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(71) **Applicant** *(for all designated States except US)*: **ELONA BIOTECHNOLOGIES** [US/US]; 1040 Sierra Drive, Suite 1000, Greenwood, Indiana 46143 (US).

(72) **Inventors; and**
(75) **Inventors/Applicants** *(for US only)*: **ZIMMERMAN, Ronald E.** [US/US]; 4465 W. Stones Crossing Road, Greenwood, Indiana 46143 (US). **STOKELL, David John** [US/US]; 5513 Floating Leaf Drive, Indianapolis, Indiana 46237 (US). **AKERS, Michael Patrick** [US/US]; 517 Kessler Blvd. E Dr., Indianapolis, Indiana 46220 (US).

(74) **Agents**: **MAYFIELD, Denise L.** et al.; Katten Muchin Rosenman LLP, 2900 K Street, NW, Suite 200, Washington, District of Columbia 20007-5118 (US).

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(54) **Title**: ASPART PROINSULIN COMPOSITIONS AND METHODS OF PRODUCING ASPART INSULIN ANALOGS

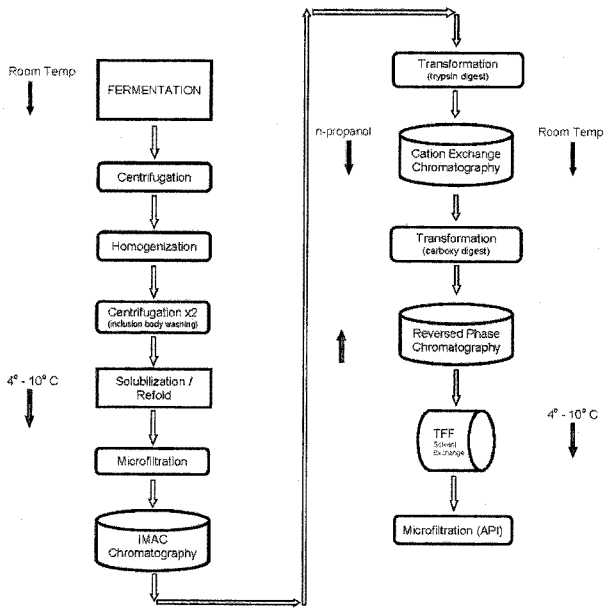

Figure 2a: Production Block Flow Diagram for Insulin Aspart

(57) **Abstract**: Aspart modified proinsulin sequences that have a modified C-peptide amino acid and/or nucleic acid modification for producing aspart insulin analogs are provided. Highly efficient processes for preparing the aspart insulin analogs and improved preparations containing the aspart insulin analogs prepared according to the methods described herein are also provided.

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ASPART PROINSULIN COMPOSITIONS AND METHODS OF PRODUCING ASPART INSULIN ANALOGS

Field of the Invention

[0001] The invention relates to compositions and preparations that comprise aspart proinsulin. in particular aspart proinsulin with modified C-peptide sequences. The invention also relates to methods of manufacture for manufacturing aspart insulin analogs from modified proinsulin sequences.

Background

[0002] Insulin is a hormone that regulates glucose metabolism in animals. Insulin is a polypeptide hormone secreted by beta-cells of the pancreas. This hormone is made up of two polypeptide chains, an A-chain of 21 amino acids, and a B-chain of 30 amino acids. These two chains are linked to one another in the mature form of the hormone by two interchain disulphide bridges. The A-chain also features one intra-chain disulphide bridge.

[0003] Insulin analogs are altered forms of native insulin that are available to the body for performing the same action as native insulin. A specific insulin analog known as aspart insulin has also been described in US Pat. Nos. 5,618,913, 5,547,930, and 5,834,422. This analog is used in the treatment of diabetes. Aspart insulin analogue has increased charge repulsion as compared with native insulin, which prevents the formation of hexamers and thus results in a faster acting insulin. This aspart insulin analog has been available commercially as NOVOLOG® (Eli Lilly). NOVOLOG® is an insulin analog wherein the molecule includes a Asp(B_{28}) amino acid sequence in place of the native insulin Pro(B_{28}). NOVOLOG® is an injectable, fast-acting insulin. NOVOLOG® is also available as mix with insulin aspart protamine and commercially referred to as NovoLog Mix 70/30, which contains 30% insulin aspart and 70% insulin aspart protamine. The insulin aspart protamine portion is a crystalline form of insulin aspart, which delays the action of the insulin, giving NovoLog Mix 70/30 a prolonged absorption profile after injection.

[0004] Native insulin is a hormone that is synthesized in the body in the form of a single-chain precursor molecule, proinsulin. Proinsulin is a molecule comprised of a prepeptide of 24 amino

acids, followed by the B-chain peptide, a C-peptide of 35 amino acids, and the A-chain peptide. The C-peptide of this precursor insulin molecule ("proinsulin") contains the two amino acids, lysine-arginine (KR) at its carboxy end (where it attaches to the A-chain), and the two amino acids, arginine-arginine (RR) at its amino end (where it attaches to the B-chain). In the mature insulin molecule, the C-peptide is cleaved away from the peptide so as to leave the A-chain and the B-chain connected directly to one another in its active form.

[0005] Molecular biology techniques have been used to produce human proinsulin. In this regard, three major methods have been used for the production of this molecule. Two of these methods involve *Escherichia coli*, with either the expression of a large fusion protein in the cytoplasm (Chance *et al.* (1981), and Frank *et al.* (1981) in Peptides: Proceedings of the 7th American Peptide Chemistry Symposium (Rich, D. and Gross, E., eds.), pp. 721-728, 729-739, respectively, Pierce Chemical Company, Rockford, IL), or the use of a signal peptide to enable secretion into the periplasmic space (Chan *et al.* (1981) P.N.A.S., USA., 78:5401-5404). A third method utilizes yeast, especially *Saccharomyces cerevisiae*, to secrete the insulin precursor into the medium (Thim, *et al.* (1986), P.N.A.S., USA., 83: 6766-6770).

[0006] Chance *et al.* reported a process for preparing insulin by producing each of the A and B chains of insulin in the form of a fusion protein by culturing *E. coli* that carries a vector compromising a DNA encoding the fusion protein, cleaving the fusion protein with cyanogen bromide to obtain the A and the B chains, sulfonating the A and B chains to obtain sulfonated chains, reacting the sulfonated B chain with an excess amount of the sulfonated A chain; and then purifying the resultant products to obtain insulin. Drawbacks associated with this process are that it requires two fermentation processes and the requirement of a reaction step for preparing the sulfonated A chain and the sulfonated B chain. This results in a low insulin yield.

[0007] Frank *et al.* described a process for preparing insulin in the form of a fusion protein in *E. coli.* In this process, proinsulin is produced in the form of a fusion protein by culturing *E. coli* which carries a vector comprising a nucleic acid sequence (DNA) encoding for the fusion protein, cutting the fusion protein with cyanogens bromide to obtain proinsulin, sulfonating the proinsulin and separation of the sulfonated proinsulin, refolding the sulfonated proinsulin to form correct disulfide bonds, treating the refolded proinsulin with trypsin and carboxypeptidase B, and then purifying the resultant product to obtain insulin. However, the yield of the refolded proinsulin having correctly folded disulfide bonds is reported to sharply decrease as the

concentration of the proinsulin increases. This is allegedly due to, at least among other reasons, misfolding of the protein, and some degree of polymerization being involved. Hence, the process entails the inconvenience of using laborious purification steps during the recovery of proinsulin.

[0008] Thim *et al.* reported a process for producing insulin in yeast, *Saccharomyces cerevisiae*. This process has the steps of producing a single chain insulin analog having a certain amino acid sequence by culturing *Saccharomyces cerevisiae* cells, and isolating insulin therefrom through the steps of: purification, enzyme reaction, acid hydrolysis and a second purification. This process, however, results in an unacceptably low yield of insulin.

[0009] The role of the native C-peptide in the folding of proinsulin is not precisely known. The dibasic terminal amino acid sequence at both ends of the C-peptide sequence has been considered necessary to preserve the proper processing and/or folding of the proinsulin molecule to insulin.

[0010] Other amino acids within the C-peptide sequence, however, have been modified with some success. For example, Chang *et al.* (1998) (Biochem. J., 329:631-635) described a shortened C-peptide of a five (5) amino acid length, -YPGDV- (SEQ ID NO: 1), that includes a preserved terminal di-basic amino acid sequence, RR at one terminal end, and KR at the other terminal end, of the peptide. Preservation of the dibasic amino acid residues at the B-chain-C peptide and C-peptide-A-chain junctures is taught as being a minimal requirement for retaining the capacity for converting the proinsulin molecule into a properly folded mature insulin protein. The production of the recombinant human insulin was described using *E. coli* with a shortened C-peptide having a dibasic amino acid terminal sequence. US Pat. No. 5,962,267 also describes dibasic terminal amino acid sequences at both ends of the C-peptide.

[0011] One of the difficulties and/or inefficiencies associated with the production of recombinant insulin employing a proinsulin construct having the conserved, terminal di-basic amino acid sequence in the C-peptide region is the presence of impurities, such as Arg(A_0)-insulin, in the reaction mixture, once enzymatic cleavage to remove the C-peptide is performed. This occurs as a result of misdirected cleavage of the proinsulin molecule so as to cleave the C-peptide sequence away from the A-chain at this juncture, by the action of trypsin. Trypsin is a typical serine protease, and hydrolyses a protein or peptide at the carboxyl terminal of an arginine or lysine residue (*Enzymes*, pp. 261-262 (1979), ed. Dixon, M. & Webb, E. C. Longman Group Ltd., London). This unwanted hydrolysis results in the unwanted Arg(A_0)-insulin by-

product, and typically constitutes about 10% of the reaction yield. Hence, an additional purification step is required. The necessity of an additional purification step makes the process much more time consuming, and thus expensive, to use. Moreover, an additional loss of yield may be expected from the necessity of this additional purification step.

[0012] Others have described the use of proinsulin constructs that do not have a conserved terminal dibasic amino acid sequence of the C-peptide region. For example, US Pat. No. 6,777,207 (Kjeldsen *et al.*) relates to a novel proinsulin peptide construct containing a shortened C-peptide that includes the two terminal amino acids, glycine-arginine or glycine-lysine at the carboxyl terminal end that connects to the A-chain of the peptide. The B-chain of the proinsulin construct described therein has a length of 29 amino acids, e.g., desthreonine, in contrast to the native 30 amino acid length of the native B-chain in human insulin. The potential effects of this change to the native amino acid sequence of the B-chain in the human insulin produced are yet unknown. Methods of producing insulin using these proinsulin constructs in yeast are also described. Inefficiencies associated with correct folding of the mature insulin molecule when yeast is used as the expression host, render this process, among other things, inefficient and more expensive and time consuming to use. In addition, yeast provides a relatively low insulin yield, due to the intrinsically low expression levels of a yeast system as compared to *E. coli*.

[0013] In addition, US Pat. No. 5,618,913 describes various insulin analogs prepared by transpeptidation of an insulin analog (B_1-B_{29})-Ala-Ala-Lys-(A_1-A_{21}) with an threonine ester and acidolysis thereof with trifluoroacetic acid.

[0014] As evidenced from the above review, a present need exists for a more efficient process for production of aspart insulin analogs that is efficient and that may also improve and/or preserve acceptable production yield requirements of the pharmaceutical industry.

[0015] The above references are incorporated by reference herein where appropriate for appropriate teachings of additional or alternative details, features and/or technical background.

Summary of the Invention

[0016] The present invention provides processes for using a modified proinsulin sequence to produce aspart insulin analogs. The modified proinsulin sequence has the formula

$$R_1\text{-}(B_1\text{-}B_{26})\text{-}B_{27}\text{-}B_{28}\text{-}B_{29}\text{-}B_{30}\text{-}R_2\text{-}R_3\text{-}X\text{-}R_4\text{-}R_5\text{-}(A_1\text{-}A_{20})\text{-}A_{21}\text{-}R_6 \qquad \text{Formula I}$$

[0017] wherein

[0018] R_1 is a tag sequence containing one or more amino acids, preferably with a C-terminal Arg or Lys, or R_1 is absent with an Arg or Lys present prior to the start of the B chain;

[0019] (B_1-B_{26}) and (A_1-A_{20}) comprise amino acid sequences of native human insulin;

[0020] B_{27} is Thr, Asp, or Glu;

[0021] B_{28} is Asp, Glu, or Pro, preferably Asp;

[0022] B_{29} is Lys, or Pro, preferably Lys;

[0023] B_{30} is Ala, Thr, or is absent, preferably Thr;

[0024] R_2, R_3 and R_5 are Arg;

[0025] R_4 is any amino acid other than Gly, Lys or Arg or is absent, preferably Ala;

[0026] X is a sequence comprises one or more amino acids or is absent, provided that X does not comprise a C-terminal Gly, Lys, or Arg when R_4 is absent;

[0027] A_{21} is Asn, Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Ser, Thr, Tyr, Asp, or Glu; and

[0028] R_6 is a tag sequence containing one or more amino acids, preferably with a N-terminal Arg or Lys, or R_6 is absent.

[0029] One aspect of the present invention is related to a process for producing aspart insulin analogs comprising the steps of culturing *E. coli* cells under conditions suitable for expression of a modified proinsulin sequence of Formula I, disrupting the cultured *E. coli* cells to provide a composition comprising inclusion bodies containing the modified proinsulin sequence, solubilizing the composition of inclusion bodies, and recovering aspart insulin analogs from the solubilized composition.

[0030] In another aspect of the present invention is related to a process for producing aspart insulin analogs comprising the steps of providing a modified proinsulin sequence of Formula I, folding the modified proinsulin sequence to provide an aspart proinsulin derivative peptide, purifying the aspart proinsulin derivative peptide using metal affinity chromatography, enzymatically cleaving the aspart proinsulin derivative peptide to remove a connecting peptide and provide an intermediate solution comprising aspart insulin analog, and purifying the intermediate solution using chromatography column(s) to yield the aspart insulin analog.

[0031] Additional advantages, objects, and features of the invention will be set forth in part in the description which follows and in part will become apparent to those having ordinary skill in the art upon examination of the following or may be learned from practice of the invention. The

objects and advantages of the invention may be realized and attained as particularly pointed out in the appended claims.

Brief Description of the Figures

[0032] The invention will be described in detail with reference to the following drawings in which like reference numerals refer to like elements wherein.

[0033] FIG. 1, according to one aspect of the invention, is a vector map of plasmid pTrcHis2A (Kan) with a aspart proinsulin gene insert.

[0034] FIG. 2a and FIG 2b, according to aspects to the invention, are process flow schemes for the purification of aspart insulin analogs.

Detailed Description

[0035] The present invention generally relates to the preparation of insulin analogs, specifically aspart insulin analog, from modified proinsulin sequences. Aspart insulin analog comprises a modified B-chain having Asp(B_{28}). Modified proinsulin sequences refer to a single-chain polypeptide that may be converted into human insulin or insulin analogs and comprise a connecting peptide (C-peptide) having at least one non-dibasic terminal amino acid sequences. In one embodiment, non-dibasic terminal amino acid sequences may comprise (any amino acid except Lys or Arg-Arg ((any except R or K)R), and more preferably (any amino acid except Gly, Lys, or Arg-Arg ((any except G, R, or K)R). In one embodiment the terminal amino acid sequence may comprise Ala-Arg. Advantageously, the positioning of these particular terminal amino acids in the C-peptide provides for an improved method for producing recombinant aspart insulin analog, having fewer steps, improved yields of the recombinant aspart insulin analog and less contaminating byproducts.

[0036] The process for producing aspart insulin analogs of the invention presents many advantages, among them the advantage of reducing and/or eliminating the presence of unwanted and contaminating cleavage by-products characteristic of conventional manufacturing processes for producing recombinant human insulin in *E. coli.* Previously undesirable by-products evident in yield mixtures using conventional methods of producing recombinant human aspart insulin analogs included, by way of example, the production of an unwanted cleavage product, Arg(A_0)-insulin analogs. A highly efficient process for the production of recombinant human insulin analogs is presented that reduces and/or eliminates the presence of this and other unwanted and

undesirable cleavage by-products, and that further presents the advantages of eliminating several time consuming, expensive, purification steps. A process having fewer technician-assisted steps is thus devised, and illustrates the additional advantage of eliminating the degree of inconsistency and/or error associated with technician assisted steps in the manufacturing process.

[0037] In one embodiment, the modified aspart proinsulin sequence of the present invention has the formula

$$R_1\text{-}(B_1\text{-}B_{26})\text{-}B_{27}\text{-}B_{28}\text{-}B_{29}\text{-}B_{30}\text{-}R_2\text{-}R_3\text{-}X\text{-}R_4\text{-}R_5\text{-}(A_1\text{-}A_{20})\text{-}A_{21}\text{-}R_6 \qquad \text{Formula I}$$

[0038] wherein

[0039] R_1 is a tag sequence containing one or more amino acids, preferably with a C-terminal Arg or Lys, or R_1 is absent with an Arg or Lys present prior to the start of the B chain;

[0040] $(B_1\text{-}B_{26})$ and $(A_1\text{-}A_{20})$ comprise amino acid sequences of native human insulin;

[0041] B_{27} is Thr, Asp, or Glu;

[0042] B_{28} is Asp, Glu, or Pro, preferably Asp;

[0043] B_{29} is Lys, or Pro, preferably Lys;

[0044] B_{30} is Ala, Thr, or is absent, preferably Thr;

[0045] R_2, R_3 and R_5 are Arg;

[0046] R_4 is any amino acid other than Gly, Lys or Arg or is absent, preferably Ala;

[0047] X is a sequence comprises one or more amino acids or is absent, provided that X does not comprise a C-terminal Gly, Lys, or Arg when R_4 is absent;

[0048] A_{21} is Asn, Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Ser, Thr, Tyr, Asp, or Glu; and

[0049] R_6 is a tag sequence containing one or more amino acids, preferably with a N-terminal Arg or Lys, or R_6 is absent.

[0050] R_1 or R_6 in the modified aspart proinsulin of Formula I comprises a pre or post-peptide that may be a native pre-peptide or an N-terminal multiple His-tag sequence, or any other commercially available tag utilized for protein purification, e.g. DSBC, Sumo, Thioredein, T7, S tag, Flag Tag, HA tag, VS epitope, Pel B tag, Xpress epitope, GST, MBP, NusA, CBP, or GFP. In one embodiment at least one of R_1 or R_6 is present in Formula I. It is preferably that the terminal amino acid of the pre or post-peptide that connects to the B-chain or A-chain comprise Arg or Lys. Native pre-peptide has the sequence of MALWMRLLPLLALLALWGPDPAAA (SEQ ID NO: 2). In preferred embodiments, the N-terminal multiple His-tagged proinsulin

construct comprises a 6-histidine N-terminal tag ("6-histidine" disclosed as SEQ ID NO: 3) and may have the sequence of MHHHHHHGGR (SEQ ID NO: 4). The modified proinsulin sequence may replace the native 24 amino acid pre-peptide with the 6-histidine N-terminal tag sequence ("6-histidine" disclosed as SEQ ID NO: 3). In some embodiments, R_1 and/or R_6 may be a sequence of one or more amino acids, e.g., preferably from 1 to 30 and more preferably from 6 to 10.

[0051] Native insulin comprises an A-chain having the sequence GIVEQCCTSICSLYQLENYCN (SEQ ID NO: 5) and a B-chain having the sequence FVNQHLCGSHLVEALYLVCGERGFFYTPKT (SEQ ID NO: 6). According to the invention, the B-chain of Formula I is modified from native insulin and contains at least one amino acid mutation, substitution, deletion, insertion, and/or addition. For aspart insulin analogs, preferably B_{28} of the B-chain is modified. The proline B_{28} of native insulin is substituted with aspartic acid. In addition B_{27}, B_{29}, and B_{30} are preferably native amino acid residues. In one embodiment, the B-chain that is modified is wild-type human insulin B-chain. In another embodiment, the B-chain that is modified is wild-type porcine insulin B-chain.

[0052] As used in the description of the present invention, the term "connecting peptide" or "C-peptide" is meant the connecting moiety "C" of the B-C-A polypeptide sequence of a single chain proinsulin molecule. As in the native human proinsulin, the N-terminus of the C-peptide connects to C-terminus of the modified B-chain, e.g., position 30 of the B-chain, and the C-terminus of the C-peptide connects to N-terminus of the A-chain, e.g., position 1 of the A-chain.

[0053] In one embodiment, the C-peptide may have a sequence of the formula:

$$R_2\text{-}R_3\text{-}X\text{-}R_4\text{-}R_5 \quad \text{Formula II}$$

[0054] wherein R_2, R_3, R_4, R_5, and X have the same meaning as in Formula I. In one embodiment, X may be a sequence having up to 40 amino acids, preferably up to 35 amino acids or more preferably up to 30 amino acids. Although X may be any amino acid sequence, in one embodiment, X is preferably not EAEALQVGQVELGGGPGAGSLQPLALEGSLQ (SEQ ID NO: 7).

[0055] The C-peptide sequences of the present invention may include:

[0056] (1) RREAEDLQVGQVELGGGPGAGSLQPLALEGSLQAR (SEQ ID NO: 8);

[0057] (2) RREAEDLQVGQVGLGGGPGAGSLQPLALEGSLQAR (SEQ ID NO: 9);

[0058] (3) RREAEALQVGQVGLGGGPGAGSLQPLALEGSLQAR (SEQ ID NO: 10);

[0059] (4) RREAEDLQVGQVELGGGPGAGSLQPLAIEGSLQAR (SEQ ID NO: 11);

[0060] (5) RREAEDLQVGQVGLGGGPGAGSLQPLAIEGSLQAR (SEQ ID NO: 12);

[0061] (6) RREAEALQVGQVGLGGGPGAGSLQPLAIEGSLQAR (SEQ ID NO: 13); or

[0062] (7) RREAEALQVGQVELGGGPGAGSLQPLALEGSLQAR (SEQ ID NO: 14).

[0063] In the above embodiments, the designation where A appears at the terminal end of the C-peptide sequence, AR cannot be replaced with KR or RR.

[0064] Preferred modified aspart-proinsulin sequences of the present invention may include:

[0065] (1)FVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGGGPG AGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCN (SEQ ID NO: 15);

[0066] (2)MHHHHHHGGRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQ VGQVELGGGPGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCN (SEQ ID NO: 16);

[0067] (3)MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGF FYTDKTRREAEDLQVGQVELGGGPGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLEN YCN (SEQ ID NO: 17);

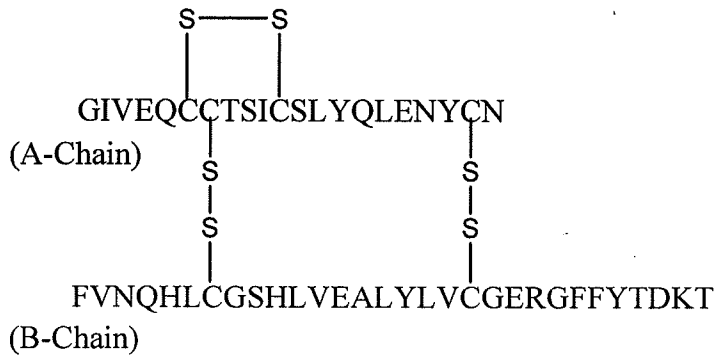
[0068] (4)MHHHHHHGGRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQ VGQVELGGGPGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCNRHHHHHH (SEQ ID NO: 18);

[0069] (5)MHHHHHHGGRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQ VGQVELGGGPGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCNKHHHHHH (SEQ ID NO: 19);

[0070] (6)MRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGG GPGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCNRHHHHHH (SEQ ID NO: 20);

[0071] (7)MRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGG GPGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCNKHHHHHH (SEQ ID NO: 21).

[0072] The single chain aspart insulin analogs of the invention will include three (3) correctly positioned, disulphide bridges, as is characteristic of the native human insulin. In some embodiments, the folded modified aspart proinsulin, or aspart proinsulin derivative peptide, may include three (3) correctly positioned, disulphide bridges. In the production, the C-peptide of the proinsulin derivative peptide is removed to produce the aspart insulin analog. Aspart insulin analogs of the invention have a sequence (SEQ ID NOS 5 and 22, respectively, in order of appearance) of the formula, where the three disulphide bridges are shown as –S–S–:



Formula III

[0073] The present invention provides modified proinsulin sequences having the modified C-peptide and methods for using these in a process to provide high yields of mature recombinant aspart insulin analog. Advantageously, the positioning of these particular terminal amino acids in the C-peptide may provide for an improved method for producing recombinant aspart insulin analog, having fewer steps, improved yields of the recombinant aspart insulin analog and less contaminating byproducts.

[0074] As used in the description of the present invention, the terms "insulin precursor" or "proinsulin" are described as a single-chain polypeptide in which, by one or more subsequent chemical and/or enzymatic processes, may be converted into human insulin or insulin analog.

[0075] A proinsulin analog or modified proinsulin is defined as a proinsulin molecule having one or more mutations, substitutions, deletions, and or additions, of the A, B and/or C chains relative to the native human proinsulin nucleic acid sequence. The proinsulin analogs are preferably such wherein one or more of the naturally occurring nucleic acids have been substituted with another nucleic acid within a triplet encoding for a particular amino acid. For purposes of convenience proinsulin analog is understood to refer to aspart proinsulin analog, unless otherwise specified.

[0076] The term "insulin analog" includes insulin molecules having one or more mutations, substitutions, deletions, additions, or modifications to one or more amino acids of a native insulin sequence. For example, in one embodiment, the native insulin sequence is porcine insulin, while in another embodiment, the native insulin sequence is human. For purposes of convenience insulin analog is understood to refer to aspart insulin analog, unless otherwise specified.

[0077] The term "a" as used in the description of the present invention is intended to mean "one or more", and is used to define both the singular and plural forms of the item or items to which it

references, or to a feature or characteristic to which it refers. The use of the singular or plural in the claims or specification is not intended to be limiting in any way and also includes the alternative form.

[0078] The term "about" is intended to be inclusive of and to encompass both an exact amount as well as an approximate amount or range of values or levels of the item, ingredient, element, activity, or other feature or characteristic to which it references. Generally, and in some embodiments, the term "about" is intended to reference a range of values relatively close to the specific numerical value specifically identified. For example, "about 3 grams to about 5 grams" is intended to encompass a measure of in or around a value of 3 grams, concentration values between 3 grams and 5 grams, concentration values in and around 5 grams, as well as concentration values that are exactly 3 grams and exactly 5 grams.

[0079] As used in the description of the present process, a high protein concentration of the proinsulin or insulin analog product is defined as a protein yield concentration of at least about 3 grams/liter, or between about 3 grams to about 5 grams per liter. The expression yield to be expected may be defined as a protein/peptide yield that is sufficient to detect via polyacrylamide gel electrophoreses (PAGE).

[0080] The invention provides a process for producing highly purified aspart insulin analog that is more efficient than current techniques. The invention in a general and overall sense relates to an improved process for preparing a heterologous recombinant protein in a prokaryotic host cell. This process is characterized in that it employs a recombinant protein that provides a useful and efficiently processed modified proinsulin sequence analog as described above.

[0081] The term "heterologous protein" is intended to mean that the protein in the prokaryotic host cell is not native, *i.e.*, it occurs as a protein in peculiar or foreign (i.e., not native to) the host prokaryotic cell.

[0082] The term "recombinant" is intended to mean produced or modified by molecular-biological methods. For example, according to one embodiment, a recombinant protein is made using genetic engineering techniques and is not found in nature.

[0083] As used in the description of the present invention, the term "heterologous recombinant protein" is defined as any protein known to the skilled person in the molecular biological arts, such as, for example, insulin, insulin analog, proinsulin, proinsulin analog, C-peptide, and proteins containing these together with any other protein or peptide fragment.

[0084] Prokaryotic host cells may be any host cells known to the skilled artisan in the molecular biological arts, and by way of example, *Escherichia coli*. Such types of cells available from public collections and useful in the practice of the present invention include, by way of example, the Deutsche Sammlung von Mikrooganismen and Zellkulturen GmbH, raunschweig, Germany, e.g., *E. coli* Strain K12 JM107 (DSM 3950).

[0085] The following reference table, Table 1, provides the triplet codons corresponding to each of the various amino acids that are used in the description of the present invention. As will be understood by those of skill in the art, the amino acid that may be used in any particularly defined position as part of any of the peptide, protein, or constructs otherwise defined herein by reference to a particular nucleotide triplet base pair may be encoded by a number of different nucleotide triplets that function to encode the same amino acid. For example, where the amino acid of the sequence defined herein is alanine (Ala, or A), the triplet codon of nucleic acids that may encode for this amino acid are: GCT, GCC, GCA, or GCG. The following table illustrates this definition of variables at and substitutions as can be applied to all of the naturally occurring amino acids sequences of the disclosure.

Table 1.

	U	C	A	G	
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGG Trp	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCG Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

[0086] It should be understood that process steps within the following description of the method may be modified, changed and/or eliminated, depending on the particular preferences of the processor and/or the particular mechanical apparatus available to the processor, as well as the specific reagents and/or materials available and/or convenience and/or economics of use.

[0087] The aspart insulin analog prepared by the present invention may be formulated as liquid aspart insulin analog or crystalline aspart insulin analog. According to an embodiment of the invention, a preparation of recombinant liquid aspart insulin analog is in a substantially liquid form and that has not been through a crystallization process. Eliminating these steps has no

negative impact on the purity of the liquid aspart insulin analog produced, but has the added advantage of reducing the amount of inactive insulin multimers in the liquid aspart insulin analog of the invention. Aspart insulin analog reconstituted from lyophilized and crystallized insulin may be contaminated with inactive insulin multimers and is less preferred.

[0088] According to one embodiment, the methods of producing aspart insulin analog described herein generally include the following steps: fermentation/expression, Inclusion body isolation, solubilization of aspart proinsulin analog; refolding processing and transformation of aspart proinsulin analog to aspart insulin analog; and purification of aspart insulin analog. FIGS. 2A and 2B illustrate flow charts of preferred processes steps in producing aspart insulin analog according to embodiments of the present invention.

[0089] Expression of aspart proinsulin analog may occur in a recombinant expression system. According to one embodiment, the recombinant expression system is an *E. coli* expression system containing aspart proinsulin analog expressing vectors. A working cell bank (WCB) of *E. coli* is provided as an example herein. The cells of the WCB may be vertebrate or invertebrate cells, such as prokaryote or eukaryote cells, and most preferably the cells may be mammalian, bacterial, insect, or yeast cells. In one embodiment, the cell is a bacterial cell and in a further embodiment, the bacteria is *E. coli*. In another embodiment, the cell is a yeast cell and in a further embodiment, the yeast cell is *S. cerevisiae or S. pombe.*

[0090] In one embodiment, *E. coli* cells may be cultured and disrupted to provide a composition comprising inclusion bodies. The inclusion bodies contain the modified proinsulin sequence. The aspart proinsulin analogs expressed by cells of the WCB according to the method of the invention may be secreted from the cells and include a secretory sequence. In other embodiments, aspart proinsulin analogs expressed by cells of the WCB are not secreted from the cells, and thus do not include a secretory sequence.

[0091] The step of solubilizing of the composition of inclusion bodies may involve adjusting the pH to achieve complete solubilization of the modified proinsulin sequences. In one embodiment, the inclusion bodies may be solubilized by adjusting the pH to at least 10.5, preferably from 10.5 to 12.5, and most preferably from 11.8-12. The pH may be adjusted by adding an alkali hydroxide such as NaOH or KOH to the composition of inclusion bodies. In addition, the step of solubilization may use one or more reducing agents and/or chaotropic agent. Suitable reducing agents may include those selected from the group consisting of 2-mercaptoethanol, L-cysteine

hydrochloride monohydrate, dithiothreitol, dithierythritol, and mixtures thereof. Suitable chaotropic agents include those selected from the group consisting of urea, thiourea, lithium perchlorate or guanidine hydrochloride, and mixtures thereof.

[0092] The solubilized inclusion bodies may be mixed in a refolding buffer, such as glycine or sodium carbonate, at a pH of 7 to 12, preferably from 10 to 11, preferably from 10.5 to 11, to refold the modified proinsulin sequences to a proinsulin derivative peptide, e.g., aspart proinsulin derivative peptide. The solution with refolded material should be pH adjusted to 7 to 9, preferably 7.8 to 8.2, with or without the addition of an alkaline salt, preferably sodium chloride to a final concentration of 100mM to 1M final concentration, preferably 500mM to 1M, preferably 700mM, and may be filtered and loaded onto a column, such as an immobilized metal-ion affinity chromatography (IMAC) column. Commercially available resins suitable for embodiments of the present invention include Nickle Sepharose 6 Fast Flow (GE Healthcare), Nickle NTA Agarose (GE Healthcare), Chelating Sepharose Fast flow(GE Healthcare), IMAC Fast Flow (GE Healthcare).

[0093] Proinsulin derivative peptide is subject to concentration by tangential flow filtration or diafiltration. Next, proinsulin derivative peptide is enzymatically cleaved, preferred by subjecting the proinsulin derivative peptide to trypsin digestion. Although embodiments of the present invention may use commercially available rat, bovine, porcine or human trypsins or other isoenzymes or derivatives or variants thereof, it is also possible to use the following enzymes: trypsin from Fusarium oxysporum and from Streptomyces (*S. griseus, S. exfoliatus, S. erythraeus, S. fradiae* and *S. albidoflavus*), tryptase, mastin, acrosin, kallikrein, hepsin, prostasin I, lysyl endopeptidase (Lysin-C) and endoproteinase Arg-C (clostripain). In one embodiment, trypsin digestion occurs at pH from about 7 to 10, and more preferably from 8.1 to 8.3. In a further embodiment, the trypsin digest is quenched by adding glacial acetic acid. While it is contemplated that other additives may be employed, acetic acid appears to be most preferred and stable for this purpose.

[0094] Trypsin is an enzyme that has specific cleavage activity at the terminal arginine residues, and to a lesser extent, lysine residues, of the C-peptide. In the transformation reaction, it is required that the terminal arginine or lysine residues of the C-peptide be removed. In native human proinsulin, when trypsin cleaves at the lysine in position 64, it will be unable to remove the arginine at position 65, due to the fact that it requires at least one residue on both sides of a

cleavage site. What results is the production of an unwanted by-product, arg(A_0)-insulin. This by-product constitutes a small loss in yield and generates an undesired contaminant. By converting this lysine 64 into another uncharged amino acid, particularly alanine, the arg(A_0)-insulin byproduct is preferentially not formed. When formed is less than 10%, and more preferably is less than 0.3% of total byproducts from the trypsin transformation reaction may be arg(A_0). This is because the trypsin no longer acts to cleave at this particular site of the proinsulin derivative peptide.

[0095] The proinsulin derivative peptide, may also be subjected to carboxypeptidase B digestion. In one embodiment, a trypsin inhibitor may be added to the insulin prior to addition of carboxypeptidase B. Trypsin inhibitor is added in an equal amount to the amount of trypsin added for the trypsin digest step. In another embodiment, a glycine solution is added to aspart proinsulin analog prior to addition of carboxypeptidase B. For example, in some embodiments, glycine is added to adjust the pH of the insulin solution to about 9.6±0.1. The target concentration of glycine is 50mM using a 1M glycine stock. In some embodiments, the carboxypeptidase B is permitted to digest for at least 1-16 hours, preferably at least 8 hours. A minimum of 10 hours is preferred, but overdigestion is rarely a significant issue so there is no maximum time limit.

[0096] After trypsin digestion the intermediate solution is preferably purified in a chromatography column, such as a ion exchange chromatography column or reverse phase chromatography. In one embodiment, the intermediate solution may be purified in a chromatography column by eluting the aspart insulin analog using a buffer comprising n-propanol or acetonitrile. The buffer may also further comprise sodium sulfate and phosphoric acid.

[0097] The manufacturing process described herein results in a preparation of aspart insulin analog in liquid active pharmaceutical ingredient (API) form. The process eliminates the need to prepare a crystallized insulin that is later reconstituted. As a result of eliminating the crystallization and drying steps, the amount of inactive insulin multimers present in the liquid formulation is reduced in comparison to the amounts otherwise present in crystallized forms of insulin and reconstituted crystallized insulin. Although crystallization is less preferred, in some embodiments, a crystallization step may be included to produce aspart insulin analog API crystals. The aspart insulin analog may be crystallized to allow for increased shelf life to the API

material. However, as mentioned the crystallization process will lead to increased levels of multimers and in turn an overall lower purity.

[0098] Aspart insulin analog may prevent the formation of non-monomeric insulin, such as dimers and hexamers. Accordingly, upon administration of the aspart insulin analog to a patient, larger amounts of active monomeric insulin are available to act in the patient. In particular, aspart insulin analog is particularly suitable for postprandial, i.e., after eating, injection as it is available immediately for use by the patient to control glucose levels. Accordingly, this analog has the advantage over native insulin in that its short delay of onset allows more flexibility with eating schedules for diabetic patients than regular insulin which requires a longer waiting period between injection and eating. According to one embodiment of the invention, the aspart insulin analog is provided to a patient in combination with a longer acting insulin to provide optimal glycemic control.

[0099] In some embodiments, the preparations comprise a pharmaceutically acceptable preparation comprising recombinant aspart insulin analog and being essentially free of modified proinsulin sequences.

[0100] It should be understood that process steps within the following description of the method may be modified, changed and/or eliminated, depending on the particular preferences of the processor and/or the particular mechanical apparatus available to the processor, as well as the specific reagents and/or materials available and/or convenience and/or economics of use.

Example 1—Preparation of an *E. coli* clone expressing aspart proinsulin

[0101] The preparation of a *E. coli* containing cells capable of expressing recombinant aspart proinsulin is carried out according to the following processes.

[0102] Step 1: Construction of a purified aspart proinsulin gene segment for insertion into the vector. The initial gene construct was synthesized in a basic cloning vector. The gene construct included the N-terminal histidine tag, MHHHHHHGGR (SEQ ID NO: 4), modified B-chain, and modified C-peptide with the alanine codon in place of the native lysine and having the amino acid sequence:
MHHHHHHGGRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGGG PGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCN (SEQ ID NO: 16). The gene was flanked by Nde1 and EcoR1 restriction sites, for subsequent subcloning into the desired

expression vector. The codons selected were optimized for expression in *E.coli*. The following sequence represents the pTrcHis2a(Kan) vector with aspart proinsulin insert (FIG. 1). The IPTG inducible promoter region which regulates the transcription rate is shown by a dotted underlined, while the aspart proinsulin insert, adjacent the promoter region, is shown by a solid underlined. The sequence indicated as bolded and italicized is the Kanamycin gene, which provides the antibiotic selection marker for the vector.

5′GTTTGACAGCTTATCATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGG
AAGCTGTGGTATGGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTC
CCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGAAATGAGC
TGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACAC
AGGAAACAGCGCCGCTGAGAAAAAGCGAAGCGGCACTGCTCTTTAACAATTTATCAGACAATCT
GTGTGGGCACTCGACCGGAATTATCGATTAACTTTATTATTAAAAATTAAAGAGGTATATATTA
ATGTATCGATTAAATAAGGAGGAATAAACCATGATGCATCATCATCATCATCATGGTGGCCGCT
TTGTGAACCAACACCTGTGCGGCTCACACCTGGTGGAAGCTCTCTACCTAGTGTGCGGGGAACG
AGGCTTCTTCTACACAGACAAGACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAG
CTGGGCGGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGCAGAAGC
GTGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCGG
CTAGGAATTCGAAGCTTGGGCCCGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGT
CGACCATCATCATCATCATCATTGAGTTTAAACGGTCTCCAGCTTGGCTGTTTTGGCGGATGAG
AGAAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACAGAATTT
GCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAACTCAGAAGTGAAACGCCGT
AGCGCCGATGGTAGTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAA
CGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCC
TGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCG
GGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGC
CTTTTTGCGTTTCTACAAACTCTTTTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCT
CATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAA
CATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAG
AAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACT
GGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGC
ACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCG
GTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTCCTGAATCGCCCCATCATCCAGCCA
GAAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTTGGTGATTTTGAAC
TTTTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAAGATGCGTGATCTGATCCTTCAACTCAG
CAAAAGTTCGATTTATTCAACAAAGCCGCCGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTGT
TACAACCAATTAACCAATTCTGA***TTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTAT***
TCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTC
ACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACA
TCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAG
TGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGG
CCAGCCATTACGCTCGTCATCAAAATCACTGCATCAACCAAACCGTTATTCATTCGTGATTGCG
CCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAA
CCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAAT
ACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGA

TAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATC
TGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTC
CCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCAT
ATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTCGAGCAAGACGTTTCCCGTTGAATATG
GCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATA
TTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTGTTGAATAAATC
GAACTTTTGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGTTCCGTGGC
AAAGCAAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTCC
CTCACTTTCTGGCTGGATGATGGGGCGATTCAGGACTCACCAGTCACAGAAAAGCATCTTACGG
ATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAA
CTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGAT
CATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTG
ACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTAC
TCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTG
CGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTC
GCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGAC
GGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATT
AAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATT
TTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACG
TGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCT
TTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTT
TGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACC
AAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCT
ACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTA
CCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTC
GTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTA
TGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCG
GAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGG
GTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGG
AAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGT
TCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATAC
CGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTG
ATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTA
CAATCTGCTCTGATGCCGCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTC
ATGGCTGCGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGG
CATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTC
ATCACCGAAACGCGCGAGGCAGCAGATCAATTCGCGCGCGAAGGCGAAGCGGCATGCATTTACG
TTGACACCATCGAATGGTGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCA
ATTCAGGGTGGTGAATGTGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCT
TATCAGACCGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAG
TGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACTGGCGGGCAA
ACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTC
GCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAA
GCGGCGTCGAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGAT
CATTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCG
GCGTTATTTCTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGACG
GTACGCGACTGGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGG

CCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAATATCTCACTCGCAAT
CAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCA
TGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCT
GGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGA
TACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTCAACCACCATCAAACAGGATTTTC
GCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGG
CAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACC
GCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAA
GCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCGCGAATTGATCTG 3′(SEQ ID NO:
23)

[0103] The modified proinsulin sequence without the tag is as follows:

TTTGTGAACCAACACCTGTGCGGCTCACACCTGGTGGAAGCTCTCTACCTAGTGTGCGGGGAAC
GAGGCTTCTTCTACACAGACAAGACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGA
GCTGGGCGGGGGCCCTGGTGCAGGCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGCAGAAG
CGTGGCATTGTGGAACAATGCTGTACCAGCATCTGCTCCCTCTACCAGCTGGAGAACTACTGCG
GCTAG (SEQ ID NO: 24)

[0104] Step 2: Generation of the pTrcHis2A(Kan) vector containing aspart proinsulin. Commercially available pTrcHis2A(Kan) vector was modified to include a Kanamycin resistance gene in the middle of the Ampicillin resistance gene to negate the Ampicillin resistance prior to insertion of the proinsulin sequence into the vector. Ampicillin resistance heightens the potential for allergic reactions to preparations made using vector constructs that include the Ampicillin resistance gene. Therefore it is preferable to eliminate the Ampicillin resistance in the constructs that are prepared and used.

[0105] The pTrcHis2A(Kan) vector was modified at the start codon in the multiple cloning site by replacing the Nco1 restriction site with an Nde1 site to simplify subsequent subcloning work.

Nco1 = CCATGG - Nde1 = CATATG

[0106] The proinsulin gene was isolated from the DNA 2.0 plasmid using Nde1 to cleave at the N-terminal side of the gene and EcoR1 to cleave at the C-terminal side of the gene. The Digested DNA was run over a 2% agarose gel to separate the plasmid DNA from the aspart proinsulin gene. A QIAquick™ (Qiagen) gel purification kit was then used to purify the gene construct.

[0107] Accordingly, a sequential digest of the vector with Nde1 and EcoR1, respectively, was performed. The vector DNA was also purified using a QIAquick gel purification kit.

Following purification of the vector and the gene, a 5' NdeI and a 3' EcoR1 ligation reaction was utilized to insert the proinsulin gene into the pTrcHis2A(Kan) vector.

[0108] Step 3: Transformation. One microliter of the ligation reaction was used to transform competent *E. coli* cells BL21 with the pTrcHis2A(Kan) plasmid containing the proinsulin gene. The transformed *E. coli* BL21 cells were plated on LB-Kan agar plates and incubated overnight at 37°C. Several clones were selected and sequenced. Clones with the correct sequence were then screened for expression.

[0109] The resulting clone is referred to as the aspart His Tagged proinsulin pTrcHis2A(Kan) vector.

[0110] Step 4: Preparation of the working cell bank (WCB). To establish the WCB, sterile growth medium was inoculated with the recombinant BL21 *E. coli* containing the His Tagged aspart proinsulin/pTrcHis2A(Kan) vector and incubated to allow cell growth. The cells were harvested in an ISO5 (class 100) environment under a biosafety cabinet via centrifugation. Sterile medium and glycerol were added to the cells. 1 mL aliquots of the cells were then dispensed into sterile ampoules and stored at -80°C. Aseptic techniques were utilized to generate the WCB.

Example 2—Product Manufacture of Aspart Insulin Analog from Modified Proinsulin Sequence

[0111] Step 1 – Culturing of *E. coli* transformed with aspart modified proinsulin sequence from the WCB of Example 1. Seed an inoculum preparation of the WCB in a sterile growth medium that includes yeastolate (purchased from VWR, Prod. # 90004-426 or – 488), select phytone, sodium chloride, purified water, sterile Kanamycin solution), and incubate until growth to an Optical density (OD_{600nm}) of 2 to 4. Prepare a fermentation media (containing select phytone, yeastolate, glycerin, BioSpumex 153K (Cognis, Inc.) in a fermentor. Add the following sterilized phosphate solutions to the Fermentor. Prepare a Phosphate flask 1 – potassium phosphate monobasic and potassium phosphate dibasic containing Kanamycin solution. Prepare a Phosphate flask 2 – potassium phosphate monobasic and potassium phosphate dibasic. Add seed inoculate of E. coli to the Fermentor – growth to O.D. (optical density) 600nm of 8 to 10 (mid log phase). Add a dioxane free IPTG (purchased from Promega, Catalog No. #PA V3953 (VWR Catalog #PAV3953) solution to the fermentor (to induce transcription of the K64A aspart proinsulin gene). Incubate for 4 hours. This results in the production of a concentrated cell

suspension containing His-tagged aspart proinsulin inclusion bodies. The cell suspension is then centrifuged to provide a cell paste for the subsequent inclusion body isolation step.

[0112] Step 2 – Disruption —Cells containing inclusion bodies expressing aspart modified proinsulin sequence are lysed in a basic Tris/salt buffer, using a Niro Soavi homogenizer (1100-1200 bar).

[0113] Step 3 - Inclusion Body Washing—Contaminant protein removal is accomplished via two sequential washes with a Tris/Triton X-100 buffer, followed by two sequential washes with a Tris/Tween-20 buffer, and finally a single wash with a Tris/NaCl buffer.

[0114] Step 4 - Solubilization—Inclusion bodies enriched with the modified proinsulin peptide are solubilized in 4-8M urea, preferably about 6-8 urea, containing reducing agents (2-mercaptoethanol, L-cysteine hydrochloride monohydrate). Complete solubilization is achieved by adjusting the pH to 10.5-12, preferably 11.8-12 with NaOH.

[0115] Step 5 - Dilution refolding—The solubilized protein is then diluted into refolding buffer (20 mM Glycine, pH 10-11 at 6-10°C.) to a final concentration of 1 mg/ml and permitted to refold for 24 to 72 hours, preferentially about 48 hours, at 6-10°C. Higher protein concentration may be used in the refold if desired, however, overall refold efficiency will decrease. Sodium Chloride and Phosphate are then added to final concentrations of 700 mM and 25 mM respectively, followed by pH adjustment to 7.9-9.0, preferably 7.9 to 8.0, with 6M HCl.

[0116] Step 6 - IMAC Chromatography—The dilute proinsulin derivative is loaded onto an IMAC column to a maximum capacity of ≤ 26.5 mg main peak protein per ml of resin. A 75mM imidizole buffer is used to isocratically strip the majority of impurities from the column. Aspart proinsulin is eluted isocratically using ≤300 mM imidizole.

[0117] Step 7 - Buffer exchange—To the IMAC main peak pool material, add EDTA to a final concentration of 20 mM. Exchange the buffer using a membrane with a suitable molecular weight cutoff (e.g. 3000 Da). The final buffer should be at least 97% exchanged to a 20 mM Tris-Cl, pH 7 to 10, preferably 8.1, at 8-10°C. A protein concentration of approximately 5 mg/ml is desirable.

[0118] Step 8 Trypsin/Carboxypeptidase B Enzymatic Transformation/Proteolysis—The buffer exchanged sample is digested with a 1500:1 mass ratio of main peak protein to trypsin and 1000:1 mass ratio of main peak protein to carboxypeptidase B, in the presence of 5mM CaCl. The ratios of trypsin and carboxypeptidase may be increased or decreased depending on the

desired length of time for the reaction. Once complete, based on HPLC, the digest is then quenched by the addition of acetic acid to ≥ 700 mM, to a pH of approximately 3-3.5.

Example 3A Final Purification

[0119] After step 8 in Example 2, the final purification may proceed using alternative processes in Examples 3A or 3B.

[0120] Step 9a - Ion Exchange Chromatography—The digested material is loaded onto a cation exchange column and eluted with a NaCl gradient, in the presence of 20% n-propanol or at pH 4.0. RP-HPLC is used to pool the appropriate fractions containing the Aspart insulin peak of interest at the desired purity level.

[0121] Step 10a - Reverse Phase Chromatography—The S-column pool containing the Aspart insulin is loaded onto an RPC30 or C18 reverse phase column and eluted using an n-propanol or acetonitrile gradient in the presence of 200mM sodium sulfate and 0.136% phosphoric acid. Fractions are immediately diluted 1:4 with 100mM phosphate at pH 7.0-9.0, preferably 7.5-8.0 as they are collected. RP-HPLC is used to pool the appropriate fractions containing the Insulin peak of interest at the desired purity level.

[0122] Step 11a - Buffer Exchange—Exchange the sample into WFI (water for injection) using a membrane with a suitable molecular weight cutoff (e.g. 3000 Da). The pH of the solution should be monitored and maintained at 7.0-9.0, preferably 7.5-8.0. The final sample is concentrated to 5.5-8 mg/ml, with an adjusted pH of 7.0-9.0, preferably 7.5-8.0 at 6-10°C. This material represents the liquid API form of the presently disclosed preparations of Aspart Insulin Analog. The API should be stored in the dark at 6-10°C.

Example 3B Final Purification

[0123] Step 10b - Reverse Phase Chromatography—The digested material containing the Aspart insulin is loaded onto a RPC30 or C18 reverse phase column and eluted using a n-propanol or acetonitrile gradient in the presence of 200mM sodium sulfate and 0.136% phosphoric acid. Fractions are immediately diluted 1:4 with 100mM phosphate buffer at pH 7.0-9.0 preferably, 7.5-8.0 as they are collected. RP-HPLC is used to pool the appropriate fractions containing the aspart insulin peak of interest at the desired purity level.

[0124] Step 11b - Buffer Exchange—Exchange the sample into WFI using a membrane with a suitable molecular weight cutoff (e.g. 3000 Da). The pH of the solution should be monitored and maintained at 7.0-9.0, preferably 7.5-8.0. The final sample is concentrated to 5.5-8 mg/ml,

with an adjusted pH of 7.0-9.0, preferably 7.5-8.0 at 6-10°C. This material represents the liquid API form of the presently disclosed preparations and formulations of Aspart Insulin Analog.

Example 4 – API Formulation

[0125] The aspart Insulin Analog purified by Examples 3A or 3B is formulated by diluting the API material with cold WFI to a final concentration of 4.3375 mg/ml. A concentrated formulation buffer stock containing 80 mg/ml glycerol, 15.75 mg/ml meta cresol, 0.0985 mg/ml zinc chloride at pH 7.5 ± 0.1 is added to the API material in a 1/5 ratio of formulation buffer stock to API. The solution is mixed, followed by sterile filtration into appropriate vials in 10 ml aliquots.

Example 5 – Working Cell Bank

[0126] The preparation of a WCB (working cell bank) for research and development containing cells capable of expressing recombinant Aspart proinsulin is carried out according to the following processes:

[0127] The cloning procedure outlined in Example 1 is utilized to create the initial vector. Purified His Tagged Aspart proinsulin pTrcHis2A(Kan) vector is transformed into competent BL21 cells and plated on sterile LB-Kan plates. From the plates, an isolated colony is used to inoculate sterile LB-Kan media (~100mls). The cells are grown at 37°C to mid log phase (about 4-5 hours) OD_{600nm} of about 1.5-2.0. Culture media containing cells is then aliquoted into sterile cryovials, combined with glycerol at a 20% final concentration. The vials are then stored at 80°C.

WHAT IS CLAIMED IS:

1. A composition comprising an aspart proinsulin sequence having the formula

R_1-(B_1-B_{26})-B_{27}-B_{28}-B_{29}-B_{30}-R_2-R_3-X-R_4-R_5-(A_1-A_{20})-A_{21}-R_6 Formula I

wherein

R_1 is a tag sequence containing one or more amino acids or R_1 is absent with an Arg or Lys present prior to the start of the B chain;

(B_1-B_{26}) and (A_1-A_{20}) comprise amino acid sequences of native human insulin;

B_{27} is Thr, Asp, or Glu;

B_{28} is Asp, Glu, or Pro;

B_{29} is Lys, or Pro;

B_{30} is Ala, Thr, or is absent;

R_2, R_3 and R_5 are Arg;

R_4 is any amino acid other than Gly, Lys or Arg or is absent;

X is a sequence comprises one or more amino acids or is absent, provided that X does not comprise a C-terminal Gly, Lys, or Arg when R_4 is absent;

A_{21} is Asn, Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Ser, Thr, Tyr, Asp, or Glu; and

R_6 is a tag sequence containing one or more amino acids or R_6 is absent.

2. The composition of claim 1, wherein R_1 and/or R_6 is present and R_1 is tag sequence of one or more amino acids with a C-terminal Arg or Lys and/or R_6 tag sequence of one or more amino acids with a N-terminal Arg or Lys.

3. The composition of claim 1, wherein R_4 is Ala.

4. The composition of claim 1, wherein the modified proinsulin sequence comprises a connecting peptide sequence of a sequence having the formula

R_2-R_3-X-R_4-R_5 Formula II

wherein

R_2, R_3, R_4, R_5, and X are defined in claim 1.

5. The composition of claim 4, wherein the connecting peptide sequence is RREAEDLQVGQVELGGGPGAGSLQPLALEGSLQAR (SEQ ID NO: 8).

6. The composition of claim 1, wherein the modified aspart proinsulin sequence is FVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGGGPGAGSLQPLAL EGSLQARGIVEQCCTSICSLYQLENYCN (SEQ ID NO: 15).

7. The composition of claim 1, wherein the modified aspart proinsulin sequence is MHHHHHHGGRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGGG PGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCN (SEQ ID NO: 16).

8. The composition of claim 1, wherein the modified aspart proinsulin sequence is MHHHHHHGGRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGGG PGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCNRHHHHHH (SEQ ID NO: 18).

9. The composition of claim 1, wherein the modified aspart proinsulin sequence is MHHHHHHGGRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGGG PGAGSLQPLALEGSLQARGIVEQCCTSICSLYQLENYCNKHHHHHH (SEQ ID NO: 19).

10. The composition of claim 1, wherein the modified aspart proinsulin sequence is MRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGGGPGAGSLQPL ALEGSLQARGIVEQCCTSICSLYQLENYCNRHHHHHH (SEQ ID NO: 20).

11. The composition of claim 1, wherein the modified aspart proinsulin sequence is MRFVNQHLCGSHLVEALYLVCGERGFFYTDKTRREAEDLQVGQVELGGGPGAGSLQPL ALEGSLQARGIVEQCCTSICSLYQLENYCNKHHHHHH (SEQ ID NO: 21).

12. An expression vector comprising the nucleic acid sequence of claim 1.

13. The expression vector of claim 12, wherein the expression vector is His Tagged Aspart proinsulin pTrcHis2A(Kan).

14. A microorganism transformed with the vector of claim 13.

15. The microorganism of claim 14, further defined as an *E. coli* transformed with plasmid His Tagged Aspart proinsulin pTrcHis2A(Kan).

16. A process for producing aspart insulin analogs comprising the steps of:

(a) culturing under conditions suitable for expression *E. coli* cells having a modified proinsulin sequence of a formula

R_1-(B_1-B_{26})-B_{27}-B_{28}-B_{29}-B_{30}-R_2-R_3-X-R_4-R_5-(A_1-A_{20})-A_{21}-R_6 Formula I

wherein

R_1 is a tag sequence containing one or more amino acids or R_1 is absent with an Arg or Lys present prior to the start of the B chain;

(B_1-B_{26}) and (A_1-A_{20}) comprise amino acid sequences of native human insulin;

B_{27} is Thr, Asp, or Glu;

B_{28} is Asp, Glu, or Pro;

B_{29} is Lys, or Pro;

B_{30} is Ala, Thr, or is absent;

R_2, R_3 and R_5 are Arg;

R_4 is any amino acid other than Gly, Lys or Arg or is absent;

X is a sequence comprises one or more amino acids or is absent, provided that X does not comprise a C-terminal Gly, Lys, or Arg when R_4 is absent;

A_{21} is Asn, Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Ser, Thr, Tyr, Asp, or Glu; and

R_6 is a tag sequence containing one or more amino acids or R_6 is absent, to provide cultured *E. coli* cells;

(b) disrupting said cultured *E. coli* cells to provide a composition comprising inclusion bodies containing the modified aspart proinsulin sequence;

(c) solubilizing said composition of inclusion bodies; and

(d) recovering the aspart insulin analogs from said solubilized composition.

17. The process of claim 16, wherein the step of recovering the aspart insulin analogs further comprises:

(e) folding said modified aspart proinsulin sequence to provide an aspart proinsulin derivative peptide;

(f) purifying said aspart proinsulin derivative peptide using metal affinity chromatography

(g) enzymatically cleaving aspart proinsulin derivative peptide to remove a connecting peptide and provide an intermediate solution comprising aspart insulin analog; and

(h) purifying said intermediate solution using chromatography column(s) to yield the aspart insulin analog.

18. The process of claim 16, wherein the solubilization of said composition of inclusion bodies further comprises adjusting the pH to at least 10.5.

19. The process of claim 16, wherein the solubilization of said composition of inclusion bodies comprises adjusting the pH to 11.8 to 12.

20. The process of claim 16, wherein the solubilization of said composition of inclusion bodies includes one or more reducing agents selected from the group consisting of 2-mercaptoethanol, L-cysteine hydrochloride monohydrate, dithiothreitol, dithierythritol, and mixtures thereof.

21. The process of claim 16, wherein the solubilization of said composition of inclusion bodies includes the use one or more chaotropic agents selected from the group consisting of urea, thiourea, lithium perchlorate or guanidine hydrochloride and mixtures thereof.

22. A process for producing aspart insulin analogs comprising the steps of:

(a) providing an *E. coli* transformed to include a modified aspart proinsulin sequence having a formula I sequence under conditions suitable for expression of said sequence, Formula I as follows:

R_1-(B_1-B_{26})-B_{27}-B_{28}-B_{29}-B_{30}-R_2-R_3-X-R_4-R_5-(A_1-A_{20})-A_{21}-R_6 Formula I

wherein

R_1 is a tag sequence containing one or more amino acids or R_1 is absent with an Arg or Lys present prior to the start of the B chain;

(B_1-B_{26}) and (A_1-A_{20}) comprise amino acid sequences of native human insulin;

B_{27} is Thr, Asp, or Glu;

B_{28} is Asp, Glu, or Pro;

B_{29} is Lys, or Pro;

B_{30} is Ala, Thr, or is absent;

R_2, R_3 and R_5 are Arg;

R_4 is any amino acid other than Gly, Lys or Arg or is absent;

X is a sequence comprises one or more amino acids or is absent, provided that X does not comprise a C-terminal Gly, Lys, or Arg when R_4 is absent;

A_{21} is Asn, Gly, Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Ser, Thr, Tyr, Asp, or Glu; and

R_6 is a tag sequence containing one or more amino acids or R_6 is absent;

(b) folding modified apart proinsulin collected from said culture to provide a proinsulin derivative peptide;

(c) purifying said aspart proinsulin derivative peptide using metal affinity chromatography;

(d) enzymatically cleaving said proinsulin derivative peptide to remove a connecting peptide and provide an intermediate solution comprising aspart insulin analog; and

(e) purifying said intermediate solution using chromatography column(s) to yield the aspart insulin analog.

23. The process of claim 22, wherein the step of purifying further comprises eluting the aspart insulin analog using a buffer.

pTrcHis2A(Kan) Vector map with Aspart proinsulin Insert:

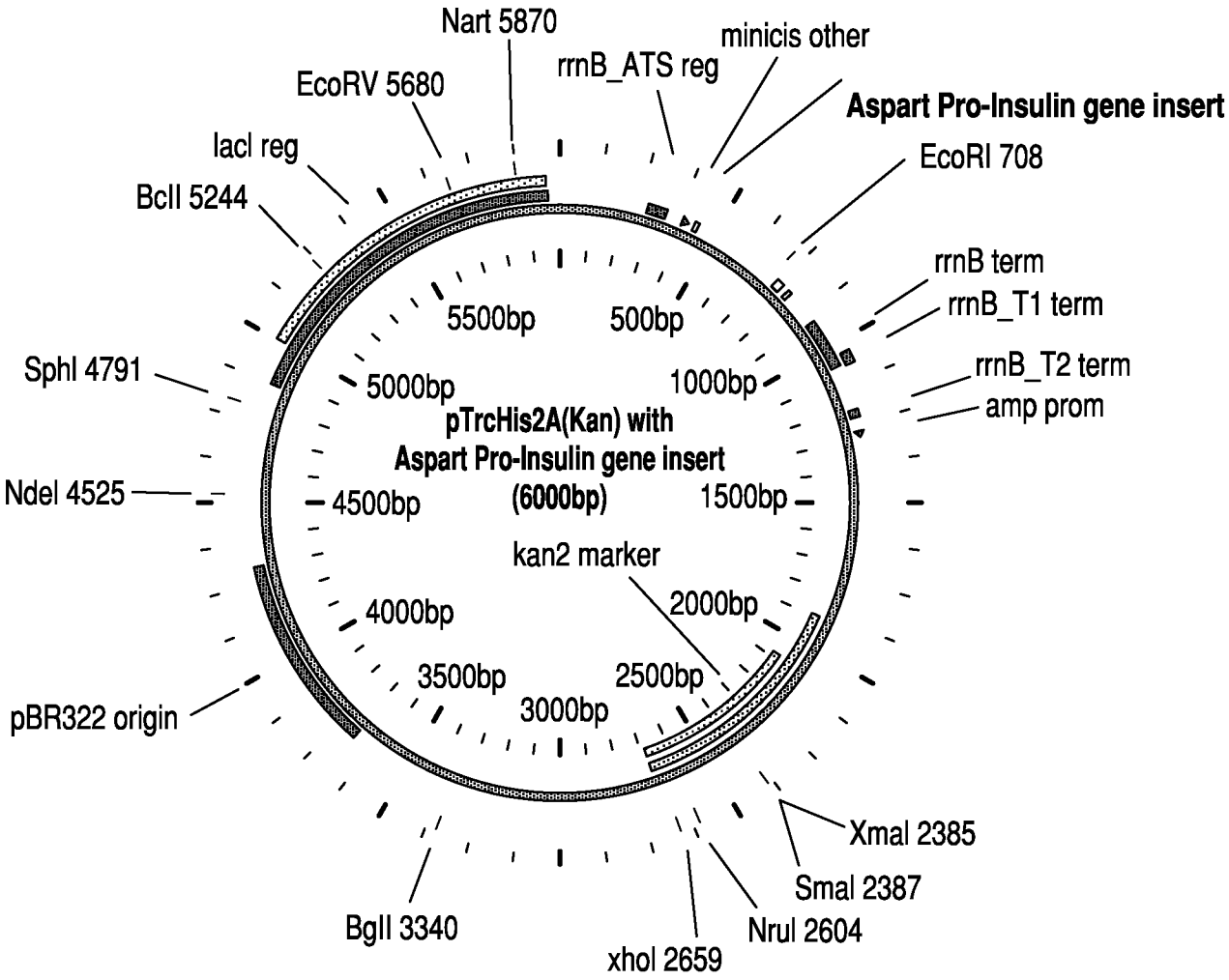


FIG. 1

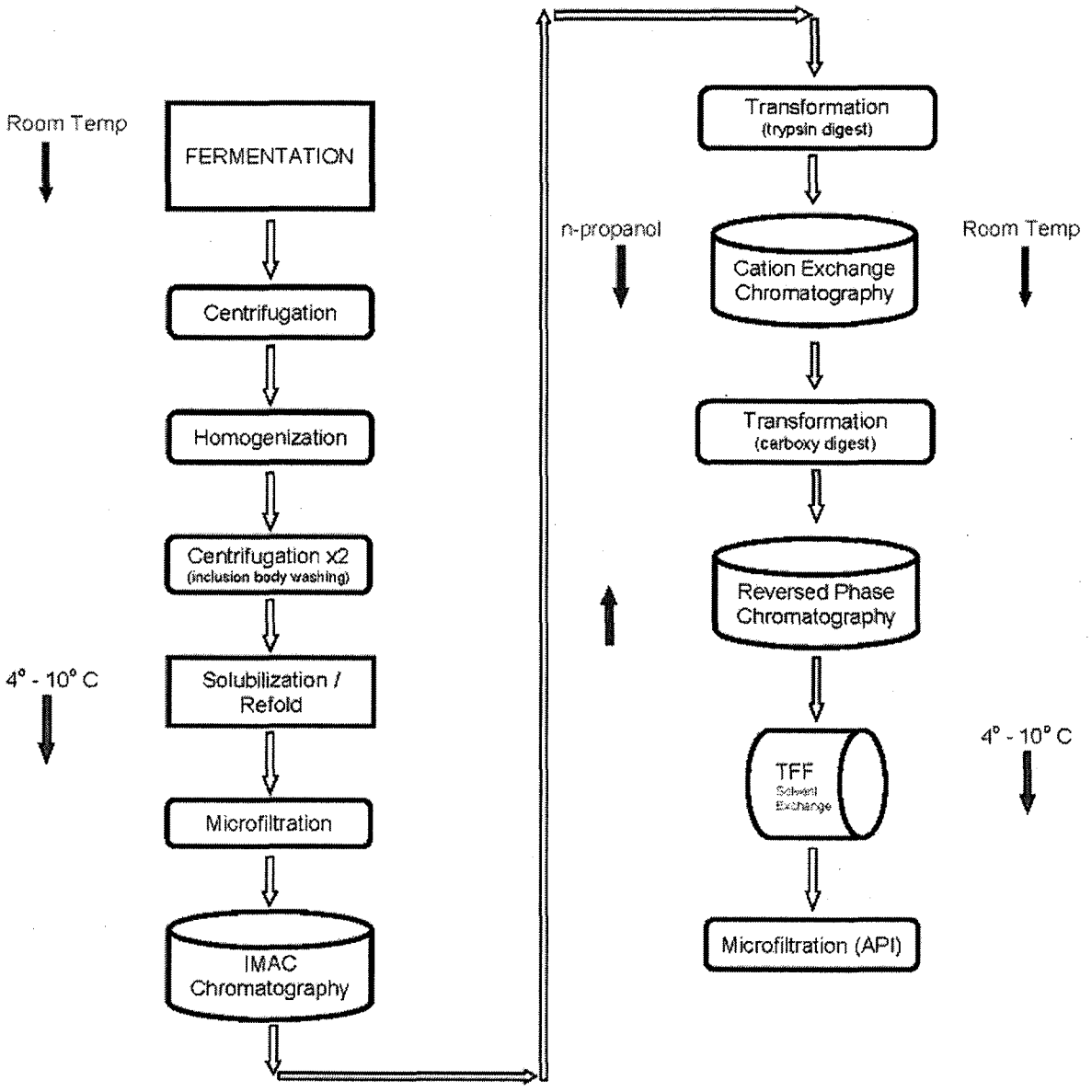


Figure 2a: Production Block Flow Diagram for Insulin Aspart

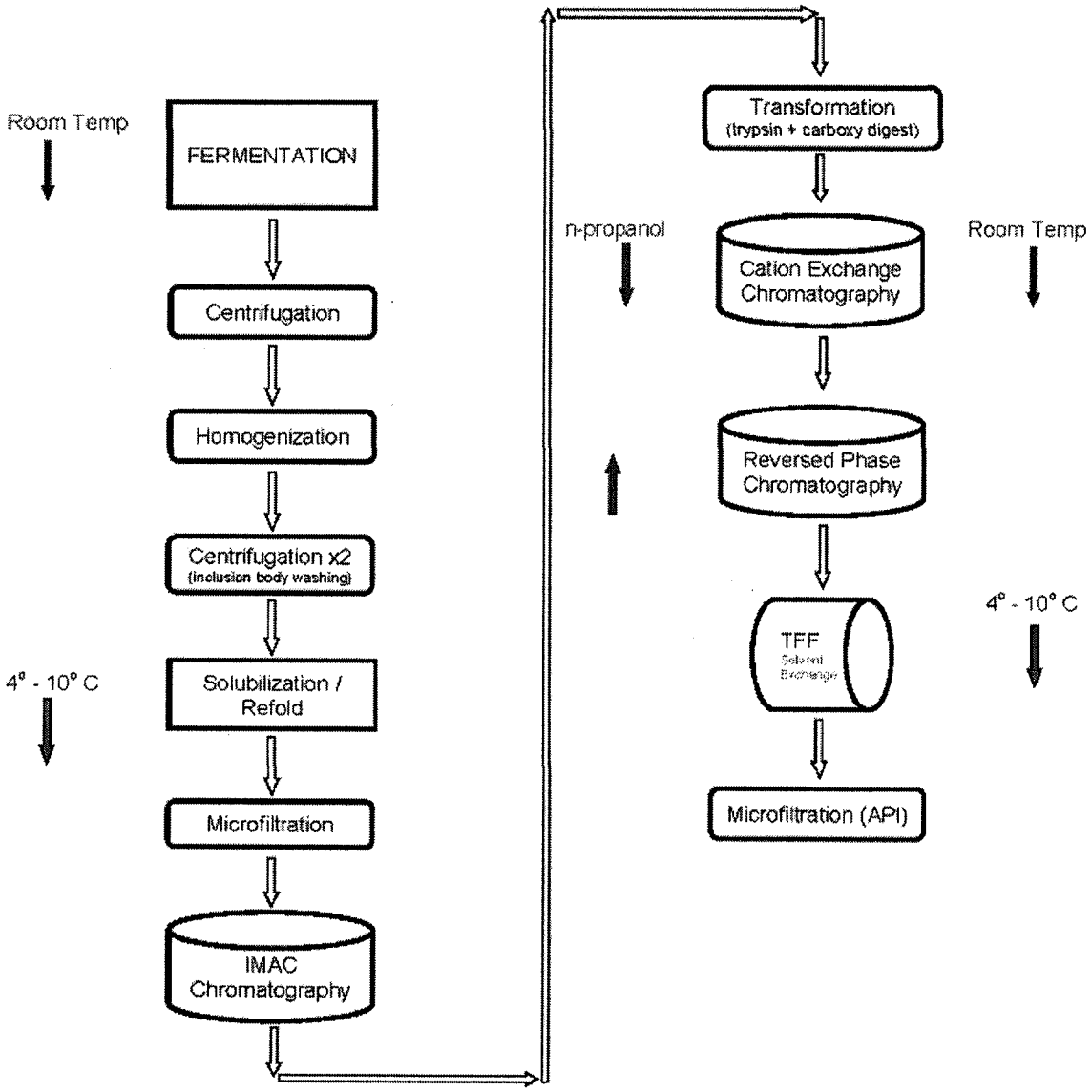


Figure 2b: Alternate Production Block Flow Diagram for Insulin Aspart

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/025918

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/28 C07K14/62
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 834 222 A (FRIEDMAN STEPHEN B [US] ET AL) 10 November 1998 (1998-11-10) *cf. abstract, col. 3, lines 5-15, col. 4, lines 34-48, claims*	1-23
X	US 2008/146492 A1 (ZIMMERMAN RONALD E [US] ET AL) 19 June 2008 (2008-06-19) *cf. abstract, pages 1/2, section [0013], amino acid sequences of section [0113] on page 8, further pro-insulin constructs of sections [0120] to [0123] of example 8 on page 9, claim 1*	1-23

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 October 2011	07/11/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Stoltner, Anton

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/025918

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 214 826 A2 (NOVO INDUSTRI AS [DK] NOVO NORDISK AS [DK]) 18 March 1987 (1987-03-18) *cf. abstract, generic formula (I) on page 3, and substitutions on page 4, line 9ff., furthermore generic formula (II) on page 6 with definitions for (R-Qn)* -----	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

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