mins using a bench top shaker. The plates were then centrifuged at 4000rpm for 2 mins and loaded into an HPLC for analysis. An Agilent eclipse XDB C18, 5µm, 2.1 x 150mm column was used to analyze the HTP samples. The flow rate was set to 0.5ml/min and the temperature was set to 50°C. Mobile phase A was water + 0.05% TFA, and mobile phase B was acetonitrile + 0.05% TFA. The runtime was 7.2 minutes, with injection

overlap enabled. The gradient was 75% mobile phase A in 0.2 minutes, 55% mobile phase A in 4.9 minutes, 5% mobile phase A in 5.4 minutes, back to 75% mobile phase A in 5.9 minutes.

EXAMPLE 4

Preparation and Analysis of Lyophilized Lysates from Shake Flask (SF) Cultures

[0168] Selected HTP cultures grown as described above were plated onto LB agar plates with 1% glucose and 30 µg/ml CAM and grown overnight at 37 °C. A single colony from each culture was transferred to 50 ml of LB with 1% glucose and 30 µg/ml CAM. The cultures were grown for 18 h at 30°C, 250 rpm, and subcultured approximately 1:10 into 250 ml of TB containing 30 µg/ml CAM, to a final OD₆₀₀ of 0.2. The cultures were grown for 135 minutes at 30°C, 250 rpm, to an OD₆₀₀ between 0.6-0.8 and induced with 1 mM IPTG. The cultures were then grown for 20 h at 30°C, 250 rpm. The cultures were centrifuged 4000 rpm x 20 min. The supernatant was discarded, and the pellets were resuspended in 30 ml of 50 mM sodium phosphate, pH 7.5. The cells were pelleted (4000 rpm x 20 min) and frozen at -80°C for 120 minutes. Frozen pellets were resuspended in 30 ml of 50 mM sodium phosphate pH 7.5, and lysed using a Microfluidizer system (Microfluidics) at 18,000 psi. The lysates were pelleted (10,000 rpm x 60 min) and the supernatants were frozen and lyophilized to generate shake flask (SF) enzymes.

[0169] The activity of selected shake flask PGA variants was evaluated based on the efficiency of the variants in removing the three phenyl acetate groups chemically attached to the A1 (glycine), B1 (phenylalanine), and B29 (lysine) residues of insulin. The shake flask reactions were carried out in 96 well deep well plates. First, 0.3ml of the reaction mixture contained 0.1M Tris-HCl, pH 8.0, 5g/L tri-protected insulin, shake flask lysate between 0.1-0.8g/L. The deep well reaction plates were incubated in Thermotron® shakers (3 mm throw, model # AJ185, Infors) at 30°C, 300rpm, for 22 or 6 hrs (22hrs for round 1 evolution and 6 hrs for round 2 evolution). The reactions were quenched with 300µl acetonitrile and mixed for 3 mins on a bench top shaker. The plates were then centrifuged at 4000rpm for 2 mins and loaded into HPLC for analysis. An Agilent eclipse XDB C18, 5µm, 2.1 x 150mm columns was used to analyze the HTP samples. The flow rate was set to 0.6ml/min and the temperature was set to 50°C. Mobile phase A was water + 0.05% TFA and mobile phase B was acetonitrile + 0.05% TFA. The runtime was 18.2 minutes with injection overlap enabled. The gradient was 80% mobile phase A in 0-1 minutes, 60% mobile phase A in 12 minutes, 5% mobile phase A in 15 minutes, back to 80% mobile phase A in 16 minutes.

EXAMPLE 5

Round 1 Evolution Backbone Selection, Construction and Screening

[0170] Following the HTP protocol described in the above Examples, the Codex® Acylase Panel (Codexis) was evaluated using the shake flask protocols described in Example 4. One of the variants, designated as "Variant 1" from the Codex® Acylase Panel (SEQ ID NO:4) generated 54% free insulin

with 0.8g/L shake flask lysate loading in 22 hrs. A substrate inhibition study was conducted using Variant 1 (See, Figure 1). With the increasing concentration of the tri-protected insulin substrate, generation of free insulin catalyzed by Variant 1 decreased significantly. In 5 hrs., free insulin generation dropped from 82% with 1g/L substrate loading to 2% with 10g/L substrate loading at a fixed enzyme loading of 0.8g/L. While it is not intended that the present invention be limited to any particular mechanism, these results suggest that higher concentrations of the tri-protected insulin substrate caused substrate inhibition. The amount of free insulin production increased at lower substrate concentration (See e.g., Wang et al., Biopolymer 25:S109-S114 [1986]). Thus, one of the advantages provided by the present invention is the production of PGA variants that overcome substrate inhibition. This Variant was chosen as the backbone for the first round evolution. A homology model of Variant 1 was built using *E. coli* PGA as template (the *E. coli* PGA has 87% sequence identity to the wild-type *K. citrophila* PGA). Tri-protected insulin was docked into the active site of Variant 1 to assess its interaction with PGA. Then, 96 positions in its amino acid sequence were chosen for the first round of evolution, covering the 1st layer (amino acid residues within 5-6Å) and part of the 2nd layer (amino acid residues within 6-12Å) of the active site and tri-protected insulin binding site. Two combinatorial libraries were also designed, based on an analysis of the PGA panel screening results and consensus mutations. All of the HTP screenings for the variants obtained in this first round of evolution were conducted using the same protocols as described above, with the final reaction time point being 22 hrs. Active mutations with corresponding fold-improvement in total activity over Variant 1 are shown in Table 5.1, below. In this Table, the positive control is Variant 1 (SEQ ID NO:4).

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants

Variant #	FIOPC	Active Mutations (as compared to Variant 1)
2	+++	V56I;V264A;A308T;T379A;D484N
3	—	G71F;D74G
4	+	Q547K;L711Q;S750G
5	—	G71F;D74G
6	+++	V264A;D484N;Q547K
7	—	G71F;D74G
8	—	G71F;D74G
9	++	V56I;A308T;T379A;D484N
10	—	F367S;L754P
11	+	V56I
12	—	G71F;D74G
13	++	D484N

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants		
Variant #	FIOPC	Active Mutations (as compared to Variant 1)
14	+++	V264A;A308T;T379A;D484N;Q547K
15	+	L754P
16	—	G71F;D74G
17	—	V264A;A308T;L711Q
18	—	G71F;D74G
19	+++	V264A;T379A;D484N;S750G;L754P
20	+	T379A;Q547K
21	++	Q547K
22	++	V56I;T379A;S750G
23	—	Q547K;L711Q
24	—	G71F;D74G
25	—	Q547K;L711Q
26	—	G71F;D74G
27	++	V56I;D484N;L711Q
28	+	Q547K;L754P
29	—	G71F;D74G
30	+	Y27F;T131N;G368D;S619K
31	—	G71F;D74G
32	+	Y27F;V28N;G368D;S372L;A616D;V618I;S619K
33	—	G71F;D74G
34	+	V28N;T131N
35	+	Y27F;G368D;S372L;S619K
36	+	Y27F;S372L;V618I;S619K
37	+	Y27F;V56L;I127V;S372L;S619K
38	+	Y27F;V28N;Q134H;A616D;S619K
39	++	I127V;Q134H;G368D;V618I;S619K
40	+	Y27F;I127V;T131N;A616D;V618I;S619K
41	+	T131N;S372L
42	—	G71F;D74G
43	+	V56L;I127V;T131N;Q134H;G368D;A616D
44	—	G71F;D74G
45	—	G71F;D74G
46	++	Y27F;V56L;G368D;S372L

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants		
Variant #	FIOPC	Active Mutations (as compared to Variant 1)
47	+	V28N;I127V;T131N;G368D;S372L
48	++	V28N;G368D;A616D;V618I;S619K
49	+	V28N;V56L;S372L
50	+	V28N;A616D;S619K
51	+	Q134H
52	++	Y27F;V28N;T131N;Q134H;G368D;A616D;V618I;S619K
53	++	Y27F;V28N;V56L
54	--	G71F;D74G
55	+	V56L;Q134H;G368D;A616D
56	--	G71F;D74G
57	++	Y27F;V28N;T131N;G368D;S619K
58	--	G71F;D74G
59	++	Y27F;S619K
60	--	G71F;D74G
61	++	Y27F;V28N;V56L;T131N;Q134H;S619K
62	+	Y27F;G368D;S372L;V618I;S619K
63	--	G71F;D74G
64	+	V56L;G368D;A616D;V618I
65	+	Y27F;V28N;I127V;T131N;G368D;A616D;V618I;S619K
66	+	V28N;T131N;S372L
67	+++	V28N;S619K
68	+	V56L;S372L;A616D;S619K
69	+++	Y27F;V28N;V56L;I127V;Q134H;G368D;S372L;S619K
70	++	V28N;T131N
71	+++	Y27F;V56L;I127V;V618I;S619K
72	+	I127V;T131N
73	--	G71F;D74G
74	++	Y27F;V28N;A616D;V618I;S619K
75	++	V56L
76	++	Y27F;V28N;V56L;G368D
77	--	G71F;D74G
78	+	Y27F;V56L;S372L
79	++	S619K

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants		
Variant #	FIOPC	Active Mutations (as compared to Variant 1)
80	--	G71F;D74G
81	+	Y27F;V28N;I127V;Q134H;A616D;S619K
82	+	V56L;I127V;T131N;S325R;V618I;S619K
83	+	Y27F;V56L;I127V;Q134H;G368D;S372L
84	+	Y27F;I127V;T131N;G368D
85	++	Y27F;I127V;G368D;S372L;V618I;S619K
86	+	V28N
87	+	Y27F;V56L;I127V;Q134H;S372L;V618I;S619K
88	+++	Y27F;V28N;V56L;V618I
89	--	G71F;D74G
90	+	G368D;S372L;V618I;S619K
91	--	G71F;D74G
92	++	D74P
93	++	G71A;D74S
94	++	G71A;D74V
95	++	G71A;D74V
96	+	D74A
97	+++	G71A;D74V
98	++	D74S
99	++	D74T
100	+++	G71A;D74N
101	+++	D74S
102	++	D74G
103	++	D74G
104	+++	D74N
105	++	G71A;D74A
106	++	D74V
107	+++	G71A;D74S
108	+++	D74N
109	++	D74G
110	+++	D74S
111	++	D74P
112	++	D74N

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants		
Variant #	FIOPC	Active Mutations (as compared to Variant 1)
113	++	D74N
114	+	P49H;M697R
115	+	D74H
116	+	T32D
117	+	P418Q
118	+	V56I;D74L
119	+++	D381F
120	+++	K369C
121	+++	N388G
122	+++	D381K
123	++	A255G
124	++	L253S
125	++	Y27T;
126	++	D381W
127	++	N348R
128	++	F254W
129	++	D381W
130	++	A373L
131	++	L257N
132	++	L253V
133	++	W370I
134	++	A255L
135	++	A373L
136	++	T384R
137	++	Q380R
138	++	D381M
139	++	T384P
140	++	D381I
141	++	W240F
142	++	D381Q
143	++	L387I
144	++	D381G
145	++	L253W

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants		
Variant #	FIOPC	Active Mutations (as compared to Variant 1)
146	++	A255M
147	++	V360A
148	++	W370F
149	++	N348H
150	++	L253W
151	++	N388S
152	++	D381C
153	++	A255R
154	++	L253F
155	++	N348R
156	++	D381L
157	++	A255V
158	++	A255Y
159	++	A132R
160	++	A255L
161	+	F454Y
162	+	T129W
163	+	N348H
164	+	L387Q
165	+	D381M
166	+	L387F
167	+	L387M
168	+	S372A
169	+	D381C
170	+	T379S
171	+	D381P
172	+	L387G
173	+	L387S
174	+	N348D
175	+	T378Q
176	+	T379S
177	+	I389P
178	+	N348S

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants		
Variant #	FIOPC	Active Mutations (as compared to Variant 1)
179	+	T129W
180	+	Q380K
181	+	T379C
182	+	A373L
183	+	T384G
184	+	T379R
185	+	T384H
186	+	D381R
187	+	D381Q
188	+	A132R
189	+	L253T
190	+	N348E
191	+	T129K
192	+	D381Y
193	+	D381I
194	+	A255L
195	+	A373Y
196	+	T133N
197	+	V126I
198	+	A365M
199	+	D381C
200	+	N348K
201	+	T378C
202	+	Y27H
203	+	A373L
204	+	D130E
205	+	L387C
206	+	A456T
207	+	H156R
208	+	T131R
209	+	T384C
210	+	D381R
211	+	T453R

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants		
Variant #	FIOPC	Active Mutations (as compared to Variant 1)
212	+	T133C
213	+	A255V
214	+	Y27G
215	+	Q380I
216	+	L387E
217	+	A255S
218	+	T379L
219	+	T133G;L557S
220	+	D381V
221	+	L387T
222	+	T133G
223	+	T133R
224	+	V391N
225	+	Q134S
226	+	T384N
227	+	T131D
228	+	D381C
229	+	A373L
230	+	T384F
231	+	T133S
232	+	L387H
233	+	F256Y
234	+	T133S
235	+	L387E
236	+	T384A
237	+	A132T
238	+	T379G
239	+	W370V
240	+	L387F
241	+	F116I
242	+	T384R;F596L
243	+	V391P
244	+	T133A

Table 5-1. Active Mutations and FIOPC Results for Round 1 Variants		
Variant #	FIOPC	Active Mutations (as compared to Variant 1)
245	+	Y27V
246	+	A160S
247	+	L387E
248	+	N348S
249	+	L257R
250	+	A373Q
251	+	T133Q
252	+	W26F
253	+	Q380C
254	+	A255F

“-”: FIOPC<0.7

“+” : FIOPC = 0.7 to 1.3

“++”: FIOPC = 1.4 to 2.0

“+++”: FIOPC≥2.1

[0171] Based on these results, Variants 6, 19, 14, 67, 88, and 53 were scaled up to shake flask volumes and their activities were analyzed using the protocols described in the previous Examples. The results are shown in Figure 2. Variant 6 generated 93% free insulin in 22 hrs at 0.8g/L enzyme loading (See, Figure 2) and achieved a better expression level in comparison with Variant 1. Variant 53 had a similar expression level as Variant 1, but generated 73% free insulin compared to 54% generated by Variant 1. Based on the results, Variant 6 (SEQ ID NO:6) was selected as a starting backbone for the next round (i.e., round 2) of evolution. This variant was also designated as an “expression hit.” Variant 53 (SEQ ID NO:8) was also selected as an alternative round 2 backbone and was designated as an “activity hit.”

EXAMPLE 6

Round2 Library Construction and Screening

[0172] The most beneficial mutations identified from round1 evolution were D484N, V264A, Q547K, V56I, S750G, V56L, S619K, V28N, V618I, and T131N. Two combinatorial libraries were designed, based on analysis of the first round results, using both Variant 6 and Variant 53 as backbones. All of the HTP screening methods used for the round 2 variants were conducted using the previously described protocols, with the final reaction time point being 6 hrs. Active mutations with

corresponding fold improvement in total activity over Variant 6 and Variant 53 are shown in Tables 6.1 and 6.2, below. Table 6.1 provides the results for variants based on Variant 6, while Table 6.2 provides the results for variants based on Variant 53.

Table 6.1 Active Mutations and FIOPC Results for Round 2 Variants Based on SEQ ID NO:6 (Variant 6)		
Variant #	FIOPC	Active Mutations (as compared to Variant 6)
255	+++	V28N;G71A;D74V;S619K
256	+++	V28N;G71A;D74V;S619K
257	+++	G71A;D74N;S619K
258	+++	V28N;V56L;G71A;D74S;S619K
259	++	G71A;D74V;V618I;S619K
260	++	G71A;D74S;S619K
261	++	V28N;V56L;G71A;D74V
262	++	V28N;G71A;D74N;S619K
263	++	G71A;D74V;S619K
264	++	G71A;D74V
265	++	G71A;D74V
266	++	G71A;D74S
267	++	S619K
268	++	V28N;S619K
269	++	V28N;S619K
270	++	V28N;S619K
271	++	V28N;S619K
272	++	V28N;G71A;D74S
273	++	S619K
274	++	D74V;S619K
275	++	G71A;S619K
276	++	S619K
277	++	V28N;S619K
278	++	S619K
279	++	S619K
280	++	S619K
281	++	S619K

Table 6.1 Active Mutations and FIOPC Results for Round 2 Variants Based on SEQ ID NO:6 (Variant 6)		
Variant #	FIOPC	Active Mutations (as compared to Variant 6)
282	++	S619K
283	+	V28N
284	+	V28N
285	+	V618I;S619K
286	+	D74V
287	+	S619K
288	+	S619K
289	+	S619K
290	+	G71A
291	—	D74T;K139I;S619K
292	—	R455W

“—”: FIOPC<0.7

“++”: FIOPC = 0.7 to 1.3

“++”: FIOPC = 1.4 to 2.0

“+++”: FIOPC≥2.1

Table 6.2 Active Mutations and FIOPC Results for Round 2 Variants Based on SEQ ID NO:8 (Variant 53)		
Variant #	FIOPC	Active Mutations (as compared to Variant 53)
293	+++	G71A;D74V;D484N;Q547K
294	+++	G71A;D74S;D484N;Q547K
295	+++	D484N;Q547K;S619K
296	+++	G71A;D74S;D484N;Q547K
297	+++	D74N;D484N
298	+++	G71A;D484N;Q547K;V618I;S619K
299	+++	D74N;V264A;D484N;Q547K
300	+++	D74T;D484N
301	++	G71A;V264A;D484N
302	++	V264A;D484N;Q547K

Table 6.2 Active Mutations and FIOPC Results for Round 2 Variants Based on SEQ ID NO:8 (Variant 53)		
Variant #	FIOPC	Active Mutations (as compared to Variant 53)
303	++	V264A;D484N
304	++	L56I;G71A;D74S;V264A;D484N;Q547K;S619K
305	++	D74S;D484N;V618I;S619K
306	++	D484N
307	++	D484N;Q547K
308	++	G71A;D74N;D484N;V618I
309	++	D484N;Q547K
310	++	F27Y;N28V;D484N;Q547K;V618I
311	++	D484N;S619K
312	++	D484N;Q547K;V618I;S619K
313	++	D484N;V618I
314	++	D74N;Q547K
315	++	Q547K;V618I;S619K
316	++	D74N;V264A
317	++	Q547K;S619K
318	++	G71A;D74V;V264A;Q547K;V618I
319	+	Q547K;S619K
320	+	V264A;V618I;S619K
321	+	Q547K;V618I
322	+	D74V;Q547K;S619K;A741T
323	+	S619K
324	+	V264A;Q547K
325	+	Q547K;V618I;S619K
326	+	Q547K
327	+	D74V
328	+	D74T
329	+	V618I;S619K
330	+	V264A;S619K
331	+	G71A;V264A;Q547K
332	+	V618I
333	—	V264A

“—”: FIOPC<0.7

“++”: FIOPC = 0.7 to 1.3

“++”: FIOPC = 1.4 to 2.0

“+++”: FIOPC ≥ 2.1

[0173] Variants 6 (SEQ ID NO:6), 258 (SEQ ID NO:12) and 261 (SEQ ID NO:10) were scaled up in shake flasks and their activities analyzed using the protocols described in the previous Examples. The results are shown in Figure 3. As indicated, Variant 258 generated >99% free insulin in 6 hrs in <0.8g/L shake flask lysate loading, thereby completely releasing substrate inhibition, whereas Variant 261 generated ~90% free insulin in 6 hrs at 0.8g/L lysate loading.

[0174] Thus, the present invention provides PGA variants having an 8x fold improvement in total activity. It was also determined that the S619K substitution largely impacts activity whereas the D484N substitution predominantly impacts expression. S619K is located in the 1st layer of insulin binding site and D484N is located in the 2nd layer of the active site.

EXAMPLE 7

DMSO Tolerance

[0175] A DMSO tolerance study was conducted on the improved PGA variants (Variant Nos. 6, 258, and 261). The reactions were carried out in presence of 0-50% v/v DMSO following the protocol described in Example 4. The results (See, Figure 4) indicate that all the variants tested lost activity upon addition of DMSO to the test reactions. For example, at 30%v/v DMSO, only 30% free insulin was generated by Variant 258.

[0176] All publications, patents, patent applications and other documents cited in this application are hereby incorporated by reference in their entireties for all purposes to the same extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference for all purposes.

[0177] While various specific embodiments have been illustrated and described, it will be appreciated that various changes can be made without departing from the spirit and scope of the invention(s).

SEQUENCE LISTING

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<120> Penicillin-G Acylases

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<150> US 62/158,118

<151> 2015-05-07

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Arg Leu Phe Gln Met Glu Met Ala Arg Arg Ser Thr Gln Gly Thr Val
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Ser Glu Val Leu Gly Lys Ala Phe Val Ser Phe Asp Lys Asp Ile Arg
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Gln Asn Tyr Trp Pro Asp Ser Ile Arg Ala Gln Ile Ala Ser Leu Ser
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Ala Glu Asp Lys Ser Ile Leu Gln Gly Tyr Ala Asp Gly Met Asn Ala
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Trp Ile Asp Lys Val Asn Ala Ser Pro Asp Lys Leu Leu Pro Gln Gln
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Phe Ser Thr Phe Gly Phe Lys Pro Lys His Trp Glu Pro Phe Asp Val
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Ala Met Ile Phe Val Gly Thr Met Ala Asn Arg Phe Ser Asp Ser Thr
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Ser Glu Ile Asp Asn Leu Ala Leu Leu Thr Ala Leu Lys Asp Lys Tyr
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Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu Val
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Tyr Asp Gln Pro Ala Pro Met Leu Asp Arg Pro Ala Lys Gly Thr Asp
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Gly Ala Leu Leu Ala Leu Thr Ala Asp Gln Asn Arg Glu Thr Ile Ala
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Ala Ile Met Val Asn Gly Pro Gln Phe Gly Trp Tyr Ala Pro Ala Tyr
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Thr Tyr Gly Ile Gly Leu His Gly Ala Gly Tyr Asp Val Thr Gly Asn
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Thr Pro Phe Ala Tyr Pro Gly Leu Val Phe Gly His Asn Gly Thr Ile
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Ser Trp Gly Ser Thr Ala Gly Phe Gly Asp Asp Val Asp Ile Phe Ala
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Glu Lys Leu Ser Ala Glu Lys Pro Gly Tyr Tyr Gln His Asn Gly Glu
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Gln Pro Glu Thr Phe Thr Val Trp Arg Thr Leu His Gly Asn Val Ile
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Lys Thr Asp Thr Ala Thr Gln Thr Ala Tyr Ala Lys Ala Arg Ala Trp
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Asp Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr His Gln Met Lys
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Pro Val Pro Gly Thr Gly Lys Trp Asp Trp Lys Gly Leu Leu Ser Phe
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Trp Asn Asn Ser Pro Gln Lys Asp Tyr Pro Ala Ser Asp Leu Phe Ala
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Gln Ala Ala Ala Lys Glu Ala Arg His Gln Ala Glu Tyr Gln Asn Arg
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Pro Phe Ala Tyr Pro Gly Leu Val Phe Gly His Asn Gly Thr Ile Ser
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Trp Gly Ser Thr Ala Gly Gly Gly Asp Asp Val Asp Ile Phe Ala Glu
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Lys Leu Ser Ala Glu Lys Pro Gly Tyr Tyr Gln His Asn Gly Glu Trp
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Val Lys Met Leu Ser Arg Lys Glu Thr Ile Ala Val Lys Asp Gly Gln
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Pro Glu Thr Phe Thr Val Trp Arg Thr Leu His Gly Asn Val Ile Lys
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Thr Asp Thr Ala Thr Gln Thr Ala Tyr Ala Lys Ala Arg Ala Trp Asp
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Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr His Gln Met Lys Ala
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Lys Asn Trp Pro Glu Trp Thr Gln Gln Ala Ala Lys Gln Ala Leu Thr
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Ile Asn Trp Tyr Tyr Ala Asp Val Asn Gly Asn Ile Gly Tyr Val His
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Thr Gly Ala Tyr Pro Asp Arg Gln Pro Gly His Asp Pro Arg Leu Pro
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Val Pro Gly Thr Gly Lys Trp Asp Trp Lys Gly Leu Leu Ser Phe Asp
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Asp Ala Thr Ala Asn Leu Ala Glu Asn Asp Pro Arg Arg Gln Leu Val
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Asp Lys Leu Ala Ser Trp Asp Gly Glu Asn Leu Val Asn Asp Asp Gly
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Lys Thr Tyr Gln Gln Pro Gly Ser Ala Ile Leu Asn Ala Trp Leu Thr
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Lys Trp Tyr Ser Ala Ser Gly Tyr Glu Thr Thr Gln Asp Gly Pro Thr
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Ser Ala Glu Asp Lys Ser Ile Leu Gln Gly Tyr Ala Asp Gly Met Asn
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Thr Ser Glu Ile Asp Asn Leu Ala Leu Leu Thr Ala Leu Lys Asp Lys
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Tyr Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu
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Tyr Gly Ile Gly Leu His Gly Ala Gly Tyr Asp Val Thr Gly Asn Thr
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Pro Phe Ala Tyr Pro Gly Leu Val Phe Gly His Asn Gly Thr Ile Ser
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Trp Gly Ser Thr Ala Gly Gly Gly Asp Asp Val Asp Ile Phe Ala Glu
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Lys Leu Ser Ala Glu Lys Pro Gly Tyr Tyr Gln His Asn Gly Glu Trp

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90

95

Val Lys Met Leu Ser Arg Lys Glu Thr Ile Ala Val Lys Asp Gly Gln
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Pro Glu Thr Phe Thr Val Trp Arg Thr Leu His Gly Asn Val Ile Lys
 115 120 125

Thr Asp Thr Ala Thr Gln Thr Ala Tyr Ala Lys Ala Arg Ala Trp Asp
 130 135 140

Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr His Gln Met Lys Ala
 145 150 155 160

Lys Asn Trp Pro Glu Trp Thr Gln Gln Ala Ala Lys Gln Ala Leu Thr
 165 170 175

Ile Asn Trp Tyr Tyr Ala Asp Val Asn Gly Asn Ile Gly Tyr Val His
 180 185 190

Thr Gly Ala Tyr Pro Asp Arg Gln Pro Gly His Asp Pro Arg Leu Pro
 195 200 205

Val Pro Gly Thr Gly Lys Trp Asp Trp Lys Gly Leu Leu Ser Phe Asp
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Leu Asn Pro Lys Val Tyr Asn Pro Gln Ser Gly Tyr Ile Ala Asn Trp
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Asn Asn Ser Pro Gln Lys Asp Tyr Pro Ala Ser Asp Leu Phe Ala Phe
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Leu Trp Gly Gly Ala Asp Arg Ala Thr Glu Ile Asp Thr Ile Leu Asp
 260 265 270

Lys Gln Pro Arg Phe Thr Ala Asp Gln Ala Trp Asp Val Ile Arg Gln
 275 280 285

Thr Ser Arg Arg Asp Leu Asn Leu Arg Leu Phe Leu Pro Ala Leu Lys
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Asp Ala Thr Ala Asn Leu Ala Glu Asn Asp Pro Arg Arg Gln Leu Val
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Asp Lys Leu Ala Ser Trp Asp Gly Glu Asn Leu Val Asn Asp Asp Gly
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Lys Thr Tyr Gln Gln Pro Gly Ser Ala Ile Leu Asn Ala Trp Leu Thr
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Ser Met Leu Lys Arg Thr Val Val Ala Ala Val Pro Ala Pro Phe Gly
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Lys Trp Tyr Ser Ala Ser Gly Tyr Glu Thr Thr Gln Asp Gly Pro Thr
370 375 380

Gly Ser Leu Asn Ile Ser Val Gly Ala Lys Ile Leu Tyr Glu Ala Leu
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Gln Gly Asp Lys Ser Pro Ile Pro Gln Ala Val Asp Leu Phe Gly Gly
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Lys Pro Gln Gln Glu Val Ile Leu Ala Ala Leu Asp Asp Ala Trp Gln
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Glu Ser Phe Gly Arg Lys Ser Leu Trp Leu Thr Pro Gln Asp Val Asp
530 535 540

Glu His Lys Glu Ser Gln Glu Val Leu Gln Val Gln Leu Asp Gln Thr
545 550 555 560

Glu Val Lys Ile Val Arg Asp Glu Tyr Gly Met Pro His Ile Tyr Ala
565 570 575

Asp Asp Thr Tyr Arg Leu Phe Tyr Gly Tyr Gly Tyr Val Val Ala Gln
580 585 590

Asp Arg Leu Phe Gln Met Glu Met Ala Arg Arg Ser Thr Gln Gly Thr
595 600 605

Val Ser Glu Val Leu Gly Lys Ala Phe Val Ser Phe Asp Lys Asp Ile
610 615 620

Arg Gln Asn Tyr Trp Pro Asp Ser Ile Arg Ala Gln Ile Ala Ser Leu
625 630 635 640

Ser Ala Glu Asp Lys Ser Ile Leu Gln Gly Tyr Ala Asp Gly Met Asn
645 650 655

Ala Trp Ile Asp Lys Val Asn Ala Ser Pro Asp Lys Leu Leu Pro Gln
660 665 670

Gln Phe Ser Thr Phe Gly Phe Lys Pro Lys His Trp Glu Pro Phe Asp
675 680 685

Val Ala Met Ile Phe Val Gly Thr Met Ala Asn Arg Phe Ser Asp Ser
690 695 700

Thr Ser Glu Ile Asp Asn Leu Ala Leu Leu Thr Ala Leu Lys Asp Lys
705 710 715 720

Tyr Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu
725 730 735

Val Asn Pro Ser Ala Pro Thr Thr Ile Ala Ala Arg Glu Ser Ala Tyr
740 745 750

Pro Leu Lys Phe Asp Leu Gln Asn Thr Gln Thr Ala
755 760

<210> 7
<211> 2295
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic PGA nucleotide sequence for variant 53

<400> 7
agcaatatgt ggggtgattgg caaaaacaaa gcccaggatg cgaaggccat tatggtcaat 60
gggccgcagt ttggttggtt taatccggcg tacacctacg gtatcggcct gcacggcgcg 120
ggctatgacg tcaccggcaa tacgccgttt gcctatccgg gcctcctggt tggtcacaac 180
ggcaccattt catggggatc caccgccggt ggtggatgat atgtcgatat ctttgccgaa 240
aaactttccg ccgagaagcc gggctattac cagcataacg gcgagtgggt gaagatgttg 300
agccgcaagg agactattgc ggtcaaagac ggccagccgg agacctttac cgtttggcgc 360
acgctgcacg gcaacgtcat taaaaccgat actgcgacgc agaccgccta tgccaaagcg 420
cgcgcctggg atggcaaaga ggtggcgtcc ctgctggcgt ggacgcacca gatgaaggcc 480
aaaaactggc cggagtggac gcagcaggcg gccaaacagg cgctgaccat taactggtac 540
tacgccgatg tgaacggcaa tatcggctat gtgcataccg gcgcctatcc ggatcgccag 600
cccggccacg acccgctttt gccggttccc ggcactggaa aatgggactg gaaagggttg 660
ctgtcgtttg atttgaatcc gaaagtgtat aaccgcagc cgggctatat cgccaactgg 720
aacaactcgc cgcaaaaaga ctaccggcc tctgatctgt tcgcgttcct gtggggcggt 780
gcggatcgag ttactgagat cgacacgatc ctcgataagc aaccgcgctt caccgccgat 840
caggcgtggg atgtgatccg ccaaaccagc cgtcgggatc tcaacctgcg gttgttctta 900
ccggcgctga aggacgccac cgcgaacctg gcggaaaacg atccgcgccg ccaactggtg 960

gataaaactgg cgagctggga cggcgaaaac cttgtcaacg atgacggaaa aacctatcag	1020
caaccgggat cggcgattct taacgcctgg ctgaccagca tgctcaagcg cacggtggtt	1080
gccgcggtcc cagcgccgtt tggtaagtgg tacagcgcca gtggctatga aaccaccag	1140
gacgggcca cggctcgt gaacatcagc gtgggggcga aaatcctcta cgaagctctg	1200
cagggtgata agtcgccaat cccgcaggcg gtcgatctgt ttggcgggaa accgcagcag	1260
gaagtaatac tggcggcgct ggacgacgct tggcagacgc tgtcaaaacg ctacggtaac	1320
gacgtcaccg gctggaaaac ccctgccatg gcgcttacct tccgggcca taacttcttc	1380
ggcgtgccgc aggcggcagc aaaagaggcg cgtcatcagg cggagtacca gaaccgcggt	1440
acggaaaacg acatgattgt cttctcaccg acgtcgggta accgcccgggt tcttgccctgg	1500
gatgtggtgg cgccggggca aagcggtttt atcgcgccgg atggcaaagc cgataagcac	1560
tatgacgatc agctgaaaat gtacgagagc tttggccgta aatcgctgtg gttaacgcct	1620
caggacgttg acgagcacca agagtctcag gaagtgtgc aggtacagtt ggatcagacc	1680
gaggttaaga tcgttcgca tgaatacggc atgccgcata ttacgccga tgatacctat	1740
cgactgtttt acggctatgg ctacgtggtg gcgcaggatc gcctgttcca gatggaaatg	1800
gcgcgccgca gtactcaggg gaccgtctcc gaggtgctgg gcaaagcttt cgtttctttt	1860
gataaagata ttcgccagaa ctactggccg gattctattc gcgcgcagat agcttccttc	1920
tccgctgagg ataaatccat tctgcagggc tatgccgatg gcatgaatgc gtggatcgat	1980
aaagtgaacg ccagccccga taagctgtta cccagcagt tctccacctt tggttttaaa	2040
ccaagcatt gggaaccgtt tgatgtggcg atgatttttg tcggcaccat ggcgaaccgt	2100
ttctctgaca gcaccagcga aattgataac ctggcgctgc tgacggcgct aaaagacaaa	2160
tacggcaagc agcagggcat ggcggtcttt aaccagctga aatggctggt taatccttcc	2220
gcgccaacca ccattgcggc gcgggaaagc gcctatccgc tgaagtttga tctgcaaaac	2280
acgcaaacgg cgtaa	2295

<210> 8

<211> 764

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic PGA protein sequence for variant 5

<400> 8

Ser Asn Met Trp Val Ile Gly Lys Asn Lys Ala Gln Asp Ala Lys Ala
1 5 10 15

Ile Met Val Asn Gly Pro Gln Phe Gly Trp Phe Asn Pro Ala Tyr Thr
20 25 30

Tyr Gly Ile Gly Leu His Gly Ala Gly Tyr Asp Val Thr Gly Asn Thr
35 40 45

Pro Phe Ala Tyr Pro Gly Leu Leu Phe Gly His Asn Gly Thr Ile Ser
50 55 60

Trp Gly Ser Thr Ala Gly Gly Gly Asp Asp Val Asp Ile Phe Ala Glu
65 70 75 80

Lys Leu Ser Ala Glu Lys Pro Gly Tyr Tyr Gln His Asn Gly Glu Trp
85 90 95

Val Lys Met Leu Ser Arg Lys Glu Thr Ile Ala Val Lys Asp Gly Gln
100 105 110

Pro Glu Thr Phe Thr Val Trp Arg Thr Leu His Gly Asn Val Ile Lys
115 120 125

Thr Asp Thr Ala Thr Gln Thr Ala Tyr Ala Lys Ala Arg Ala Trp Asp
130 135 140

Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr His Gln Met Lys Ala
145 150 155 160

Lys Asn Trp Pro Glu Trp Thr Gln Gln Ala Ala Lys Gln Ala Leu Thr
165 170 175

Ile Asn Trp Tyr Tyr Ala Asp Val Asn Gly Asn Ile Gly Tyr Val His
180 185 190

Thr Gly Ala Tyr Pro Asp Arg Gln Pro Gly His Asp Pro Arg Leu Pro

195	200	205														
Val	Pro	Gly	Thr	Gly	Lys	Trp	Asp	Trp	Lys	Gly	Leu	Leu	Ser	Phe	Asp	
210						215					220					
Leu	Asn	Pro	Lys	Val	Tyr	Asn	Pro	Gln	Ser	Gly	Tyr	Ile	Ala	Asn	Trp	
225					230					235					240	
Asn	Asn	Ser	Pro	Gln	Lys	Asp	Tyr	Pro	Ala	Ser	Asp	Leu	Phe	Ala	Phe	
				245					250					255		
Leu	Trp	Gly	Gly	Ala	Asp	Arg	Val	Thr	Glu	Ile	Asp	Thr	Ile	Leu	Asp	
			260					265					270			
Lys	Gln	Pro	Arg	Phe	Thr	Ala	Asp	Gln	Ala	Trp	Asp	Val	Ile	Arg	Gln	
		275					280					285				
Thr	Ser	Arg	Arg	Asp	Leu	Asn	Leu	Arg	Leu	Phe	Leu	Pro	Ala	Leu	Lys	
	290					295					300					
Asp	Ala	Thr	Ala	Asn	Leu	Ala	Glu	Asn	Asp	Pro	Arg	Arg	Gln	Leu	Val	
305					310					315					320	
Asp	Lys	Leu	Ala	Ser	Trp	Asp	Gly	Glu	Asn	Leu	Val	Asn	Asp	Asp	Gly	
				325					330					335		
Lys	Thr	Tyr	Gln	Gln	Pro	Gly	Ser	Ala	Ile	Leu	Asn	Ala	Trp	Leu	Thr	
			340					345					350			
Ser	Met	Leu	Lys	Arg	Thr	Val	Val	Ala	Ala	Val	Pro	Ala	Pro	Phe	Gly	
		355					360					365				
Lys	Trp	Tyr	Ser	Ala	Ser	Gly	Tyr	Glu	Thr	Thr	Gln	Asp	Gly	Pro	Thr	
	370					375					380					
Gly	Ser	Leu	Asn	Ile	Ser	Val	Gly	Ala	Lys	Ile	Leu	Tyr	Glu	Ala	Leu	
385					390					395					400	
Gln	Gly	Asp	Lys	Ser	Pro	Ile	Pro	Gln	Ala	Val	Asp	Leu	Phe	Gly	Gly	
				405					410					415		

Lys Pro Gln Gln Glu Val Ile Leu Ala Ala Leu Asp Asp Ala Trp Gln
420 425 430

Thr Leu Ser Lys Arg Tyr Gly Asn Asp Val Thr Gly Trp Lys Thr Pro
435 440 445

Ala Met Ala Leu Thr Phe Arg Ala Asn Asn Phe Phe Gly Val Pro Gln
450 455 460

Ala Ala Ala Lys Glu Ala Arg His Gln Ala Glu Tyr Gln Asn Arg Gly
465 470 475 480

Thr Glu Asn Asp Met Ile Val Phe Ser Pro Thr Ser Gly Asn Arg Pro
485 490 495

Val Leu Ala Trp Asp Val Val Ala Pro Gly Gln Ser Gly Phe Ile Ala
500 505 510

Pro Asp Gly Lys Ala Asp Lys His Tyr Asp Asp Gln Leu Lys Met Tyr
515 520 525

Glu Ser Phe Gly Arg Lys Ser Leu Trp Leu Thr Pro Gln Asp Val Asp
530 535 540

Glu His Gln Glu Ser Gln Glu Val Leu Gln Val Gln Leu Asp Gln Thr
545 550 555 560

Glu Val Lys Ile Val Arg Asp Glu Tyr Gly Met Pro His Ile Tyr Ala
565 570 575

Asp Asp Thr Tyr Arg Leu Phe Tyr Gly Tyr Gly Tyr Val Val Ala Gln
580 585 590

Asp Arg Leu Phe Gln Met Glu Met Ala Arg Arg Ser Thr Gln Gly Thr
595 600 605

Val Ser Glu Val Leu Gly Lys Ala Phe Val Ser Phe Asp Lys Asp Ile
610 615 620

Arg Gln Asn Tyr Trp Pro Asp Ser Ile Arg Ala Gln Ile Ala Ser Leu
625 630 635 640

Ser Ala Glu Asp Lys Ser Ile Leu Gln Gly Tyr Ala Asp Gly Met Asn
645 650 655

Ala Trp Ile Asp Lys Val Asn Ala Ser Pro Asp Lys Leu Leu Pro Gln
660 665 670

Gln Phe Ser Thr Phe Gly Phe Lys Pro Lys His Trp Glu Pro Phe Asp
675 680 685

Val Ala Met Ile Phe Val Gly Thr Met Ala Asn Arg Phe Ser Asp Ser
690 695 700

Thr Ser Glu Ile Asp Asn Leu Ala Leu Leu Thr Ala Leu Lys Asp Lys
705 710 715 720

Tyr Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu
725 730 735

Val Asn Pro Ser Ala Pro Thr Thr Ile Ala Ala Arg Glu Ser Ala Tyr
740 745 750

Pro Leu Lys Phe Asp Leu Gln Asn Thr Gln Thr Ala
755 760

<210> 9
<211> 2295
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic PGA nucleotide sequence for variant 261

<400> 9
agcaatatgt gggtgattgg caaaaacaaa gccaggatg cgaaggccat tatggtcaat 60
gggccgcagt ttggttggtta taatccggcg tatacctacg gtatcggcct gcacggcgcg 120
ggctatgacg tcaccggcaa tacgccgttt gcctatccgg gcctcctttt tggtcacaac 180
ggcaccattt catggggatc caccgccggt gccggtgatg tcgtcgatat ctttgccgaa 240

aaactttccg ccgagaagcc gggctattac cagcataacg gcgagtgggt gaagatgttg	300
agccgcaagg agactattgc ggtcaaagac ggccagccgg agacctttac cgtttggcgc	360
acgtgcacg gcaacgtcat taaaaccgat actgcgacgc agaccgccta tgccaaagcg	420
cgcgcctggg atggcaaaga ggtggcgtcc ctgctggcgt ggacgcacca gatgaaggcc	480
aaaaactggc cggagtggac gcagcaggcg gccaaacagg cgctgaccat caactggtac	540
tacgccgatg tgaacggcaa tatcggctat gtgcataccg gcgcctatcc ggatcgccag	600
cccggccacg acccgcttt gccggttccc ggactggaa aatgggactg gaaagggttg	660
ctgtcgtttg atttgaatcc gaaagtgtat aaccgcagc cgggctatat cgccaactgg	720
aacaactcgc cgcaaaaaga ctaccggcc tctgatctgt tcgcgttcct gtggggcggt	780
gcggatcgag cgactgagat cgacacgatc ctcgataagc aaccgcgctt caccgccgat	840
caggcgtggg atgtgatccg ccaaaccagc cgtcgggatc tcaacctgcg gttgttctta	900
ccggcgctga aggacgccac cgccaacctg gcggaaaacg atccgcgccg ccaactggtg	960
gataaactgg cgagctggga cggcgaaaac cttgtcaacg atgacggaaa aacctatcag	1020
caaccgggat cggcgattct taacgcctgg ctgaccagca tgctcaagcg cacggtggtt	1080
gccgcggtcc cagcgccgtt tggttaagtgg tacagcgcca gtggctatga aaccaccag	1140
gacggggcaa ccggctcgct gaacatcagc gtgggggcga aaatcctcta cgaagctctg	1200
cagggtgata agtcgccaat cccgcaggcg gtcgatctgt ttggcgggaa accgcagcag	1260
gaagtaatac tggcggcgct ggacgacgct tggcagacgc tgtcaaaacg ctacggtaac	1320
gacgtcaccg gctggaaaac ccctgccatg gcgcttacct tccgggcaa taacttcttc	1380
ggcgtgccgc aggcggcagc aaaagaggcg cgtcatcagg cggagtacca gaaccgcggt	1440
acggaaaaca acatgattgt cttctcaccg acgtcgggta accgcccggg tcttgccctgg	1500
gatgtgggtg cgccggggca aagcggtttt atcgcgccgg atggcaaagc cgataagcac	1560
tatgacgatc agctgaaaat gtacgagagc tttggccgta aatcgctgtg gttaacgcct	1620
caggacgttg acgagcacia agagtctcag gaagtgtgc aggtacagtt ggatcagacc	1680
gaggtaaga tcgttcgcga tgaatacggc atgccgcata ttacgccga tgataacctat	1740
cgactgtttt acggctatgg ctacgtggtg gcgcaggatc gcctgttcca gatggaaatg	1800

gcgcgccgca gtactcaggg gaccgtctcc gaggtgctgg gcaaagcatt cgtttcattt 1860
gataaagata ttccgagaa ctactggccg gattctattc gcgcgcagat agcttccttc 1920
tccgctgagg ataaatccat tctgcagggc tatgccgatg gcatgaatgc gtggatcgat 1980
aaagtgaacg ccagccccga taagctgtta cccagcagt tctccacctt tggtttttaa 2040
cccaagcatt gggaaccgtt tgatgtggcg atgatttttg tcggcaccat ggcgaaccgt 2100
ttttctgaca gcaccagcga aattgataac ctggcgctgc tgacggcgct aaaagacaaa 2160
tacggcaagc agcagggcat ggcgggtcttt aaccagctga aatggctggg taatccttcc 2220
gcgccaacca ccattgcggc gcgggaaagc gcctatccgc tgaagtttga tctgcaaaac 2280
acgcaaacgg cgtaa 2295

<210> 10
<211> 764
<212> PRT
<213> Artificial Sequence

<220>
<223> synthetic PGA protein sequence for variant 261

<400> 10

Ser Asn Met Trp Val Ile Gly Lys Asn Lys Ala Gln Asp Ala Lys Ala
1 5 10 15

Ile Met Val Asn Gly Pro Gln Phe Gly Trp Tyr Asn Pro Ala Tyr Thr
20 25 30

Tyr Gly Ile Gly Leu His Gly Ala Gly Tyr Asp Val Thr Gly Asn Thr
35 40 45

Pro Phe Ala Tyr Pro Gly Leu Leu Phe Gly His Asn Gly Thr Ile Ser
50 55 60

Trp Gly Ser Thr Ala Gly Ala Gly Asp Val Val Asp Ile Phe Ala Glu
65 70 75 80

Lys Leu Ser Ala Glu Lys Pro Gly Tyr Tyr Gln His Asn Gly Glu Trp
85 90 95

Val Lys Met Leu Ser Arg Lys Glu Thr Ile Ala Val Lys Asp Gly Gln
100 105 110

Pro Glu Thr Phe Thr Val Trp Arg Thr Leu His Gly Asn Val Ile Lys
115 120 125

Thr Asp Thr Ala Thr Gln Thr Ala Tyr Ala Lys Ala Arg Ala Trp Asp
130 135 140

Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr His Gln Met Lys Ala
145 150 155 160

Lys Asn Trp Pro Glu Trp Thr Gln Gln Ala Ala Lys Gln Ala Leu Thr
165 170 175

Ile Asn Trp Tyr Tyr Ala Asp Val Asn Gly Asn Ile Gly Tyr Val His
180 185 190

Thr Gly Ala Tyr Pro Asp Arg Gln Pro Gly His Asp Pro Arg Leu Pro
195 200 205

Val Pro Gly Thr Gly Lys Trp Asp Trp Lys Gly Leu Leu Ser Phe Asp
210 215 220

Leu Asn Pro Lys Val Tyr Asn Pro Gln Ser Gly Tyr Ile Ala Asn Trp
225 230 235 240

Asn Asn Ser Pro Gln Lys Asp Tyr Pro Ala Ser Asp Leu Phe Ala Phe
245 250 255

Leu Trp Gly Gly Ala Asp Arg Ala Thr Glu Ile Asp Thr Ile Leu Asp
260 265 270

Lys Gln Pro Arg Phe Thr Ala Asp Gln Ala Trp Asp Val Ile Arg Gln
275 280 285

Thr Ser Arg Arg Asp Leu Asn Leu Arg Leu Phe Leu Pro Ala Leu Lys
290 295 300

Asp Ala Thr Ala Asn Leu Ala Glu Asn Asp Pro Arg Arg Gln Leu Val

305		310		315		320									
Asp	Lys	Leu	Ala	Ser	Trp	Asp	Gly	Glu	Asn	Leu	Val	Asn	Asp	Asp	Gly
				325					330					335	
Lys	Thr	Tyr	Gln	Gln	Pro	Gly	Ser	Ala	Ile	Leu	Asn	Ala	Trp	Leu	Thr
			340					345					350		
Ser	Met	Leu	Lys	Arg	Thr	Val	Val	Ala	Ala	Val	Pro	Ala	Pro	Phe	Gly
		355					360					365			
Lys	Trp	Tyr	Ser	Ala	Ser	Gly	Tyr	Glu	Thr	Thr	Gln	Asp	Gly	Pro	Thr
	370					375					380				
Gly	Ser	Leu	Asn	Ile	Ser	Val	Gly	Ala	Lys	Ile	Leu	Tyr	Glu	Ala	Leu
385					390					395					400
Gln	Gly	Asp	Lys	Ser	Pro	Ile	Pro	Gln	Ala	Val	Asp	Leu	Phe	Gly	Gly
				405					410					415	
Lys	Pro	Gln	Gln	Glu	Val	Ile	Leu	Ala	Ala	Leu	Asp	Asp	Ala	Trp	Gln
			420					425					430		
Thr	Leu	Ser	Lys	Arg	Tyr	Gly	Asn	Asp	Val	Thr	Gly	Trp	Lys	Thr	Pro
		435					440					445			
Ala	Met	Ala	Leu	Thr	Phe	Arg	Ala	Asn	Asn	Phe	Phe	Gly	Val	Pro	Gln
	450					455					460				
Ala	Ala	Ala	Lys	Glu	Ala	Arg	His	Gln	Ala	Glu	Tyr	Gln	Asn	Arg	Gly
465					470					475					480
Thr	Glu	Asn	Asn	Met	Ile	Val	Phe	Ser	Pro	Thr	Ser	Gly	Asn	Arg	Pro
				485					490					495	
Val	Leu	Ala	Trp	Asp	Val	Val	Ala	Pro	Gly	Gln	Ser	Gly	Phe	Ile	Ala
			500					505					510		
Pro	Asp	Gly	Lys	Ala	Asp	Lys	His	Tyr	Asp	Asp	Gln	Leu	Lys	Met	Tyr
		515					520					525			

Glu Ser Phe Gly Arg Lys Ser Leu Trp Leu Thr Pro Gln Asp Val Asp
530 535 540

Glu His Lys Glu Ser Gln Glu Val Leu Gln Val Gln Leu Asp Gln Thr
545 550 555 560

Glu Val Lys Ile Val Arg Asp Glu Tyr Gly Met Pro His Ile Tyr Ala
565 570 575

Asp Asp Thr Tyr Arg Leu Phe Tyr Gly Tyr Gly Tyr Val Val Ala Gln
580 585 590

Asp Arg Leu Phe Gln Met Glu Met Ala Arg Arg Ser Thr Gln Gly Thr
595 600 605

Val Ser Glu Val Leu Gly Lys Ala Phe Val Ser Phe Asp Lys Asp Ile
610 615 620

Arg Gln Asn Tyr Trp Pro Asp Ser Ile Arg Ala Gln Ile Ala Ser Leu
625 630 635 640

Ser Ala Glu Asp Lys Ser Ile Leu Gln Gly Tyr Ala Asp Gly Met Asn
645 650 655

Ala Trp Ile Asp Lys Val Asn Ala Ser Pro Asp Lys Leu Leu Pro Gln
660 665 670

Gln Phe Ser Thr Phe Gly Phe Lys Pro Lys His Trp Glu Pro Phe Asp
675 680 685

Val Ala Met Ile Phe Val Gly Thr Met Ala Asn Arg Phe Ser Asp Ser
690 695 700

Thr Ser Glu Ile Asp Asn Leu Ala Leu Leu Thr Ala Leu Lys Asp Lys
705 710 715 720

Tyr Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu
725 730 735

Val Asn Pro Ser Ala Pro Thr Thr Ile Ala Ala Arg Glu Ser Ala Tyr
740 745 750

Pro Leu Lys Phe Asp Leu Gln Asn Thr Gln Thr Ala
755 760

<210> 11
<211> 2295
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic PGA nucleotide sequence for variant 258

<400> 11
agcaatatgt gggtgattgg caaaaacaaa gcccaggatg cgaaggccat tatggtcaat 60
gggccgcagt ttggttggtta taatccggcg tataacctac gtatcggcct gcacggcgcg 120
ggctatgacg tcaccggcaa tacgccgttt gcctatccgg gcctcctttt tggtcacaac 180
ggcaccattt catggggatc caccgccggt gccggtgata gcgtcgatat ctttgccgaa 240
aaactttccg ccgagaagcc gggctattac cagcataacg gcgagtgggt gaagatgttg 300
agccgcaagg agactattgc ggtcaaagac ggccagccgg agacctttac cgtttggcgc 360
acgctgcacg gcaacgtcat taaaaccgat actgcgacgc agaccgccta tgccaaagcg 420
cgcgcctggg atggcaaaga ggtggcgtcc ctgctggcgt ggacgcacca gatgaaggcc 480
aaaaactggc cggagtggac gcagcaggcg gccaaacagg cgctgaccat caactggtac 540
tacgccgatg tgaacggcaa tatcggctat gtgcataccg gcgcctatcc ggatcgccag 600
cccggccacg acccgcttt gccggttccc ggcactggaa aatgggactg gaaagggttg 660
ctgtcgtttg atttgaatcc gaaagtgtat aaccgcagc cgggctatat cgccaactgg 720
aacaactcgc cgcaaaaaga ctaccggcc tctgatctgt tcgcgttcct gtggggcggt 780
gcggatcgag cgactgagat cgacacgatc ctcgataagc aaccgcgctt caccgccgat 840
caggcgtggg atgtgatccg ccaaaccagc cgtcgggatc tcaacctgcg gttgttctta 900
ccggcgctga aggacggcac cgccaacctg gcggaaaacg atccgcgccg ccaactgggtg 960
gataaactgg cgagctggga cggcgaaaac cttgtcaacg atgacggaaa aacctatcag 1020
caaccgggat cggcgattct taacgcctgg ctgaccagca tgctcaagcg caccgttggtt 1080

gccgcggtcc cagcgccgtt tggtagtgg tacagcgcca gtggctatga aaccacccag	1140
gacggggccaa ccggctcgct gaacatcagc gtggggggcga aaatcctcta cgaagctctg	1200
cagggtgata agtcgccaat cccgcaggcg gtcgatctgt ttggcgggaa accgcagcag	1260
gaagtaatac tggcggcgct ggacgacgct tggcagacgc tgtcaaaacg ctacggtaac	1320
gacgtcaccg gctggaaaac ccctgccatg gcgcttacct tccggggccaa taactttcttc	1380
ggcgtgccgc aggcggcagc aaaagaggcg cgtcatcagg cggagtacca gaaccgcggt	1440
acggaaaaca acatgattgt cttctcaccg acgtcgggta accgcccggg tcttgccctgg	1500
gatgtgggtg cgccggggca aagcggtttt atcgcgccgg atggcaaagc cgataagcac	1560
tatgacgatc agctgaaaat gtacgagagc tttggccgta aatcgctgtg gttaacgcct	1620
caggacgttg acgagcacia agagtctcag gaagtgtgc aggtacagtt ggatcagacc	1680
gagggttaaga tcgttcgcga tgaatacggc atgccgcata ttacgccga tgatacctat	1740
cgactgtttt acggctatgg ctacgtgggt gcgcaggatc gcctgtttca gatggaaatg	1800
gcgcgccgca gtactcaggg gaccgtctcc gaggtgctgg gcaaagcatt cgттаagttt	1860
gataaagata ttcgccagaa ctactggccg gattctattc gcgcgcagat agcttccttc	1920
tccgctgagg ataaatccat tctgcagggc tatgccgatg gcatgaatgc gtggatcgat	1980
aaagtgaacg ccagccccga taagctgtta cccagcagt tctccacctt tggtttttaa	2040
cccaagcatt gggaaccgtt tgatgtggcg atgatttttg tcggcaccat ggcgaaccgt	2100
ttttctgaca gcaccagcga aattgataac ctggcgctgc tgacggcgct aaaagacaaa	2160
tacggcaagc agcagggcat ggcggtcttt aaccagctga aatggctggt taatccttcc	2220
gcgccaacca ccattgcggc gcgggaaagc gcctatccgc tgaagtttga tctgcaaaac	2280
acgcaaacgg cgtaa	2295

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Ser Asn Met Trp Val Ile Gly Lys Asn Lys Ala Gln Asp Ala Lys Ala
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Ile Met Val Asn Gly Pro Gln Phe Gly Trp Tyr Asn Pro Ala Tyr Thr
 20 25 30

Tyr Gly Ile Gly Leu His Gly Ala Gly Tyr Asp Val Thr Gly Asn Thr
 35 40 45

Pro Phe Ala Tyr Pro Gly Leu Leu Phe Gly His Asn Gly Thr Ile Ser
 50 55 60

Trp Gly Ser Thr Ala Gly Ala Gly Asp Ser Val Asp Ile Phe Ala Glu
 65 70 75 80

Lys Leu Ser Ala Glu Lys Pro Gly Tyr Tyr Gln His Asn Gly Glu Trp
 85 90 95

Val Lys Met Leu Ser Arg Lys Glu Thr Ile Ala Val Lys Asp Gly Gln
 100 105 110

Pro Glu Thr Phe Thr Val Trp Arg Thr Leu His Gly Asn Val Ile Lys
 115 120 125

Thr Asp Thr Ala Thr Gln Thr Ala Tyr Ala Lys Ala Arg Ala Trp Asp
 130 135 140

Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr His Gln Met Lys Ala
 145 150 155 160

Lys Asn Trp Pro Glu Trp Thr Gln Gln Ala Ala Lys Gln Ala Leu Thr
 165 170 175

Ile Asn Trp Tyr Tyr Ala Asp Val Asn Gly Asn Ile Gly Tyr Val His
 180 185 190

Thr Gly Ala Tyr Pro Asp Arg Gln Pro Gly His Asp Pro Arg Leu Pro
 195 200 205

Val Pro Gly Thr Gly Lys Trp Asp Trp Lys Gly Leu Leu Ser Phe Asp
210 215 220

Leu Asn Pro Lys Val Tyr Asn Pro Gln Ser Gly Tyr Ile Ala Asn Trp
225 230 235 240

Asn Asn Ser Pro Gln Lys Asp Tyr Pro Ala Ser Asp Leu Phe Ala Phe
245 250 255

Leu Trp Gly Gly Ala Asp Arg Ala Thr Glu Ile Asp Thr Ile Leu Asp
260 265 270

Lys Gln Pro Arg Phe Thr Ala Asp Gln Ala Trp Asp Val Ile Arg Gln
275 280 285

Thr Ser Arg Arg Asp Leu Asn Leu Arg Leu Phe Leu Pro Ala Leu Lys
290 295 300

Asp Ala Thr Ala Asn Leu Ala Glu Asn Asp Pro Arg Arg Gln Leu Val
305 310 315 320

Asp Lys Leu Ala Ser Trp Asp Gly Glu Asn Leu Val Asn Asp Asp Gly
325 330 335

Lys Thr Tyr Gln Gln Pro Gly Ser Ala Ile Leu Asn Ala Trp Leu Thr
340 345 350

Ser Met Leu Lys Arg Thr Val Val Ala Ala Val Pro Ala Pro Phe Gly
355 360 365

Lys Trp Tyr Ser Ala Ser Gly Tyr Glu Thr Thr Gln Asp Gly Pro Thr
370 375 380

Gly Ser Leu Asn Ile Ser Val Gly Ala Lys Ile Leu Tyr Glu Ala Leu
385 390 395 400

Gln Gly Asp Lys Ser Pro Ile Pro Gln Ala Val Asp Leu Phe Gly Gly
405 410 415

Lys Pro Gln Gln Glu Val Ile Leu Ala Ala Leu Asp Asp Ala Trp Gln

420

425

430

Thr Leu Ser Lys Arg Tyr Gly Asn Asp Val Thr Gly Trp Lys Thr Pro
 435 440 445

Ala Met Ala Leu Thr Phe Arg Ala Asn Asn Phe Phe Gly Val Pro Gln
 450 455 460

Ala Ala Ala Lys Glu Ala Arg His Gln Ala Glu Tyr Gln Asn Arg Gly
 465 470 475 480

Thr Glu Asn Asn Met Ile Val Phe Ser Pro Thr Ser Gly Asn Arg Pro
 485 490 495

Val Leu Ala Trp Asp Val Val Ala Pro Gly Gln Ser Gly Phe Ile Ala
 500 505 510

Pro Asp Gly Lys Ala Asp Lys His Tyr Asp Asp Gln Leu Lys Met Tyr
 515 520 525

Glu Ser Phe Gly Arg Lys Ser Leu Trp Leu Thr Pro Gln Asp Val Asp
 530 535 540

Glu His Lys Glu Ser Gln Glu Val Leu Gln Val Gln Leu Asp Gln Thr
 545 550 555 560

Glu Val Lys Ile Val Arg Asp Glu Tyr Gly Met Pro His Ile Tyr Ala
 565 570 575

Asp Asp Thr Tyr Arg Leu Phe Tyr Gly Tyr Gly Tyr Val Val Ala Gln
 580 585 590

Asp Arg Leu Phe Gln Met Glu Met Ala Arg Arg Ser Thr Gln Gly Thr
 595 600 605

Val Ser Glu Val Leu Gly Lys Ala Phe Val Lys Phe Asp Lys Asp Ile
 610 615 620

Arg Gln Asn Tyr Trp Pro Asp Ser Ile Arg Ala Gln Ile Ala Ser Leu
 625 630 635 640

Ser Ala Glu Asp Lys Ser Ile Leu Gln Gly Tyr Ala Asp Gly Met Asn
645 650 655

Ala Trp Ile Asp Lys Val Asn Ala Ser Pro Asp Lys Leu Leu Pro Gln
660 665 670

Gln Phe Ser Thr Phe Gly Phe Lys Pro Lys His Trp Glu Pro Phe Asp
675 680 685

Val Ala Met Ile Phe Val Gly Thr Met Ala Asn Arg Phe Ser Asp Ser
690 695 700

Thr Ser Glu Ile Asp Asn Leu Ala Leu Leu Thr Ala Leu Lys Asp Lys
705 710 715 720

Tyr Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu
725 730 735

Val Asn Pro Ser Ala Pro Thr Thr Ile Ala Ala Arg Glu Ser Ala Tyr
740 745 750

Pro Leu Lys Phe Asp Leu Gln Asn Thr Gln Thr Ala
755 760

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 <223> Xaa can be Gln, Cys, Ile, Lys, or Arg

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<223> Xaa can be Ala or Asp

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<223> Xaa can be Val or Ile

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<222> (697)..(697)

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Ser Asn Met Trp Val Ile Gly Lys Asn Lys Ala Gln Asp Ala Lys Ala
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Ile Met Val Asn Gly Pro Gln Phe Gly Xaa Xaa Xaa Pro Ala Tyr Xaa
20 25 30

Tyr Gly Ile Gly Leu His Gly Ala Gly Tyr Asp Val Thr Gly Asn Thr
35 40 45

Xaa Phe Ala Tyr Pro Gly Leu Xaa Phe Gly His Asn Gly Thr Ile Ser
50 55 60

Trp Gly Ser Thr Ala Gly Xaa Gly Asp Xaa Val Asp Ile Phe Ala Glu
65 70 75 80

Lys Leu Ser Ala Glu Lys Pro Gly Tyr Tyr Gln His Asn Gly Glu Trp
85 90 95

Val Lys Met Leu Ser Arg Lys Glu Thr Ile Ala Val Lys Asp Gly Gln
100 105 110

Pro Glu Thr Xaa Thr Val Trp Arg Thr Leu His Gly Asn Xaa Xaa Lys
115 120 125

Xaa Xaa Xaa Xaa Xaa Xaa Thr Ala Tyr Ala Lys Ala Arg Ala Trp Asp
130 135 140

Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr Xaa Gln Met Lys Xaa
145 150 155 160

Lys Asn Trp Pro Glu Trp Thr Gln Gln Ala Ala Lys Gln Ala Leu Thr
165 170 175

Ile Asn Trp Tyr Tyr Ala Asp Val Asn Gly Asn Ile Gly Tyr Val His
180 185 190

Thr Gly Ala Tyr Pro Asp Arg Gln Pro Gly His Asp Pro Arg Leu Pro
195 200 205

Val Pro Gly Thr Gly Lys Trp Asp Trp Lys Gly Leu Leu Ser Phe Asp
210 215 220

Leu Asn Pro Lys Val Tyr Asn Pro Gln Ser Gly Tyr Ile Ala Asn Xaa
225 230 235 240

Asn Asn Ser Pro Gln Lys Asp Tyr Pro Ala Ser Asp Xaa Xaa Xaa Xaa
245 250 255

Xaa Trp Gly Gly Ala Asp Arg Xaa Thr Glu Ile Asp Thr Ile Leu Asp
260 265 270

Lys Gln Pro Arg Phe Thr Ala Asp Gln Ala Trp Asp Val Ile Arg Gln
275 280 285

Thr Ser Arg Arg Asp Leu Asn Leu Arg Leu Phe Leu Pro Ala Leu Lys
290 295 300

Asp Ala Thr Xaa Asn Leu Ala Glu Asn Asp Pro Arg Arg Gln Leu Val
305 310 315 320

Asp Lys Leu Ala Ser Trp Asp Gly Glu Asn Leu Val Asn Asp Asp Gly
325 330 335

Lys Thr Tyr Gln Gln Pro Gly Ser Ala Ile Leu Xaa Ala Trp Leu Thr
340 345 350

Ser Met Leu Lys Arg Thr Val Xaa Ala Ala Val Pro Xaa Pro Xaa Xaa
355 360 365

Xaa Xaa Tyr Xaa Xaa Ser Gly Tyr Glu Xaa Xaa Xaa Xaa Gly Pro Xaa
370 375 380

Gly Ser Xaa Xaa Xaa Ser Xaa Gly Ala Lys Ile Leu Tyr Glu Ala Leu
385 390 395 400

Gln Gly Asp Lys Ser Pro Ile Pro Gln Ala Val Asp Leu Phe Gly Gly
405 410 415

Lys Xaa Gln Gln Glu Val Ile Leu Ala Ala Leu Asp Asp Ala Trp Gln
420 425 430

Thr Leu Ser Lys Arg Tyr Gly Asn Asp Val Thr Gly Trp Lys Thr Pro
435 440 445

Ala Met Ala Leu Xaa Xaa Arg Xaa Asn Asn Phe Phe Gly Val Pro Gln
450 455 460

Ala Ala Ala Lys Glu Ala Arg His Gln Ala Glu Tyr Gln Asn Arg Gly
465 470 475 480

Thr Glu Asn Xaa Met Ile Val Phe Ser Pro Thr Ser Gly Asn Arg Pro
485 490 495

Val Leu Ala Trp Asp Val Val Ala Pro Gly Gln Ser Gly Phe Ile Ala

500					505					510					
Pro	Asp	Gly	Lys	Ala	Asp	Lys	His	Tyr	Asp	Asp	Gln	Leu	Lys	Met	Tyr
		515					520					525			
Glu	Ser	Phe	Gly	Arg	Lys	Ser	Leu	Trp	Leu	Thr	Pro	Gln	Asp	Val	Asp
	530					535					540				
Glu	His	Xaa	Glu	Ser	Gln	Glu	Val	Leu	Gln	Val	Gln	Xaa	Asp	Gln	Thr
545					550					555					560
Glu	Val	Lys	Ile	Val	Arg	Asp	Glu	Tyr	Gly	Met	Pro	His	Ile	Tyr	Ala
				565					570					575	
Asp	Asp	Thr	Tyr	Arg	Leu	Phe	Tyr	Gly	Tyr	Gly	Tyr	Val	Val	Ala	Gln
			580					585					590		
Asp	Arg	Leu	Xaa	Gln	Met	Glu	Met	Ala	Arg	Arg	Ser	Thr	Gln	Gly	Thr
		595					600					605			
Val	Ser	Glu	Val	Leu	Gly	Lys	Xaa	Phe	Xaa	Xaa	Phe	Asp	Lys	Asp	Ile
	610					615					620				
Arg	Gln	Asn	Tyr	Trp	Pro	Asp	Ser	Ile	Arg	Ala	Gln	Ile	Ala	Ser	Leu
625					630					635					640
Ser	Ala	Glu	Asp	Lys	Ser	Ile	Leu	Gln	Gly	Tyr	Ala	Asp	Gly	Met	Asn
				645					650					655	
Ala	Trp	Ile	Asp	Lys	Val	Asn	Ala	Ser	Pro	Asp	Lys	Leu	Leu	Pro	Gln
			660					665					670		
Gln	Phe	Ser	Thr	Phe	Gly	Phe	Lys	Pro	Lys	His	Trp	Glu	Pro	Phe	Asp
		675					680					685			
Val	Ala	Met	Ile	Phe	Val	Gly	Thr	Xaa	Ala	Asn	Arg	Phe	Ser	Asp	Ser
	690					695					700				
Thr	Ser	Glu	Ile	Asp	Asn	Xaa	Ala	Leu	Leu	Thr	Ala	Leu	Lys	Asp	Lys
705					710					715					720

Tyr Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu
725 730 735

Val Asn Pro Ser Ala Pro Thr Thr Ile Ala Ala Arg Glu Xaa Ala Tyr
740 745 750

Pro Xaa Lys Phe Asp Leu Gln Asn Thr Gln Thr
755 760

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<220>
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<400> 14

Ser	Asn	Met	Trp	Val	Ile	Gly	Lys	Asn	Lys	Ala	Gln	Asp	Ala	Lys	Ala
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Ile	Met	Val	Asn	Gly	Pro	Gln	Phe	Gly	Trp	Tyr	Xaa	Pro	Ala	Tyr	Thr
			20					25					30		

Tyr	Gly	Ile	Gly	Leu	His	Gly	Ala	Gly	Tyr	Asp	Val	Thr	Gly	Asn	Thr
		35					40					45			

Pro	Phe	Ala	Tyr	Pro	Gly	Leu	Xaa	Phe	Gly	His	Asn	Gly	Thr	Ile	Ser
	50					55					60				

Trp	Gly	Ser	Thr	Ala	Gly	Xaa	Gly	Asp	Xaa	Val	Asp	Ile	Phe	Ala	Glu
65					70					75					80

Lys	Leu	Ser	Ala	Glu	Lys	Pro	Gly	Tyr	Tyr	Gln	His	Asn	Gly	Glu	Trp
				85					90					95	

Val	Lys	Met	Leu	Ser	Arg	Lys	Glu	Thr	Ile	Ala	Val	Lys	Asp	Gly	Gln
			100					105					110		

Pro	Glu	Thr	Phe	Thr	Val	Trp	Arg	Thr	Leu	His	Gly	Asn	Val	Ile	Lys
		115					120					125			

Thr	Asp	Thr	Ala	Thr	Gln	Thr	Ala	Tyr	Ala	Xaa	Ala	Arg	Ala	Trp	Asp
	130					135					140				

Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr His Gln Met Lys Ala
 145 150 155 160

Lys Asn Trp Pro Glu Trp Thr Gln Gln Ala Ala Lys Gln Ala Leu Thr
 165 170 175

Ile Asn Trp Tyr Tyr Ala Asp Val Asn Gly Asn Ile Gly Tyr Val His
 180 185 190

Thr Gly Ala Tyr Pro Asp Arg Gln Pro Gly His Asp Pro Arg Leu Pro
 195 200 205

Val Pro Gly Thr Gly Lys Trp Asp Trp Lys Gly Leu Leu Ser Phe Asp
 210 215 220

Leu Asn Pro Lys Val Tyr Asn Pro Gln Ser Gly Tyr Ile Ala Asn Trp
 225 230 235 240

Asn Asn Ser Pro Gln Lys Asp Tyr Pro Ala Ser Asp Leu Phe Ala Phe
 245 250 255

Leu Trp Gly Gly Ala Asp Arg Ala Thr Glu Ile Asp Thr Ile Leu Asp
 260 265 270

Lys Gln Pro Arg Phe Thr Ala Asp Gln Ala Trp Asp Val Ile Arg Gln
 275 280 285

Thr Ser Arg Arg Asp Leu Asn Leu Arg Leu Phe Leu Pro Ala Leu Lys
 290 295 300

Asp Ala Thr Ala Asn Leu Ala Glu Asn Asp Pro Arg Arg Gln Leu Val
 305 310 315 320

Asp Lys Leu Ala Ser Trp Asp Gly Glu Asn Leu Val Asn Asp Asp Gly
 325 330 335

Lys Thr Tyr Gln Gln Pro Gly Ser Ala Ile Leu Asn Ala Trp Leu Thr
 340 345 350

Ser Met Leu Lys Arg Thr Val Val Ala Ala Val Pro Ala Pro Phe Gly

355

360

365

Lys Trp Tyr Ser Ala Ser Gly Tyr Glu Thr Thr Gln Asp Gly Pro Thr
 370 375 380

Gly Ser Leu Asn Ile Ser Val Gly Ala Lys Ile Leu Tyr Glu Ala Leu
 385 390 395 400

Gln Gly Asp Lys Ser Pro Ile Pro Gln Ala Val Asp Leu Phe Gly Gly
 405 410 415

Lys Pro Gln Gln Glu Val Ile Leu Ala Ala Leu Asp Asp Ala Trp Gln
 420 425 430

Thr Leu Ser Lys Arg Tyr Gly Asn Asp Val Thr Gly Xaa Lys Thr Pro
 435 440 445

Ala Met Ala Leu Thr Phe Arg Ala Asn Asn Phe Phe Gly Val Pro Gln
 450 455 460

Ala Ala Ala Lys Glu Ala Arg His Gln Ala Glu Tyr Gln Asn Arg Gly
 465 470 475 480

Thr Glu Asn Asn Met Ile Val Phe Ser Pro Thr Ser Gly Asn Arg Pro
 485 490 495

Val Leu Ala Trp Asp Val Val Ala Pro Gly Gln Ser Gly Phe Ile Ala
 500 505 510

Pro Asp Gly Lys Ala Asp Lys His Tyr Asp Asp Gln Leu Lys Met Tyr
 515 520 525

Glu Ser Phe Gly Arg Lys Ser Leu Trp Leu Thr Pro Gln Asp Val Asp
 530 535 540

Glu His Lys Glu Ser Gln Glu Val Leu Gln Val Gln Leu Asp Gln Thr
 545 550 555 560

Glu Val Lys Ile Val Arg Asp Glu Tyr Gly Met Pro His Ile Tyr Ala
 565 570 575

Asp Asp Thr Tyr Arg Leu Phe Tyr Gly Tyr Gly Tyr Val Val Ala Gln
580 585 590

Asp Arg Leu Phe Gln Met Glu Met Ala Arg Arg Ser Thr Gln Gly Thr
595 600 605

Val Ser Glu Val Leu Gly Lys Ala Phe Xaa Xaa Phe Asp Lys Asp Ile
610 615 620

Arg Gln Asn Tyr Trp Pro Asp Ser Ile Arg Ala Gln Ile Ala Ser Leu
625 630 635 640

Ser Ala Glu Asp Lys Ser Ile Leu Gln Gly Tyr Ala Asp Gly Met Asn
645 650 655

Ala Trp Ile Asp Lys Val Asn Ala Ser Pro Asp Lys Leu Leu Pro Gln
660 665 670

Gln Phe Ser Thr Phe Gly Phe Lys Pro Lys His Trp Glu Pro Phe Asp
675 680 685

Val Ala Met Ile Phe Val Gly Thr Met Ala Asn Arg Phe Ser Asp Ser
690 695 700

Thr Ser Glu Ile Asp Asn Leu Ala Leu Leu Thr Ala Leu Lys Asp Lys
705 710 715 720

Tyr Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu
725 730 735

Val Asn Pro Ser Ala Pro Thr Thr Ile Ala Ala Arg Glu Ser Ala Tyr
740 745 750

Pro Leu Lys Phe Asp Leu Gln Asn Thr Gln Thr Ala
755 760

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<220>
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 <223> Xaa can be Asp or Asn

<220>
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<220>
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<222> (547)..(547)
<223> Xaa can be Gln or Lys

<220>
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<223> Xaa can be Val or Ile

<220>
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<220>
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<223> Xaa can be Ala or Thr

<400> 15

Ser Asn Met Trp Val Ile Gly Lys Asn Lys Ala Gln Asp Ala Lys Ala
1 5 10 15

Ile Met Val Asn Gly Pro Gln Phe Gly Trp Xaa Xaa Pro Ala Tyr Thr
20 25 30

Tyr Gly Ile Gly Leu His Gly Ala Gly Tyr Asp Val Thr Gly Asn Thr
35 40 45

Pro Phe Ala Tyr Pro Xaa Leu Leu Phe Gly His Asn Gly Thr Ile Ser
50 55 60

Trp Gly Ser Thr Ala Gly Xaa Gly Asp Xaa Val Asp Ile Phe Ala Glu
65 70 75 80

Lys Leu Ser Ala Glu Lys Pro Gly Tyr Tyr Gln His Asn Gly Glu Trp
85 90 95

Val Lys Met Leu Ser Arg Lys Glu Thr Ile Ala Val Lys Asp Gly Gln
100 105 110

Pro Glu Thr Phe Thr Val Trp Arg Thr Leu His Gly Asn Val Ile Lys
115 120 125

Thr Asp Thr Ala Thr Gln Thr Ala Tyr Ala Lys Ala Arg Ala Trp Asp

130

135

140

Gly Lys Glu Val Ala Ser Leu Leu Ala Trp Thr His Gln Met Lys Ala
 145 150 155 160

Lys Asn Trp Pro Glu Trp Thr Gln Gln Ala Ala Lys Gln Ala Leu Thr
 165 170 175

Ile Asn Trp Tyr Tyr Ala Asp Val Asn Gly Asn Ile Gly Tyr Val His
 180 185 190

Thr Gly Ala Tyr Pro Asp Arg Gln Pro Gly His Asp Pro Arg Leu Pro
 195 200 205

Val Pro Gly Thr Gly Lys Trp Asp Trp Lys Gly Leu Leu Ser Phe Asp
 210 215 220

Leu Asn Pro Lys Val Tyr Asn Pro Gln Ser Gly Tyr Ile Ala Asn Trp
 225 230 235 240

Asn Asn Ser Pro Gln Lys Asp Tyr Pro Ala Ser Asp Leu Phe Ala Phe
 245 250 255

Leu Trp Gly Gly Ala Asp Arg Xaa Thr Glu Ile Asp Thr Ile Leu Asp
 260 265 270

Lys Gln Pro Arg Phe Thr Ala Asp Gln Ala Trp Asp Val Ile Arg Gln
 275 280 285

Thr Ser Arg Arg Asp Leu Asn Leu Arg Leu Phe Leu Pro Ala Leu Lys
 290 295 300

Asp Ala Thr Ala Asn Leu Ala Glu Asn Asp Pro Arg Arg Gln Leu Val
 305 310 315 320

Asp Lys Leu Ala Ser Trp Asp Gly Glu Asn Leu Val Asn Asp Asp Gly
 325 330 335

Lys Thr Tyr Gln Gln Pro Gly Ser Ala Ile Leu Asn Ala Trp Leu Thr
 340 345 350

Ser Met Leu Lys Arg Thr Val Val Ala Ala Val Pro Ala Pro Phe Gly
355 360 365

Lys Trp Tyr Ser Ala Ser Gly Tyr Glu Thr Thr Gln Asp Gly Pro Thr
370 375 380

Gly Ser Leu Asn Ile Ser Val Gly Ala Lys Ile Leu Tyr Glu Ala Leu
385 390 395 400

Gln Gly Asp Lys Ser Pro Ile Pro Gln Ala Val Asp Leu Phe Gly Gly
405 410 415

Lys Pro Gln Gln Glu Val Ile Leu Ala Ala Leu Asp Asp Ala Trp Gln
420 425 430

Thr Leu Ser Lys Arg Tyr Gly Asn Asp Val Thr Gly Trp Lys Thr Pro
435 440 445

Ala Met Ala Leu Thr Phe Arg Ala Asn Asn Phe Phe Gly Val Pro Gln
450 455 460

Ala Ala Ala Lys Glu Ala Arg His Gln Ala Glu Tyr Gln Asn Arg Gly
465 470 475 480

Thr Glu Asn Asp Met Ile Val Phe Ser Pro Thr Ser Gly Asn Arg Pro
485 490 495

Val Leu Ala Trp Asp Val Val Ala Pro Gly Gln Ser Gly Phe Ile Ala
500 505 510

Pro Asp Gly Lys Ala Asp Lys His Tyr Asp Asp Gln Leu Lys Met Tyr
515 520 525

Glu Ser Phe Gly Arg Lys Ser Leu Trp Leu Thr Pro Gln Asp Val Xaa
530 535 540

Glu His Xaa Glu Ser Gln Glu Val Leu Gln Val Gln Leu Asp Gln Thr
545 550 555 560

Glu Val Lys Ile Val Arg Asp Glu Tyr Gly Met Pro His Ile Tyr Ala
565 570 575

Asp Asp Thr Tyr Arg Leu Phe Tyr Gly Tyr Gly Tyr Val Val Ala Gln
580 585 590

Asp Arg Leu Phe Gln Met Glu Met Ala Arg Arg Ser Thr Gln Gly Thr
595 600 605

Val Ser Glu Val Leu Gly Lys Ala Phe Xaa Xaa Phe Asp Lys Asp Ile
610 615 620

Arg Gln Asn Tyr Trp Pro Asp Ser Ile Arg Ala Gln Ile Ala Ser Leu
625 630 635 640

Ser Ala Glu Asp Lys Ser Ile Leu Gln Gly Tyr Ala Asp Gly Met Asn
645 650 655

Ala Trp Ile Asp Lys Val Asn Ala Ser Pro Asp Lys Leu Leu Pro Gln
660 665 670

Gln Phe Ser Thr Phe Gly Phe Lys Pro Lys His Trp Glu Pro Phe Asp
675 680 685

Val Ala Met Ile Phe Val Gly Thr Met Ala Asn Arg Phe Ser Asp Ser
690 695 700

Thr Ser Glu Ile Asp Asn Leu Ala Leu Leu Thr Ala Leu Lys Asp Lys
705 710 715 720

Tyr Gly Lys Gln Gln Gly Met Ala Val Phe Asn Gln Leu Lys Trp Leu
725 730 735

Val Asn Pro Ser Xaa Pro Thr Thr Ile Ala Ala Arg Glu Ser Ala Tyr
740 745 750

Pro Leu Lys Phe Asp Leu Gln Asn Thr Gln Thr Ala
755 760

The Claims defining the Invention are as follows:

1. An engineered non-naturally occurring penicillin G acylase capable of removing the A1/B1/B29 tri-phenyl acetate protecting groups from insulin to produce free insulin, wherein said penicillin G acylase comprises SEQ ID NO:12.
2. The engineered penicillin G acylase of Claim 1, wherein said penicillin G acylase is encoded by the polynucleotide sequence set forth in SEQ ID NO:11.

ABSTRACT

The present invention provides engineered penicillin G acylase (PGA) enzymes having improved properties, polynucleotides encoding such enzymes, compositions including the enzymes, and methods of using the enzymes.

FIGURE 1

FIGURE 1.

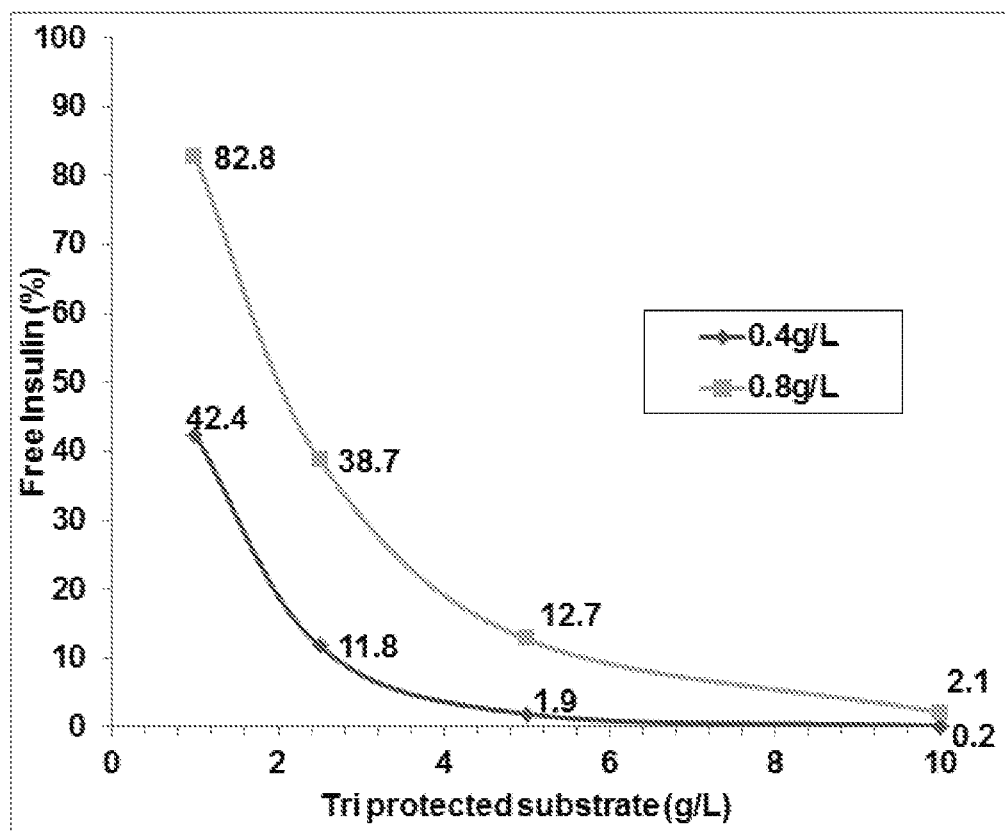


FIGURE 2.

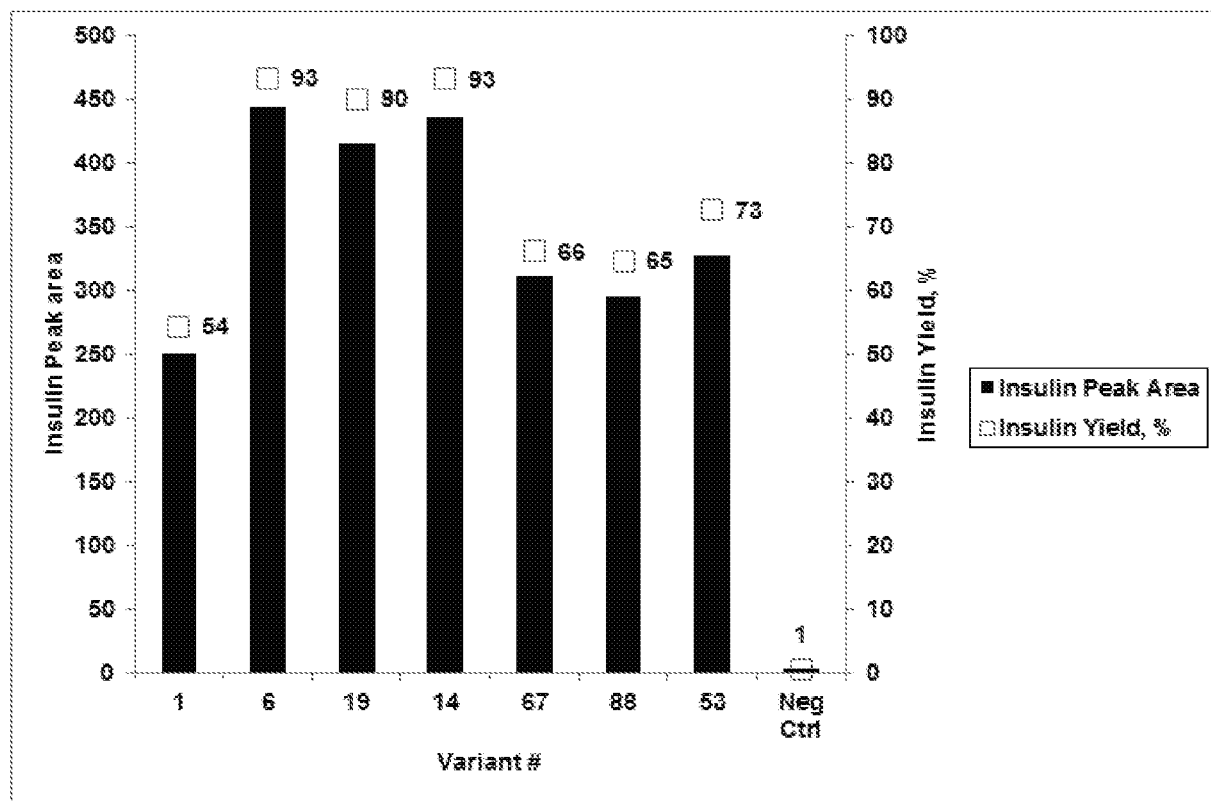


FIGURE 3.

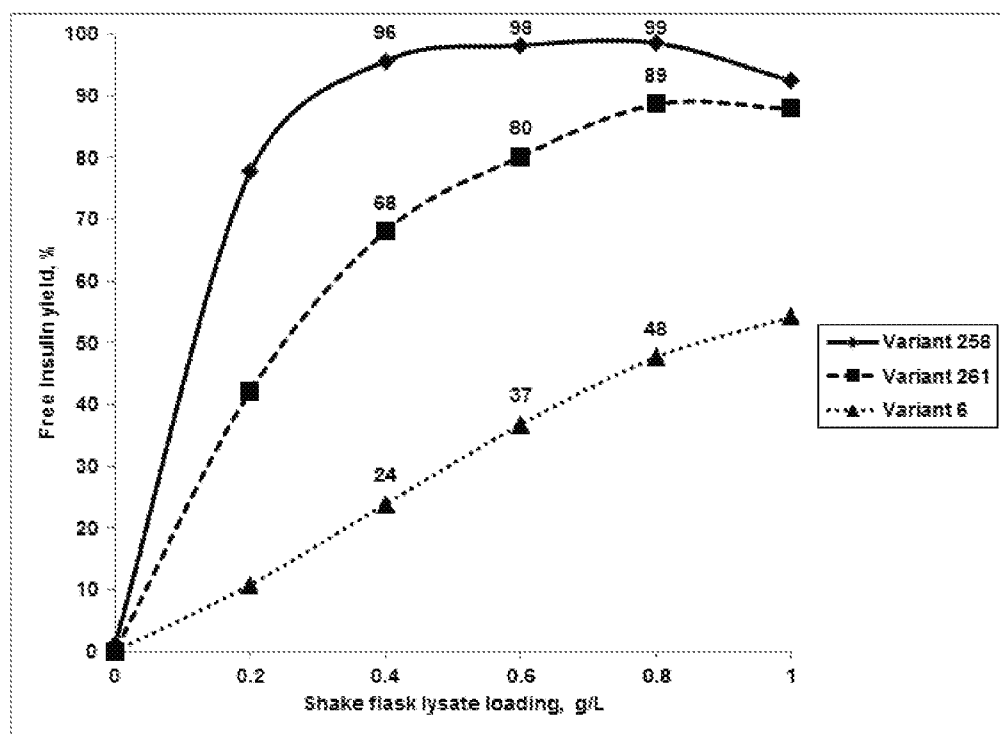


FIGURE 4.

