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**NANA A. YEBOAH ET AL: "A Rapid Purification Method for Soybean Bowman-Birk Protease Inhibitor Using Hydrophobic Interaction Chromatography", PROTEIN EXPRESSION AND PURIFICATION, vol. 7, no. 3, 1 May 1996 (1996-05-01), pages 309-314, XP055071879, ISSN: 1046-5928, DOI: 10.1006/prep.1996.0044**

# DESCRIPTION

Description

## FIELD

**[0001]** Provided herein are methods and compositions for oral administration of therapeutic proteins, improved protease inhibitor preparations, methods for producing same, and compositions comprising same.

## BACKGROUND

**[0002]** Protein/peptide-based drugs are typically susceptible to degradation in the gastrointestinal tract and/or are not efficiently absorbed into the bloodstream from the small intestine in bioactive form. Orally delivered formulations for protein-based drugs such as insulin are being developed (Ziv et al 1994; Nissan et al 2000, Kidron et al 2004, Eldor et al 2010A, Eldor et al 2010B). One such oral insulin product is scheduled to be tested in Phase II trials and is currently being reviewed for IND status.

**[0003]** Trypsin inhibitors derived from soybean (*Glycine max*) are readily available and are considered to be safe for human consumption. They include SBTI (soybean trypsin inhibitor), which is composed of KTI (Kunitz Trypsin Inhibitor), which inhibits trypsin, and BBI (Bowman-Birk inhibitor), which inhibits trypsin and chymotrypsin. Such trypsin inhibitors are available for example from Sigma-Aldrich, St. Louis, Mo., USA. Methods for preparing BBI are described for example in US 7,404,973.

**[0004]** Relevant prior art documents are:

WO2013/102899A1;

Morishita M. et al., "Novel oral microspheres of insulin with protease inhibitor protecting from enzymatic degradation", International Journal of Pharmaceutics, 78(1-3), p. 1-7, 1992;

Nana A. Y. et al., "A rapid purification method for soybean Bowman-Birk protease inhibitor using hydrophobic interaction chromatography", 7(3), p. 309-314, 1996;

WO2009/118722A2.

## SUMMARY

**[0005]** The present inventor has discovered that commercially available SBTI preparations produced highly variable results when used in pharmaceutical compositions. It was postulated that the activities of KTI and BBI should be individually optimized in order to improve the activity of the pharmaceutical compositions. To this end, SBTI was obtained from a commercial source as separate preparations of KTI and BBI. However, each of these preparations was found to be contaminated with the other activity. Compounding the issue, significant variability was found in the BBI activity of the preparations, particularly large-scale preparations, as evidenced by variable ability to prevent degradation of proteins (e.g. insulin) by intestinal enzymes.

**[0006]** Accordingly, in accordance with certain embodiments described herein, an improved method for the purification of SBTI was developed, in which each product was prepared under its own specifications to high levels of activity, and levels of high molecular weight (MW)-contaminants were minimized. In accordance with other embodiments, the process avoids the use of PEG and a second chromatography step and is characterized by higher yield. In accordance with still other embodiments, the product is particularly suitable for use in pharmaceutical compositions, for example compositions for oral administration of therapeutic proteins. In addition, in accordance with yet other embodiments, the complete separation of the KTI and BBI activities allows more precise modulation of the anti-trypsin and anti-chymotrypsin activities of pharmaceutical compositions comprising a therapeutic protein and the BBI and/or KTI, allowing for more robust and/or more reproducible *in vivo* activity of the pharmaceutical compositions.

**[0007]** It was further discovered that emulsifiers were required to conveniently prepare large-scale preparations of peptide/protein-containing drugs. However, it was necessary to empirically test whether addition of particular emulsifiers could effectively prevent precipitation in the oil-based preparations without affecting the oral efficacy of the formulations. Other described embodiments thus relate to the presence of particular emulsifiers or combinations of emulsifiers, together with improved SBTI, in oil-based pharmaceutical compositions containing therapeutic peptides and proteins.

**[0008]** The terms "protein" and "peptide" are used interchangeably herein. Neither term is intended to confer a limitation of the number of amino acids present, except where a limitation is explicitly indicated.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0009]** The following figures are by way of illustrative example and are not meant to be taken as limiting the claimed invention.

**Figure 1. Flow diagram of SBTI purification. A.** SBTI intermediate production. **B.** Downstream purification to produce BBI and KTI.

**Figure 2. Chromatogram of DEAE Sepharose™ column separation.** Fractions of 0.3 L were collected during DEAE column elution.

**Figure 3. SDS-PAGE analysis of purified BBI and KTI.** Electrophoresis was performed, the gel was scanned, and bands were quantified using an ImageScanner™ III in conjunction with ImageQuant™ TL (both from General Electric). Samples of 1 milligram per milliliter (mg./ml.) were loaded on a 20% Phastgel™. Lane 1: Control (old SBTI preparation). Lane 2: Purified KTI, Band quantity = 100%. Lane 3: Purified BBI, lower band quantity = 89.2%, upper band quantity = 10.8%.

**Figure 4. Small-scale column chromatography run report using improved procedure.** Squares, circles, and triangles denote conductivity plot, pH, and $OD_{280}$, respectively. Vertical axes: conductivity plot (milliSiemens), pH, and $OD_{280}$ (arbitrary units). Horizontal axis: column volume and fraction number.

**Figure 5**. 20% SDS-PAGE of fractions from chromatography shown in **Figure 4. A.** Left-to-right presentation of fractions 4-18 and pooled fractions 5-11. "C" denotes prior art trypsin inhibitor (Sigma-Aldrich cat. no. T9003). **B.** Right-to-left presentation of fractions 6-15, including the wash + flowthrough (W+FT) and standard (Trypsin inhibitor from *Glycine max* (soybean); "ST").

**Figure 6. SDS-PAGE analysis of steps of additional purification of BBI only.** ST = standard (see Figure 5 legend), 2mg/ml. Lanes: 1: BBI pool from column. 2: Permeate of 30 KDa filtration. 3: Retentate of 30 KDa filtration diluted 1:40. 4: Retentate of 5 KDa concentration diluted 1:4.

**Figure 7. SDS-PAGE analysis of 1 mg/ml. of the final product of purification of BBI only.** ST = standard (see Figure 5 legend), 1mg/ml.

**Figure 8.** Flowchart of revised protocol for downstream purification of BBI and KTI.

**Figure 9.** Column chromatography run report using alternative procedure.

**Figure 10.** SDS-PAGE analysis of duplicate samples of BBI purified using alternative procedure (lanes 1-2) vs. standards (lane 3).

**Figure 11. Testing results of various emulsifier formulations.** Foam buildup score was from 1-5, where 1 indicates no foam, and 5 indicates no liquid visible because of the foam. For the suspension test, numbers 1-5 indicate full phase separation; partial phase separation with some larger oil bubbles; small oil bubble, milky consistency; no bubbles initially, with later phase separation; and stable emulsion, respectively.

**Figure 12. Blood glucose profiles following administration of oral insulin formulations**

**containing various emulsifiers. A.** Formulations A (upper left), B (lower left), C (upper right), and D (lower right). **B.** Formulations E (left) and F (right).

**Figure 13. Patient record sheets. A.** Blood Sugar Record. **B.** Questionnaire.

**Figure 14. Hypoglycemia Review Sheet.** Points are awarded for each occurrence of documented hypoglycemia, with extra points given depending on the neuroglycopenic symptoms experienced or lack thereof. Examples for the definition of symptoms were as follows: visual, eyes won't focus, impaired vision, double vision; behavioral, unable to sleep, irritable, stressed out, nervous, "want to sit down and do nothing", other neurological, lightheaded, dizzy, weakness, tiredness, sleepy, difficulty walking or speaking, slow responses, delayed motor skills, loss of balance; confusion, inability to perform simple math, feeling "out of it". No points are awarded if there were autonomic symptoms that gave adequate warning of impending hypoglycemia, even if some neuroglycopenic symptoms were also present. Additional points are given for the need for outside help to either recognize or treat the event.

**Figure 15.** Glucose responses in subjects treated with various doses of oral insulin.

## DETAILED DESCRIPTION OF THE EMBODIMENTS

**[0010]** In one aspect, a BBI isolated from a soybean product is provided, wherein the BBI is at least 85% pure as measured, in various embodiments, by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Brilliant Blue staining, or imager quantitation. In certain embodiments, the soybean product is soy flour.

**[0011]** In yet another aspect is provided a BBI isolated from soy flour, wherein the protein content of the BBI is greater than 95% by BCA (bicinchoninic acid) assay.

**[0012]** In yet another aspect is provided a BBI isolated from soy flour, wherein the BBI contains less than 0.1% high-MW contaminants, for example as assessed by SDS-PAGE and imager quantitation.

**[0013]** In other embodiments, the ratio of the anti-trypsin activity to the anti-chymotrypsin activity present in the isolated BBI is between 1.5:1 and 1:1 inclusive. In more specific embodiments, the ratio may be between 1.4:1 - 1.1:1, inclusive. In more specific embodiments, the ratio may be between 1.35:1 - 1.2:1, inclusive. In more specific embodiments, the ratio may be 1.28:1.

**[0014]** Unless indicated otherwise, anti-chymotrypsin activity referred to herein is measured using chymotrypsin having an activity of 40 BTEE units per mg. of chymotrypsin, and is expressed in mg. of chymotrypsin inhibited per mg. of protein being tested. BTEE refers to N-Benzoyl-L-Tyrosine Ethyl Ester (see the directions for Sigma-Aldrich Product No. B6125).

**[0015]** Unless indicated otherwise, anti-trypsin activity referred to herein is measured using trypsin having an activity of 10,000 BAEE units per mg. of trypsin, and is expressed in mg. of trypsin inhibited per mg. of protein being tested. BAEE refers to Na-Benzoyl-L-Arginine Ethyl Ester Solution (see the directions for Sigma-Aldrich Product No. B4500). For example, in a typical assay, one unit corresponds to the amount of inhibitor that reduces the trypsin activity by one benzoyl-L-arginine ethyl ester unit (BAEE-U). One BAEE-U is the amount of enzyme that increases the absorbance at 253 nm by 0.001 per minute at pH 7.6 and 25°C. See, for example, K. Ozawa, M. Laskowski, 1966, J. Biol. Chem. 241:3955; and Y. Birk, 1976, Meth. Enzymol. 45:700.

**[0016]** Those skilled in the art will appreciate that each of the above purity requirements, regarding its protein content, level of contaminants, or potency, is typically assessed prior to the BBI being mixed with one or more other components of the pharmaceutical composition.

**[0017]** In an additional aspect, a KTI3 isolated from soy flour is provided, wherein the KTI3 is at least 85% pure as measured, in various embodiments, by SDS-PAGE, Brilliant Blue staining, or imager quantitation.

**[0018]** In yet another aspect is provided a KTI3 isolated from soy flour, wherein the protein content of the KTI3 is greater than 95% as measured by BCA assay.

**[0019]** In yet another aspect is provided a KTI3 isolated from soy flour, wherein the KTI3 contains less than 0.1% high-MW contaminants, for example as assessed by SDS-PAGE and imager quantitation.

**[0020]** Those skilled in the art will appreciate that each of the above purity requirements, regarding its protein content, level of contaminants, or potency, is typically assessed prior to the KTI3 being mixed with one or more other components of the pharmaceutical composition.

**[0021]** In certain embodiments, the KTI3-containing pharmaceutical composition further comprises a BBI that meets at least one of the above purity requirements prior to its being mixed with one or more other components of the pharmaceutical composition.

**[0022]** The above-described pharmaceutical compositions are formulated for oral administration. In specific embodiments, the pharmaceutical composition further comprises a coating that resists degradation in the stomach. In even more specific embodiments, the coating is a pH-sensitive capsule, or alternatively, is a soft gelatin capsule.

**[0023]** The above-described pharmaceutical compositions further comprise a therapeutic protein of up to 100 kilodaltons as an active ingredient, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue). In other embodiments, the active ingredient is a non-protein molecule that is sensitive to degradation or inactivation in the human digestive tract.

**[0024]** An oral pharmaceutical composition is provided, comprising an oil-based liquid formulation, wherein the oil-based liquid formulation comprises a therapeutic protein of up to 100 kilodaltons (kDa), a chelator of divalent cations, and an isolated BBI, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue). In other embodiments, the liquid formulation consists essentially of a therapeutic protein of up to 100 kDa, a chelator of divalent cations, an isolated BBI, and an oil, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue).

**[0025]** In other embodiments, the liquid formulation consists essentially of a therapeutic protein of up to 100 kDa, a chelator of divalent cations, an isolated BBI, an oil, and an emulsifier, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue).

**[0026]** In other embodiments, the liquid formulation consists essentially of a therapeutic protein of up to 100 kDa, a chelator of divalent cations, an isolated BBI, an oil, and two emulsifiers, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue). In another aspect is provided an oral pharmaceutical composition comprising an oil-based liquid formulation, wherein the oil-based liquid formulation comprises a therapeutic protein of up to 100 kDa, a chelator of divalent cations, and a soybean-derived BBI, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue), and wherein said liquid formulation contains less than 0.05% of soybean-derived substances having a MW of greater than 30,000.

**[0027]** In another aspect is provided an oral pharmaceutical composition comprising an oil-based liquid formulation, wherein the oil-based liquid formulation comprises a therapeutic protein of up to 100 kDa, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue), and a chelator of divalent cations, and said liquid formulation has an anti-chymotrypsin activity of at least 50 mg chymotrypsin inhibited per ml. of the liquid formulation. In other embodiments, the liquid formulation has an anti-chymotrypsin activity of at least 35, 40, 45, 55 or 60 mg. chymotrypsin inhibited per ml. of the liquid formulation. In still other embodiments, the liquid formulation has an anti-chymotrypsin activity in the range of 35-70, 40-70, 45-70, 50-70, or 40-60 mg. of chymotrypsin inhibited per ml. of the liquid formulation. In other embodiments, the liquid formulation further comprises an anti-trypsin activity of at least 25 mg. of trypsin inhibited per ml. of the liquid formulation. In other embodiments, the liquid formulation further comprises an anti-trypsin activity of at least 30, 35, 40, 45, or 50 mg. trypsin inhibited per ml. of the liquid formulation. Alternatively, the liquid formulation further comprises an anti-trypsin activity in the range of 25-50, 30-50, 35-50, 25-40, or 25-45 mg. trypsin inhibited per ml. of the liquid formulation.

**[0028]** In another aspect is provided a method for making a pharmaceutical composition, comprising the steps of (a) providing a preparation of isolated BBI, a therapeutic protein of up to 100 kilodaltons, and a chelator of divalent cations, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue); and (b) mixing said isolated BBI, therapeutic protein, and chelator into an oil-based liquid formulation. Each of the embodiments described herein of the identity, purity, and potency of isolated BBI may be incorporated into this method. In addition, each of the embodiments described herein of the other ingredients, and of additional ingredients that may be present, may be incorporated into this method. In other embodiments, a pharmaceutical composition made by this method is provided.

**[0029]** In another aspect is provided a method for making a pharmaceutical composition, comprising the step of mixing an isolated BBI, a therapeutic protein of up to 100 kilodaltons, and a chelator of divalent cations into an oil-based liquid formulation, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue). Each of the embodiments described herein of the identity, purity, and potency of isolated BBI may be incorporated into this method. In addition, each of the embodiments described herein of the other ingredients, and of additional ingredients that may be present, may be incorporated into this method. In other embodiments, a pharmaceutical composition made by this method is provided.

**[0030]** "Liquid" as used herein refers to a composition that has a viscosity within the range of 1-1000 millipascal seconds, inclusive, at 20°C. Fish oil, for instance, is a liquid under ambient conditions. The term includes oil-based solutions, suspensions, and combinations thereof.

**[0031]** "Isolated" BBI as used herein refers to a preparation enriched in BBI relative to other components. In more specific embodiments, BBI refers to Bowman-Birk inhibitor; Uniprot number P01055 [database accessed on January 28, 2013]).

**[0032]** A representative precursor sequence of BBI is:
MVVLKVCLVL LFLVGGTTSA NLRLSKLGLL MKSDHQHSND DESSKPCCDQ CACTKSNPPQ CRCSDMRLNS CHSACKSCIC ALSYPAQCFC VDITDFCYEP CKPSEDDKEN (SEQ ID NO: 1).

**[0033]** Of these 110 residues, residues 1-19 are the signal peptide, 20-39 are a propeptide, and the mature chain BBI chain is composed of residues 40-110 (71 AA).

**[0034]** In various embodiments, the preparation of BBI utilized in the described methods and compositions is at least 85%, 90%, 92%, 94%, or 95% pure as assessed by SDS-PAGE, Brilliant Blue staining, or imager quantitation (e.g. according to the protocol described herein). In the context of a pharmaceutical composition, this value refers to characteristics of the BBI prior to its being mixed with one or more other components of the pharmaceutical composition. In alternative embodiments, SDS-PAGE and silver staining, optionally followed by imager quantitation, may be utilized.

**[0035]** In other embodiments, the isolated BBI has an anti-chymotrypsin activity of at least 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, or 1.4 mg. chymotrypsin inhibited per mg. inhibitor. In other embodiments, the anti-chymotrypsin activity is in the range of 0.8-1.8, 0.9-1.8, 1.0-1.8, 1.1-1.8, 1.2-1.8, 1.3-1.8, or 1.4-1.8 mg. chymotrypsin inhibited per mg. inhibitor. In more specific embodiments, the activity is 0.8-1.8 mg. chymotrypsin inhibited per mg. inhibitor. In other embodiments, the activity is in the range of 0.8-1.5 mg. chymotrypsin inhibited per mg. inhibitor. In the context of a pharmaceutical composition, this value refers to characteristics of the BBI prior to its being mixed with one or more other components of the pharmaceutical composition.

**[0036]** In various embodiments, the BBI preparation contains 5% or less KTI as assessed by SDS-PAGE, Brilliant Blue staining, or imager quantitation (according to the protocol described herein). In alternative embodiments, SDS-PAGE and silver staining may be utilized.

**[0037]** In more specific embodiments, KTI as used herein refers to KTI3 (Uniprot number P01070; database accessed on January 3, 2013). A representative precursor sequence of KTI3 is:
MKSTIFFLFL FCAFTTSYLP SAIADFVLDN EGNPLENGGT YYILSDITAF
GGIRAAPTGN ERCPLTVVQS RNELDKGIGT IISSPYRIRF IAEGHPLSLK
FDSFAVIMLC VGIPTEWSVV EDLPEGPAVK IGENKDAMDG WFRLERVSDD
EFNNYKLVFC PQQAEDDKCG DIGISIDHDD GTRRLVVSKN KPLVVQFQKL
DKESLAKKNH GLSRSE SEQ ID NO: 2)

**[0038]** Of the above sequence, residues 1-24 are the signal peptide, 206-216 are the propeptide, and the mature KTI chain is composed of residues 25-205 (181 AA).

**[0039]** In other embodiments, the protein content of the BBI preparation is greater than 95% by BCA assay (for example using the Micro BCA Protein Assay Kit [cat. #23225, Thermo Scientific Rockford, IL]). In the context of a pharmaceutical composition, this value refers to its characteristics prior to its being mixed with one or more other components of the pharmaceutical composition. In other embodiments, the BBI preparation contains less than 0.1% high-MW contaminants (in other words, substances having a MW of greater than 30,000). In other embodiments, the BBI has been isolated without the use of polyethyleneglycol (PEG).

**[0040]** In other embodiments, the isolated BBI in the described methods and compositions is a recombinant BBI, for example BBI produced by a microorganism such as a bacterium that has been engineered to express it and subsequently isolated. In still other embodiments, the BBI is a synthetic BBI. An example of a synthetic BBI is BBI that has been produced in a cell-free apparatus such as a peptide synthesizer. Peptide synthesizers, for example automated peptide synthesizers, are well known in the art and are available commercially. Pharmaceutical compositions comprising recombinant BBI are also provided herein. Pharmaceutical compositions comprising synthetic BBI are also provided herein.

**[0041]** In certain embodiments, the described BBI is the only protease inhibitor in the described methods and compositions. While lower-potency SBTI requires an additional protease inhibitor, e.g. aprotinin, to efficiently protect certain therapeutic proteins in the human digestive tract, the described isolated BBI is believed to be capable of reducing the need for additional protease inhibitors in this regard.

**Additional Protease Inhibitors**

**[0042]** In certain embodiments, the oil-based liquid formulation utilized in the described methods and compositions further comprises a trypsin inhibitor other than the isolated BBI. In other embodiments, the oil-based liquid formulation utilized in the described methods and compositions further comprises a trypsin inhibitor other than the isolated KTI3. Those skilled in the art will appreciate in light of the present disclosure that a variety of trypsin inhibitors may be utilized. In the case of trypsin inhibitors that are proteins, the size will typically be up to 100 kDa.

**[0043]** As used herein, the term "trypsin inhibitor" refers to any agent capable of inhibiting the action of trypsin on a substrate. The ability of an agent to inhibit trypsin can be measured using assays well known in the art.

**[0044]** Some trypsin inhibitors known in the art are specific to trypsin, while others inhibit trypsin and other proteases such as chymotrypsin. Trypsin inhibitors can be derived from animal or vegetable sources: for example, soybean, corn, lima and other beans, squash, sunflower, bovine and other animal pancreas and lung, chicken and turkey egg white, soy-based infant formula, and mammalian blood. Trypsin inhibitors can also be of microbial origin: for example, antipain; see, for example, H. Umezawa, 1976, Meth. Enzymol. 45, 678. A trypsin inhibitor can also be an arginine or lysine mimic or other synthetic compound: for example arylguanidine, benzamidine, 3,4-dichloroisocoumarin, diisopropylfluorophosphate, gabexate mesylate, or phenylmethanesulfonyl fluoride. As used herein, an arginine or lysine mimic is a compound that is capable of binding to the $P^1$ pocket of trypsin and/or interfering with trypsin active site function.

**[0045]** In certain embodiments, the additional trypsin inhibitor utilized in the described methods and compositions is selected from the group consisting of lima bean trypsin inhibitor, aprotinin, (a.k.a. pancreatic trypsin inhibitor or basic pancreatic trypsin inhibitor [BPTI]; Uniprot No. P00974 [database accessed on January 2, 2013]), Kazal inhibitor (pancreatic secretory trypsin inhibitor),, Kazal inhibitor (pancreatic secretory trypsin inhibitor), ovomucoid, Alpha 1-antitrypsin, Cortisol binding globulin, Centerin ([SERPINA9/GCET1 (germinal centre B-cell-expressed transcript 1)], PI-6 (Sun et al 1995), PI-8 (Sprecher et al 1995), Bomapin, a clade A serpin [for example Serpina3 (NCBI Gene ID: 12), Serpina6 (NCBI Gene ID: 866), Serpina12 (NCBI Gene ID: 145264); Serpina10 (NCBI Gene ID: 51156); Serpina7 (NCBI Gene ID: 6906); Serpina9 (NCBI Gene ID: 327657); Serpina11 (NCBI Gene ID: 256394); Serpina13 (NCBI Gene ID: 388007); Serpina2 (NCBI Gene ID: 390502); and Serpina4 (NCBI Gene ID: 5104)] Yukopin (Serpinb12; Gene ID: 89777), antipain, benzamidine, 3,4-dichloroisocoumarin, diisopropylfluorophosphate, and gabexate mesylate. In other embodiments, one of the above inhibitors is selected.

**[0046]** A representative precursor sequence of aprotinin is:
MKMSRLCLSV ALLVLLGTLA ASTPGCDTSN QAKAQRPDFC LEPPYTGPCK ARIIRYFYNA KAGLCQTFVY GGCRAKRNNF KSAEDCMRTC GGAIGPWENL (SEQ ID NO: 3).

**[0047]** Of these 100 residues, residues 1-21 are the signal peptide, 22-35 and 94-100 are propeptides, and the mature chain BBI chain is composed of residues 36-93 (58 AA).

**[0048]** In other embodiments, an oil-based liquid formulation utilized in the described methods and compositions comprises both isolated BBI and isolated KTI, in more specific embodiments both isolated BBI and isolated KTI3. "Isolated" KTI as used herein refers to a preparation enriched in KTI relative to other components. In various embodiments, the preparation of KTI utilized in the described methods and compositions is at least 85% pure as assessed by SDS-PAGE, Brilliant Blue staining, or imager quantitation (e.g. according to the protocol described herein). In other embodiments, the protein content of the KTI preparation is greater than 95% by BCA assay. In the context of a pharmaceutical composition, these values refer to characteristics of the KTI prior to its being mixed with one or more other components of the pharmaceutical composition. In other embodiments, the KTI preparation contains 5% or less BBI as assessed by SDS-PAGE. In other embodiments, the KTI preparation contains less than 0.1% high-MW contaminants (in other words, substances having a MW of greater than 30,000). In other embodiments, the KTI has been isolated without the use of PEG.

**[0049]** In still more specific embodiments, the described methods and compositions comprise the described BBI and KTI, in more specific embodiments BBI and KTI3, as the only protease inhibitors. In other embodiments, the described methods and compositions comprise KTI and aprotinin, in more specific embodiments KTI3 and aprotinin, as the only protease inhibitors. In other embodiments, isolated BBI, isolated KTI, and aprotinin are all present in the oil-based liquid formulation.

**[0050]** In other embodiments, the isolated KTI3 has an activity of at least 0.8, 0.9, 1.0, 1.1, 1.2, or 1.3 mg. trypsin inhibited per mg. inhibitor. In other embodiments, the activity of the KTI3 is in the range of 0.8-1.8, 0.9-1.8, 1.0-1.8, 1.1-1.8, 1.2-1.8, or 1.3-1.8 mg. trypsin inhibited per mg. inhibitor. In more particular embodiments, the activity of the KTI3 is 0.8-1.7 mg. trypsin inhibited per mg. inhibitor. In other embodiments, the activity is range of 0.8-1.4 mg. trypsin inhibited per mg. inhibitor.

**[0051]** In other preferred embodiments, the isolated KTI is a recombinant KTI, for example KTI produced by a microorganism such as a bacterium that has been engineered to express it. In still other preferred embodiments, the KTI is a synthetic KTI. An example of a synthetic KTI is KTI that has been produced in a cell-free apparatus such as a peptide synthesizer.

**[0052]** Other embodiments concern the ratio of the anti-chymotrypsin activity present in the described pharmaceutical composition to the anti-trypsin activity of the composition. In some embodiments, this parameter is between 1.5:1 and 1:1 inclusive. In more specific embodiments, the ratio may be between 1.4:1 - 1.1:1, inclusive. In more specific embodiments, the ratio may be between 1.35:1 - 1.2:1, inclusive.

**[0053]** In certain embodiments, the BBI utilized in the described methods and compositions, and/or KTI, if present, has been stored with a preservative. In other embodiments, the BBI and/or KTI has been prepared and stored without use of a preservative.

**[0054]** In certain embodiments, the BBI utilized in the described methods and compositions, and/or the KTI, if present, is obtained from soy flour. The term "soy flour" refers to flour obtained from the species *Glycine max.* In other embodiments, any species from the genus Glycine may be utilized. Methods for obtaining soy flour are well known in the art. The described methods are believed be applicable to any type of soy flour, no matter how it was produced.

**Therapeutic Proteins**

**[0055]** Therapeutic proteins for compositions and methods described herein, wherein said therapeutic proteins are insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue), are in some embodiments isolated prior to inclusion in the described pharmaceutical compositions. "Isolated" in this regard excludes provision of the therapeutic protein as a homogenized tissue preparation or other form containing substantial amounts of contaminating proteins. A preferred example of an isolated protein or peptide is a recombinant protein or peptide. An even more preferred embodiment is a synthetic protein, in other words a protein produced in a cell-free apparatus. Those skilled in the art will appreciate in light of the present disclosure that both wild-type and mutated therapeutic proteins may be utilized.

**[0056]** Certain proteins and peptides are known to be specific inhibitors of trypsin and/or chymotrypsin, including but not limited to those described herein as being trypsin and/or chymotrypsin inhibitors. Such proteins are not intended for use as the therapeutic component in the described compositions, and are excluded from the definition of "therapeutic proteins" as used herein.

**[0057]** The therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue) in the described methods and compositions. In certain embodiments, the therapeutic protein is up to 100 kilodaltons (kDa) in size, typically between 1-100 kDa, inclusive. In more specific embodiments, the size is up to 90 kDa. In other embodiments, the size is up to 80 kDa. In other embodiments, the size is up to 70 kDa. In other embodiments, the size is up to 60 kDa. In other embodiments, the size is up to 50 kDa. Preferably, the size is between 1-90 kDa, inclusive. In other embodiments, the size is between 1-80 kDa, inclusive. In other embodiments, the size is between 1-70 kDa, inclusive. In other embodiments, the size is between 1-60 kDa, inclusive. In other embodiments, the size is between 1-50 kDa, inclusive.

**[0058]** In still more specific embodiments, the therapeutic protein is insulin. Alternatively, the therapeutic protein may be a GLP-1 inhibitor. In other embodiments, both insulin and exenatide are present in the described composition. In other embodiments, the liquid formulation consists essentially of insulin, exenatide, a chelator of divalent cations, an isolated BBI, and an oil. In other embodiments, the liquid formulation consists essentially of insulin, exenatide, a chelator of divalent cations, an isolated BBI, at least one emulsifier, and an oil. In still other embodiments, the liquid formulation consists essentially of insulin, exenatide, a chelator of divalent cations, an isolated KTI3, aprotinin, and an oil. In yet embodiments, the liquid formulation consists essentially of insulin, exenatide, a chelator of divalent cations, an isolated KTI3, aprotinin, at least one emulsifier, and an oil.

**[0059]** A person skilled in the art will appreciate in light of the present disclosure that various types of insulin are suitable for the described methods and compositions. Exemplary insulin proteins include but are not limited to both wild-type and mutated insulin proteins, including synthetic human insulin, synthetic bovine insulin, synthetic porcine insulin, synthetic whale insulin, and metal complexes of insulin, such as zinc complexes of insulin, protamine zinc insulin, and globin zinc insulin.

**[0060]** Various classes of insulin may also be utilized, for example fast-acting insulin, lente insulin, semilente insulin, ultralente insulin, NPH insulin, glargine insulin, lispro insulin, aspart insulin, or combinations of two or more of the above types of insulin.

**[0061]** In a particularly preferred embodiment, the insulin of the described methods and compositions is wild-type human insulin (Uniprot ID P01308; SEQ ID NO: 4). Of the 110 amino acids, 1-24 is the signal peptide, 25-54 forms the insulin B chain, 57-87 forms C peptide, and 90-110 forms the insulin A chain. In one preferred embodiment, human insulin is produced as a recombinant protein in bacterial cells. In another preferred embodiment, human insulin is produced synthetically.

**[0062]** GLP-1 analogues are also referred to in the art as GLP-1 mimetics. A person of skill in the art will appreciate in light of the present disclosure that the described compositions may include at least one of the following GLP-1 analogues: exenatide (Byetta™; CAS no. 141732-76-5; SEQ ID NO: 5), lixisenatide (CAS no. 320367-13-3), liraglutide (CAS no. 204656-20-2), exendin-9 (CAS no. 133514-43-9), AC3174 ([Leu(14)]exendin-4, Amylin Pharmaceuticals, Inc.), taspoglutide (CAS no. 275371-94-3), albiglutide (CAS no. 782500-75-8), semaglutide (CAS no. 910463-68-2), LY2189265 (dulaglutide™; CAS no. 923950-08-7), and CJC-1134-PC (a modified Exendin-4 analogue conjugated to recombinant human albumin manufactured by ConjuChem™). All CAS records were accessed on December 19, 2011. Thus, in certain embodiments, the described method or composition utilizes any of the above-listed GLP-1 analogues. In other embodiments, one of the above-listed GLP-1 analogues is selected. Those of skill in the art will appreciate in light of the findings presented herein that other GLP-1 analogues can also be utilized in the described methods and compositions.

**Emulsifiers**

**[0063]** "Weight/weight" percentages of emulsifiers and detergents referred to herein utilize the amount of oil base in the formulation, for example fish oil, as the denominator; thus, 60 mg of Gelucire in 500 mg fish oil is considered as 12% w/w, regardless of the weight of the other components. Similarly, 50 mg. Tween-80 mixed with 500 mg fish oil is considered as 10% Tween-80.

**[0064]** In certain embodiments, the oil-based liquid formulation utilized in the described methods and pharmaceutical compositions, or in other embodiments, each of the oil-based liquid formulation that is present, comprises, in addition to the therapeutic protein, chelator, and BBI, a polyethylene glycol (PEG) ester of a fatty acid, for example a PEG ester of a monoglyceride, a diglyceride, a triglyceride, or a mixture thereof. In more specific embodiments, the PEG ester may be provided as a mixture of (a) a free monoacylglycerol, a free diacylglycerol, a free triacylglycerol, or a mixture thereof; and (b) a PEG ester of a fatty acid, for example a PEG ester of a monoglyceride, a diglyceride, a triglyceride, or a mixture thereof. In this regard, each of the terms "monoacylglycerol", "diacylglycerol", and "triacylglycerol" need not refer to a single compound, but rather can include mixtures of compounds, for example mixtures of monoacylglycerols, diacylglycerols, or triacylglycerols having fatty acids of varying lengths. In certain preferred embodiments, monoacylglycerols, diacylglycerols, or triacylglycerols utilized in the described methods and compositions, for example those used to general PEG esters, are from an oil source that is Generally Recognized As Safe (GRAS). Examples of GRAS oils are coconut oil, corn oil, peanut oil, soybean oil, Myvacet 9-45 (Diacetylated monoacylglycerols of C-18 fatty acids). A more specific embodiment of (a) is a mixture of $C_8$-$C_{18}$ monoacylglycerols, diacylglycerols, and triacylglycerols. A more specific embodiment of component (b) is a mixture of PEG monoesters and diesters of one or more $C_8$-$C_{18}$ fatty acids.

**[0065]** In more specific embodiments, the liquid formulation further comprises, in addition to the PEG ester of a fatty acid, a free PEG. In still more specific embodiments, an additional non-ionic detergent, for example a polysorbate-based detergent, is present in addition to the PEG ester and free PEG.

**[0066]** In a still more specific embodiment of the described methods and compositions, a liquid formulation comprises: (a) a mixture of $C_8$-$C_{18}$ monoacylglycerols, diacylglycerols, and triacylglycerols; (b) PEG-32 monoesters and diesters of a mixture of $C_8$-$C_{18}$ fatty acids; and (c) free PEG-32. In even more specific embodiments, the weight/weight ratio of component (a) to the sum of components (b) + (c) is between 10:90-30:70 inclusive; more specifically between 15:85-25:75 inclusive; more specifically 20:80. In certain embodiments, components (a)-(c) together constitute 8-16% weight/weight inclusive of the oil-based liquid formulation. In more specific embodiments, the amount is 9-15% inclusive. In more specific embodiments, the amount is 10-14% inclusive. In more specific embodiments, the amount is 11-13% inclusive. In more specific embodiments, the amount is 12%.

**[0067]** In other embodiments, an oil-based liquid formulation utilized in the described methods and pharmaceutical compositions comprises, in addition to the therapeutic protein, chelator, and BBI, a self-emulsifying component. While some embodiments of self-emulsifying components are the mixtures of components described in the preceding paragraphs, these mixtures do not limit the definition of the term "self-emulsifying components" as used herein. "Self-emulsifying component" as used herein refers to a component that spontaneously forms an emulsion. Typically, such components will form an emulsion on contact with aqueous media, forming a fine dispersion i.e. a microemulsion (SMEDDS). Certain embodiments of such components comprise a triacylglycerol mixture of triacylglycerols and a high hydrophile/lipophile balance (HLB; see Griffin WC: "Calculation of HLB Values of Non-Ionic Surfactants," J Soc Cosmetic Chemists 5:259 (1954)) surfactant. Other embodiments of the self-emulsifying component have a waxy, semi solid consistency.

**[0068]** Preferably, the HLB of a self-emulsifying component utilized in the described methods and compositions is 10 or greater. In other embodiments, it is between 11-19 inclusive. In other embodiments, it is between 12-18 inclusive. In other embodiments, it is between 12-17 inclusive. In other embodiments, it is between 12-16 inclusive, which is indicative of an oil-in-water (O/W) emulsifier. In other embodiments, it is between 13-15 inclusive. In other embodiments, it is 14. Still more specific embodiments of self-emulsifying components have an HLB of 12-16 inclusive and comprise medium- and long-chain triacylglycerols conjugated to PEG, free triacylglycerols, and free PEG. In other embodiments, the self-emulsifying component has an HLB of 12-16 inclusive and consists of a mixture of medium- and long-chain triacylglycerols conjugated to PEG, free triacylglycerols, and free PEG. In other embodiments, the self-emulsifying component has an HLB of 14 and comprises medium- and long-chain triacylglycerols conjugated to PEG, free triacylglycerols, and free PEG. In other embodiments, the self-emulsifying component has an HLB of 14 and consists of a mixture of medium- and long-chain triacylglycerols conjugated to PEG, free triacylglycerols, and free PEG.

**[0069]** Certain, more specific embodiments utilize self-emulsifying components that comprise (a) a monoacylglycerol, a diacylglycerol, a triacylglycerol, or a mixture thereof; and (b) a polyethylene glycol (PEG) ester of a fatty acid. In this regard, each of the terms "monoacylglycerol", "diacylglycerol", and "triacylglycerol" need not refer to a single compound, but rather can include mixtures of compounds, for example mixtures of monoacylglycerols, diacylglycerols, or triacylglycerols having fatty acids of varying lengths. A more specific embodiment is a mixture of $C_8$-$C_{18}$ monoacylglycerols, diacylglycerols, and triacylglycerols. A more specific embodiment of component (b) is a mixture of PEG monoesters and diesters of a mixture of $C_8$-$C_{18}$ fatty acids.

**[0070]** In other, more specific embodiments, the self-emulsifying component further comprises free PEG.

**[0071]** Certain PEG moieties for use in the described compositions and methods contain between 5-100 monomers. In more specific embodiments, the PEG may contain between 15-50 monomers. In still more specific embodiments, the PEG may contain between 25-40 monomers. In more specific embodiments, the PEG may contain 32 monomers.

**[0072]** In still more specific embodiments of the described methods and compositions, a self-emulsifying component used therein comprises: (a) a mixture of $C_8$-$C_{18}$ monoacylglycerols, diacylglycerols, and triacylglycerols; (b) PEG-32 monoesters and diesters of a mixture of $C_8$-$C_{18}$ fatty acids; and (c) free PEG-32; and the weight/weight ratio of component (a) to components (b) + (c) is 20:80. In certain embodiments, such a component constitutes 8-16% weight/weight inclusive of the oil-based liquid formulation. In more specific embodiments, the amount is 9-15% inclusive. In more specific embodiments, the amount is 10-14% inclusive. In more specific embodiments, the amount is 11-13% inclusive. In more specific embodiments, the amount is 12%.

**[0073]** Examples of self-emulsifying components meeting the above specifications are Gelucire™ 44/14, Gelucire™ 53/10, and Gelucire™ 50/13. A more specific embodiment is Gelucire™ 44/14. The suffixes 44 and 14 refer respectively to its melting point and its hydrophilic/lypophilic balance (HLB). Gelucire™ 44/14 (Gattefossé SAS, Saint-Priest, France) is obtained by polyglycolysis of hydrogenated coconut oil (medium- and long-chain triacylglycerols with PEG-32. It has a hydrophile/lipophile balance of 14. It is composed of a defined admixture of $C_8$-$C_{18}$ mono-, di- and triacylglycerols (20% w/w); PEG-32 mono- and diesters and free PEG-32 (80% w/w). The main fatty acid present is lauric acid, accounting for 45% on average of the total fatty acid content. It is a solid dispersion composed of a PEG ester fraction under a lamellar phase of 120 Å with a helical conformation and an acylglycerol fraction under a hexagonal packing. The main products of simulated gastrointestinal lipolysis of Gelucire™ 44/14 are PEG-32 mono and diesters. In more specific embodiments, the described compositions comprise about 12% Gelucire 44/14 as the only emulsifier, or, in other embodiments, together with another emulsifier. In other embodiments, the described compositions comprise about 12% Gelucire 44/14 and about 10% Tween-80.

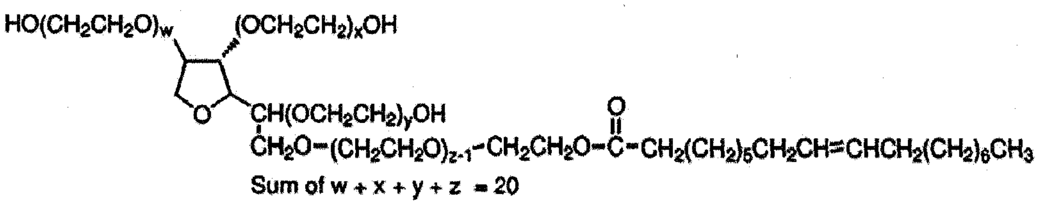
<u>Non-ionic Detergents</u>

**[0074]** In certain embodiments, an oil-based liquid formulation utilized in the described methods and pharmaceutical compositions further comprises a non-ionic detergent in addition to the self-emulsifying component. In certain embodiments, the non-ionic detergent is selected from the group consisting of polysorbate-20, polysorbate-40, polysorbate-80, lauromacrogol 400, polyoxyl 40 stearate, polyoxyethylene hydrogenated castor oil 10, 50 and 60, glycerol monostearate, polysorbate 40, 60, 65 and 80, sucrose fatty acid ester, methyl cellulose, carboxymethyl cellulose, n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-

N-methylglucamide, decanoyl-N-methylglucamide, Triton™-X-100, Triton™-X-114, Thesit™, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), and N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate. In other embodiments, one of the above-listed non-ionic detergents is selected.

**[0075]** In certain, more specific embodiments, a non-ionic detergent used in the described methods and compositions is a polysorbate-based detergent. Examples of polysorbate-based detergents are detergents derived by covalently bonding polyethoxylated sorbitan to a fatty acid. More specific embodiments of polysorbate-based detergents are polysorbate-20, polysorbate-40, and polysorbate-80.

**[0076]** For example, polysorbate 80 (Tween-80) is a non-ionic detergent derived from polyethoxylated sorbitan and oleic acid and having the following structure:




**[0077]** In the case of polysorbate 80, the moiety shown on the right side is a mixture of fatty acids, containing 60-70% oleic acid (as depicted), with the balance being primarily linoleic, palmitic, and stearic acids.

**[0078]** In a more specific embodiment, the polysorbate 80 constitutes 3-15% weight/weight inclusive of an oil-based liquid formulation used in the described methods and compositions. In a more specific embodiment, the percentage is 5-14% inclusive. In a more specific embodiment, the percentage is 7-12% inclusive. In more specific embodiments, the percentage is 10%, or alternatively 5%.

## Chelators of Divalent Cations

**[0079]** The chelator of divalent cations utilized in the described methods and compositions is, in one embodiment, any physiologically acceptable compound having a high affinity for at least one of calcium, magnesium, and manganese ions. In another embodiment, the chelator is selected from the group consisting of citrate or a salt thereof; ethylenediamine tetracetic acid (EDTA) or a salt thereof (for example disodium EDTA and calcium disodium EDTA); EGTA (ethylene glycol tetraacetic acid) or a salt thereof; diethylene triamine pentaacetic acid (DTPA) or a salt thereof; and BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) or a salt thereof. In other embodiments, one of the above-listed chelators is utilized. In more specific embodiments, the chelator is EDTA.

**Oils**

**[0080]** Those of skill in the art will appreciate, in light of the present findings, that various oils may be utilized as the basis of their liquid phase of the described compositions. In certain embodiments, the oil may be any physiologically acceptable oil that is liquid at ambient temperature.

**[0081]** In more specific embodiments, the oil comprises an omega-3 fatty acid. In other embodiments, the omega-3 fatty acid is an omega-3 polyunsaturated fatty acid. In another embodiment, the omega-3 fatty acid is DHA, an omega-3, polyunsaturated, 22-carbon fatty acid also referred to as 4, 7, 10, 13, 16, 19-docosahexaenoic acid. In another embodiment, the omega-3 fatty acid is --linolenic acid (9, 12, 15-octadecatrienoic acid). In another embodiment, the omega-3 fatty acid is stearidonic acid (6, 9, 12, 15-octadecatetraenoic acid). In another embodiment, the omega-3 fatty acid is eicosatrienoic acid (ETA; 11, 14, 17-eicosatrienoic acid). In another embodiment, the omega-3 fatty acid is eicsoatetraenoic acid (8, 11, 14, 17-eicosatetraenoic acid). In one embodiment, the omega-3 fatty acid is eicosapentaenoic acid (EPA; 5, 8, 11, 14, 17-eicosapentaenoic acid). In another embodiment, the omega-3 fatty acid is eicosahexaenoic acid (also referred to as 5, 7, 9, 11, 14, 17-eicosahexaenoic acid). In another embodiment, the omega-3 fatty acid is docosapentaenoic acid (DPA; 7, 10, 13, 16, 19-docosapenatenoic acid). In another embodiment, the omega-3 fatty acid is tetracosahexaenoic acid (6, 9, 12, 15, 18, 21-tetracosahexaenoic acid).

**[0082]** In other embodiments, the oil is a naturally-occurring oil comprising an omega-3 fatty acid. In more specific embodiments, the oil is selected from the group consisting of a fish oil, canola oil, flaxseed oil, algal oil and hemp seed oil. In more specific embodiments, the oil is a fish oil. Several types of fish oil have been tested in the described compositions and have all been found to work equally well.

**[0083]** In other embodiments, a liquid formulation utilized in the described method or composition is water-free. "Water-free" refers, in certain embodiments, to a formulation into which no aqueous components have been intentionally added. It does not preclude the presence of trace amounts of water that have been absorbed from the atmosphere into the components thereof. In another embodiment, the liquid formulation is free of aqueous components. In yet other embodiments, one or more oils are the only liquid components of the liquid formulation. In more specific embodiments, fish oil is the only liquid component of the liquid formulation.

**Coatings**

**[0084]** Those of skill in the art will appreciate, in light of the present findings, that various pH-sensitive coatings may be utilized in the described methods and compositions. In certain embodiments, any coating that inhibits digestion of the composition in the stomach of a subject may be utilized.

**[0085]** In other embodiments, the coating comprises a biodegradable polysaccharide. In other embodiments, a hydrogel is utilized. In other embodiments, the coating comprises one of the following excipients: chitosan, an aquacoat ECD coating, an azo-crosslinked polymer, cellulose acetate phthalate, cellulose acetate trimellitate (CAT), cellulose acetate butyrate, hydroxypropylmethyl cellulose phthalate, or poly vinyl acetate phthalate.

**[0086]** In other embodiments, a timed-release system such as Pulsincap™ is utilized.

**[0087]** In preferred embodiments, the described coated dosage forms release the core (containing the oil-based formulation) when pH reaches the range found in the intestines, which is alkaline relative to that in the stomach. In more specific embodiments, the coating comprises a pH-sensitive polymer. In various embodiments, either mono-layer or multi-layer coatings may be utilized.

**[0088]** In one embodiment, the coating is an enteric coating. Methods for enteric coating are well known in the art (see, for example, Siepmann F et al 2005). In more specific embodiments, a Eudragit™ coating is utilized as the enteric coating. Eudragit™ coatings are acrylic polymers, the use of which is well known in the art.

**[0089]** In another embodiment, microencapsulation is used as a stomach-resistant coating in the described compositions. Methods for microencapsulation are well known in the art.

**[0090]** In other embodiments, the coating is a capsule, of which gelatin capsules are a more specific embodiment. Methods for inserting an oil-based formulation into a gelatin capsule are well known in the art. In still other embodiments, the coating is a soft-gel, enteric-coated capsule.

**[0091]** In another embodiment, an oral pharmaceutical composition is provided, comprising an oil-based liquid formulation, wherein the oil-based liquid formulation comprises a therapeutic protein of up to 100 kilodaltons, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue), a chelator of divalent cations, an isolated chymotrypsin/trypsin inhibitor, an isolated trypsin inhibitor, and a PEG ester of a fatty acid. In other embodiments, the liquid formulation has an anti-chymotrypsin activity of at least 50 mg. chymotrypsin inhibited per ml. of the liquid formulation. In other embodiments, the liquid formulation has both an anti-chymotrypsin activity of at least 50 mg. chymotrypsin inhibited per ml. of the liquid formulation and an anti-trypsin activity of at least 25 trypsin inhibited per ml. of the liquid formulation. In other embodiments, a free PEG is also present. In other embodiments, a non-ionic detergent is also present. In other embodiments, the liquid formulation consists essentially of a therapeutic protein of up to 100 kilodaltons, a chelator of divalent cations, a chymotrypsin/trypsin inhibitor, a trypsin inhibitor, and a PEG ester of a fatty acid. In other embodiments, the liquid formulation consists essentially of a therapeutic protein of up to 100 kilodaltons, a chelator of divalent cations, a chymotrypsin/trypsin inhibitor, a trypsin inhibitor, a PEG ester of a fatty acid, and a free PEG. In other embodiments, the liquid formulation consists essentially of a therapeutic protein of up to 100 kilodaltons, a chelator of divalent cations, a chymotrypsin/trypsin inhibitor, a trypsin inhibitor, a PEG ester of a fatty acid, a free PEG, and a non-ionic detergent.

## Representative Formulations

**[0092]** Certain representative insulin formulations comprise insulin, Gelucire 44/14, EDTA, SBTI, and aprotinin in fish oil, coated in a capsule. One more specific embodiment is a formulation having the following components: 8-20% Gelucire 44/14; 50-100 mg. per capsule isolated BBI, or isolated BBI/isolated KTI mixture; 20-30 mg. per capsule Aprotinin; and 100-200 mg EDTA; with a therapeutic protein, which may be 8-32 mg. per capsule insulin, all combined into 0.5 - 0.7 ml. fish oil. Another representative liquid insulin formulation contains insulin, Gelucire 44/14, EDTA, BBI, KTI, and aprotinin in fish oil. In other embodiments, the liquid formulation consists essentially of insulin, Gelucire 44/14, EDTA, BBI, KTI, aprotinin, and fish oil. More specific formulations contain 8 mg insulin, 12% Gelucire 44/14, 150 mg EDTA, 75 mg total of BBI and KTI, and 24 mg aprotinin in 0.5-0.7 ml. fish oil; 16 mg insulin, 12% Gelucire 44/14, 150 mg EDTA, 75 mg total of BBI and KTI, and 24 mg aprotinin in 0.5-0.7 ml. fish oil; and 16 mg insulin, 12% Gelucire 44/14, 150 mg EDTA, 150 mg total of BBI and KTI, and 24 mg aprotinin in 0.5-0.7 ml. fish oil. In a still more specific formulation, the ratio of the anti-trypsin activity to the anti-chymotrypsin activity of the composition is about 1.28:1. In other embodiments, the above composition further comprises a non-ionic detergent. In more specific embodiments, the non-ionic detergent is a polysorbate-based detergent. In even more specific embodiments, the polysorbate-based detergent is polysorbate 80. Preferably, the polysorbate 80 constitutes 3-10% weight/weight inclusive of the oil-based liquid formulation. The liquid formulation may be coated by a soft-gel, enteric-coated capsule. Specific formulations described in this paragraph also encompass, in certain embodiments, scaled-up and scaled-down formulation containing the same components in the same ratios.

## Therapeutic indications

**[0093]** In another aspect is provided use of a BBI described herein in the preparation of a medicament for orally administering an active ingredient to a subject. In another aspect is provided use of a KTI3 described herein in the preparation of a medicament for orally administering an active ingredient.

**[0094]** In still another aspect is provided a method for making a pharmaceutical composition, comprising the steps of: (a) producing a mixture comprising a BBI described herein and an active ingredient; and (b) formulating the mixture into a pharmaceutically acceptable formulation.

**[0095]** In still another aspect is provided a method for making a pharmaceutical composition, comprising the steps of: (a) producing a mixture comprising a KTI3 described herein and an active ingredient; and (b) formulating the mixture into a pharmaceutically acceptable formulation.

**[0096]** As referred to herein, the step of formulating comprises the steps of optionally adding excipients, milling, coating, and the like, as appropriate for the desired pharmaceutical composition. These steps are well within the ability of those skilled in the art.

**[0097]** In certain embodiments, an active ingredient as referred to herein is sensitive to degradation or inactivation in the human digestive tract.

**[0098]** In another aspect is provided an oral pharmaceutical composition described herein for orally administering a therapeutic protein, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue), and

**[0099]** In another aspect is provided an oral pharmaceutical composition for use as a medicament.

**[0100]** In another aspect is provided a pharmaceutical composition described herein for use in treating diabetes in a human, where, in some embodiments, the therapeutic protein is in various embodiments insulin.

**[0101]** In an additional aspect is provided a pharmaceutical composition described herein for use in treating unstable diabetes in a human. In another aspect, a pharmaceutical composition described herein is provided for use in treating an elevated fasting blood glucose level in a human.

**Unstable Diabetes**

**[0102]** Physicians skilled in the art will appreciate that unstable diabetes, also known as glycemic lability, can be diagnosed by a number of acceptable standard procedures. One such procedure appears in Ryan et al, 2004. In this procedure, subjects are asked to monitor their glucose levels with a minimum of 2 capillary glucose readings a day. Patients record all measured glucose values and details about hypoglycemic occurrences over a 4-week period on sheets (Figure 11A-B). On any occasion that glucose is recorded as <3.0 mmol/l, the subject is asked to describe the details of the event on the questionnaire (Figure 12), including which symptoms occur and whether outside help from a third party is obtained to either recognize or treat the hypoglycemic reaction. A reaction is considered severe if the individual had lost control of the situation and required outside help to treat the hypoglycemic event. Other such methods involve calculation of the MAGE index (Service et al 1970) or the "M value" (Schlichtkrull et al) or utilize continuous glucose monitoring systems (Kessler et al).

Unstable diabetes is typically associated with elevated fasting blood glucose and/or hypoglycemic episodes.

**Methods of isolating protease inhibitors**

**[0103]** In yet another aspect is provided a method for purifying BBI from a soybean product, e.g. soy flour, comprising the steps of: a) obtaining a liquid mixture comprising an extract from the soybean product, wherein the liquid mixture is a pH-buffered aqueous solution having a pH of between 5.5-7.5 inclusive, with a salt concentration of less than 50 mM; and b). subjecting the solution to size-exclusion chromatography, utilizing a stationary phase that comprises a positively charged resin and a polysaccharide polymer material, wherein the size-exclusion chromatography comprises a discontinuous step salt gradient, wherein the salt concentration of the solution used in the first step is less than 40 mM, preferably less than 20 mM, and the salt concentration of a solution used in a subsequent step (typically but not necessarily the second step) is between 40-85 mM, more preferably 50-80 mM, more preferably 60-80 mM, more preferably 65-75 mM, most preferably 70 mM.

**[0104]** Alternatively or in addition, the above method further comprises the previous step of extracting soy flour at buffer with a pH of 4.5, in the presence of 80-120 mM NaCl, more preferably 100 mM NaCl, and in some embodiments subsequently adjusting the pH to 4.5, followed, alternatively or in addition, by clarification in a filter press. Alternatively or in addition, the method further comprises the previous step (before the aforementioned step of obtaining a liquid mixture having a pH of between 5.5-7.5, but subsequent to extracting the soy flour) of precipitating the initial liquid preparation of the soy flour using 30-40% saturation ammonium sulfate (AS), more preferably 35% saturation, in some embodiments under cooling, for example at a temperature of 12-17 °C. In further embodiments, the precipitate is then collected by centrifugation, for example in a continuous tubular centrifuge. In still other embodiments, the AS pellet is extracted, in certain embodiments in a phosphate buffer of pH 7.0-8.0, then optionally clarified by centrifugation. In various other embodiments, the resulting supernatant is dialyzed against water, or alternatively 10 mM sodium phosphate, pH 6.0-7.0 buffer, clarified by centrifugation, and/or lyophilized.

**[0105]** In more specific embodiments, the above method further comprises the steps of isolating KTI by washing the column with a buffer of 90-120 mM, more preferably 100 mM, and eluting KTI with a buffer of over 150 mM, preferably 180 mM. Alternatively or in addition, the method further comprises the step of removing microorganisms through filtration (a non-limiting example is a 0.45/0.2 μm filter). Alternatively or in addition, the method further comprises the step of filtration though a 20,000-40,000 MWCO filter, preferably 30,000 MWCO, and keeping the permeate. This step is preferably done in the presence of a 0.15-0.5 M salt concentration, more preferably 0.18 M. Alternatively or in addition, the method further comprises the step of concentrating the resulting solution with a 5000 MWCO filter and keeping the retentate. Alternatively or in addition, the method further comprises the step of diafiltering the resulting solution with a 10,000 MWCO filter and keeping the retentate, for example in sodium phosphate buffer having a pH of 7.0-8.0. Alternatively or in addition, the method further comprises the step of lyophilizing the final liquid product.

**[0106]** In still another aspect is provided a method for purifying KTI3 (Kunitz Trypsin Inhibitor 3) from a soybean product, comprising the steps of: a). obtaining a liquid mixture comprising an extract from the soybean product, wherein the liquid mixture is a pH-buffered aqueous solution having a pH of between 6-7 inclusive, with a salt concentration of less than 50 mM; and b) subjecting the solution to size-exclusion chromatography, utilizing a stationary phase that comprises a positively charged resin and a polysaccharide polymer material, wherein the size-exclusion chromatography comprises a discontinuous step salt gradient, wherein the salt concentration of the solution used in a wash step (either the first step or a subsequent step) is more than 80 mM, and the salt concentration of a subsequent step (wherein the KTI3 is eluted) is between 140-250 mM inclusive, preferably between 160-220 mM inclusive, more preferably between 170-200 mM inclusive, more preferably 180 mM.

**[0107]** Alternatively or in addition, the above method further comprises the previous step of extracting soy flour at buffer with a pH of 4.5, in the presence of 80-120 mM NaCl, more preferably 100 mM NaCl. Alternatively or in addition, the method further comprises the previous step of precipitating the initial liquid preparation of the soy flour using 30-40% saturation ammonium sulfate, more preferably 35% saturation. Alternatively or in addition, the method further comprises the step of removing microorganisms through filtration (a non-limiting example is a 0.45/0.2 μm filter). Alternatively or in addition, the method further comprises the step of concentrating the resulting solution with a 5000 MWCO filter and keeping the retentate. Alternatively or in addition, the method further comprises the step of diafiltering the resulting solution with a 10,000 MWCO filter and keeping the retentate. Alternatively or in addition, the method further comprises the step of lyophilizing the final liquid product.

**[0108]** The "liquid mixture" referred to above may in certain embodiments be a solution, a suspension, or a combination thereof. In preferred embodiments, its salt concentration is less than 40 mM, more preferably less than 30 mM, more preferably less than 20 mM, more preferably less than 10 mM. Most preferably, no salt has been added. In other embodiments, the pH of the liquid mixture is between 6-7 inclusive.

**[0109]** The positively charged resin used in the above methods may be in some embodiments a diethylaminoethyl (DEAE) tertiary amine functional group. Alternatively or in addition, the polysaccharide polymer material is crosslinked through lysine side chains. One non-limiting example of such a material is Sepharose™, a crosslinked, beaded-form of a polysaccharide polymer material extracted from seaweed.

**[0110]** In certain embodiments, the described method of isolating protease inhibitors does not utilize a PEG-containing reagent.

**Methods of producing pharmaceutical compositions**

**[0111]** Also provided herein are methods of producing pharmaceutical compositions. In certain embodiments, the method comprises the steps of optionally combining molten Gelucire (for example Gelucire 44/14) with fish oil, cooling the mixture, then adding, in powder form, EDTA, SBTI, aprotinin, and a therapeutic protein, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue). In other embodiments, the method comprises the steps of optionally combining molten Gelucire (for example Gelucire 44/14) with fish oil, cooling the mixture of, then adding, in powder form, EDTA, BBI, and a therapeutic protein, wherein said therapeutic protein is insulin or a glucagon-like peptide-1 analogue (GLP-1 analogue). In certain embodiments, the powder components are added in the listed order. In other embodiments, the resulting mixture is optionally mixed and/or homogenized.

**[0112]** "Consisting essentially of", as used herein, limits the scope of the invention to the specified materials or steps and those that do not materially affect the basic and novel characteristics of the claimed invention.

## EXPERIMENTAL DETAILS SECTION

## EXAMPLE 1: Small-Scale Production of Improved KTI and BBI formulations

### Overview

**[0113]** Previous protocols for producing SBTI from soybean flour generated crude protease inhibitors at a yield of 1%. The following experiments were performed to develop a production method of separate bulk materials of KTI (Kunitz Trypsin Inhibitor) with a Trypsin inhibitor (TI) activity and BBI (Bowman-Birk Inhibitor) with a Trypsin/Chymotrypsin inhibitor (CTI) activity, where each product is prepared under its own specifications to high levels of enzymatic activity, the yield is improved, and high molecular weight (MW)-contaminants are minimized.

**[0114]** The production process involves two major phases:

Part 1: Production of SBTI Intermediate from soy flour. Crude SBTI is precipitated from the supernatant with ammonium sulfate (AS) and collected by centrifugation. The protein is extracted and dialyzed to remove excess AS. The dialyzed extract is lyophilized, analyzed and termed the "SBTI intermediate" (Figure 1A).

Part 2: Downstream purification of SBTI intermediate to produce purified BBI and KTI. The SBTI intermediate is loaded on a DEAE-Sepharose™ fast flow column. KTI and BBI are separately eluted by salt step gradients. The BBI is further purified to reduce the high-MW contaminants by filtration through a 30,000 molecular weight cut-off (MWCO) filter. The final formulation is prepared in phosphate buffer before lyophilization (Figure 1B).

**[0115]** Various parameters of the processes shown in Figure 1 were altered, including: the type of soy flour used as a starting material, the pH and salt level of the extraction buffer, the percentages of AS used for precipitation, the pH and salt level of the buffer used for column loading, and the elution conditions (continuous salt gradient vs. step gradient).

**[0116]** 7B soy flour (de-fatted and minimally heat processed soy flour, produced by Archer Daniels Midland Company Decatur, IL) was used. The preferred pH for extraction was 4.5, which yielded a much lower level of non-relevant proteins, while keeping the level of KTI and BBI relatively high. A continuous material precipitation was observed in the absence of salt; the addition of salt to the extraction buffer improved the process considerably by preventing the precipitation. Use of 35% AS for protein precipitation resulted in a much lower level of high-MW proteins, while the level of KTI and BBI was minimally affected. Use of pH-6.5 column loading buffer reduced the amount of non-relevant proteins bound to the DEAE-Sepharose column. Elution in salt gradient steps resulted in a better separation of BBI from KTI and other non-relevant proteins. The BBI was efficiently purified by filtering through a 30,000 MWCO filter in the presence of 0.5M NaCl.

**EXAMPLE 2: Larger-Scale Improved SBTI Production**

**[0117]** Three larger-scale experiments using 25 kilograms (kg) soy flour were performed. Extract collection was performed with the use of a disk stack centrifuge (Westfalia; first two experiments) or with a filter press (third experiment). 50% of the material was lost with the Westfalia centrifuge, while no loss of extract was observed with the filter press. The protocol and the analytic results of the third experiment are shown in Tables 1-2, respectively.

**Table 1.** Large-scale SBTI Intermediate preparation

| Process Step | Results |
|---|---|
| **Flour extraction:** Mix 25 Kg 7B flour with 250 L extraction buffer, containing 25 mM sodium phosphate and 100 mM NaCl, at pH 4.5, for one hour at room temperature, using an overhead stirrer. Adjust pH to 4.5 with concentrated HCl. | |
| **Clarification:** add 7.5 kg. Hyflo and filter using filter press. Wash filter press with 2 x 50 L extraction buffer. | Clear yellowish solution |
| **Ammonium sulfate precipitation:** 209 grams (gr)/L final concentration (=35% percent saturation). Mix 2 hours at RT, incubate overnight at 12-17 °C. To 490-540 L, with a cooling coil, gradually add the AS. | |

| Process Step | Results |
|---|---|
| **Precipitation of AS pellet:** Collect AS precipitate by centrifugation in a continuous tubular centrifuge (Alfa Laval) at 1.8-2.2 L/min under suction. | 1.4 Kg of firm brownish pellet |
| **Extraction of AS pellet:** Mix pellet with 12L of 10 mM sodium phosphate, pH 7.6 buffer for 30 min and centrifuge in a Sorvall centrifuge, keeping the supernatant. Repeat this step twice with 8L and 4L of buffer. | 4 cycles of extraction were required to extract all the brownish solution from the pellet. Total volume = 15 L |
| **Dialysis:** Pool supernatants at and dialyze in 3,500 MWCO bags against a total of 1 L water, for 40-48 hours at 4-17 °C. | Beige, very hazy solution. Conductivity at the end of dialysis was 800 uSi |
| **Clarification:** by Sorvall centrifuge, 15 minutes at 4800 RPM. | Light brown clear suspension |
| **Lyophilization:** for 48-72 hours | Light brown powder, 444 gr. Final product* |
| * Calculated value based on dialysis of 2.1 liter (L) aliquot. | |

**Table 2.** Analytical Results of SBTI intermediate.

| TEST | RESULTS |
|---|---|
| Appearance | Brown solid |
| Protein percentage by BCA | ≥102% |
| Trypsin inhibitor activity | 1 mg of protein inhibits 1.34 mg of Trypsin having an activity of approx. 10,000 BAEE units per mg. of protein |
| Chymotrypsin inhibitor activity | 1 mg should inhibit 0.98 mg of Chymotrypsin having an activity of approx. 40 BTEE units per mg. of protein |

**[0118]** Next, the SBTI intermediate was subjected to the downstream purification process shown in Table 3. The analytical results of the obtained KIT and BBI are shown in Tables 4-5, respectively.

**Table 3.** Large-scale downstream preparation of purified BBI and KTI.

| Step | | Result |
|---|---|---|
| **Solubilize SBTI Intermediate:** 20 mg/ml. in 1250 ml. of buffer A: 10 mM sodium phosphate, pH 6.5 | | Clear brownish solution, 1250 ml |
| **DEAE Sepharose column chromatography:** | | |
| | Conductivity of charge | 3.09 mS/cm |
| | pH of charge | Adjust to pH 6.5 |
| | Resin amount | 840 ml |
| | Column size (diameter x height) | 10 cm x 10.7 cm |

| **DEAE Sepharose column chromatography:** | | |
|---|---|---|
| | Column volume (CV) | 840 mL |
| | Flow rate | 28 mL/min |
| | Volume of charge | 1250 mL |
| | Volume of wash | 8 L |
| Elution with buffer A containing salt as follows: | | Collect fractions of 0.3L |
| 5 L (6 x CV) of 0 to 0.14 M NaCl (gradient) | | |
| 5L (6 x CV) of 0.14 M NaCl (step) | | |
| 5 L (6 x CV) of 0.14 to 0.18 M NaCl (gradient) | | |
| 5L (6 x CV) of 0.18 M NaCl (step) | | |
| 3L of 0.4 M NaCl (step) | | |
| Collecting BBI pool | | 3.1 L clear solution |
| Collecting KTI pool | | 4.5 L clear solution |
| **Filtration of BBI pool (only 840 ml):** Add NaCl to final concentration of 0.5 M. Filter through TFF 30,000 MWCO and collect permeate | | 830 ml. clear light yellow solution. Recovery by $OD_{280}$ = 61% |
| **Dialysis** of KTI pool and filtered BBI pool in 1000 MWCO bags against water for 2 days | | Clear solutions. Conductivity of both pools is < 100 uSi |
| **Formulation:** Add 0.5 M sodium phosphate, pH 7.6 to the protein suspension so that the protein will be 90% in solid | | |
| **Lyophilization:** in Lyoguard trays for 48 hours | | *White powder of both proteins: 10.2 gr of KTI and 2.25 gr of BBI |

**Table 4.** Analytical results of purified KTI.

| TEST | ANALYTICAL RESULT |
|---|---|
| Appearance | White |
| Protein by BCA | 99% |
| Trypsin inhibitor activity | 1 mg inhibits 1.38 mg of Trypsin with activity of approx. |
| | 10,000 BAEE units per mg of protein |
| Purity by SDS-PAGE and imager Quantitation | 100% |
| | High molecular weight contaminants: <0.1%. |

**Table 5.** Analytical results of purified BBI.

| TEST | ANALYTICAL RESULT |
|---|---|
| Appearance | White |
| Protein by BCA | 99% |

| TEST | ANALYTICAL RESULT |
|---|---|
| Chymotrypsin inhibitor activity | 1 mg inhibits 1.49 mg. of Chymotrypsin with activity of approx. 40 BTEE units per mg of protein. |
| Purity by SDS-PAGE and imager quantitation | 89% BBI |
| | High molecular weight contaminants: <0.1%. |

**[0119]** A clear separation of the KTI and BBI fractions was achieved (Figure 2). The elution was performed with NaCl gradient from 0 to 0.14 M, from 0.14 to 0.18 M and from 0.18 to 0.4 M. Conductivity and $OD_{280}$ were determined for each fraction. The pool of BBI was collected from fractions 18-28. The pool of KTI was collected from fractions 42-57.

**[0120]** The products of each pool were analyzed by SDS-PAGE. A high-purity product was obtained in each case (Figure 3).

**[0121]** Thus, significant improvements in the synthetic method and the resulting product were achieved.

**Table 6**: A summary of the characteristics of the different preparations.

| SBTI intermediate preparation | | | |
|---|---|---|---|
| **Step** | **Previous procedure** | **Small-scale prep** | **Large-scale prep** |
| Starting material (flour) | 1,013 Kg | 0.2 Kg | 25 Kg |
| Clarification of flour extraction | _ | 7.84 gr protein (3.92%) | 1,862 gr (7.5%) |
| SBTI intermediate | 10 kg | 3.5 gr | 444 gr |
| Protein yield | 0.98% | 1.75% | 1.78% |
| Downstream SBTI purification | | | |
| Protein Loaded on column | 2,000 gr | 3.5 gr | 30 gr |
| Final product (BBI+KTI) | | 0.449 | 2.31 gr BBI<br>10.35 gr KTI |
| Purification yield | 6.2% for BBI and 61% for KTI | 13-17% of a mixture of both proteins | 7.4% of BBI and 34% of KTI |
| Degree of BBI purification | + | +++ | +++ |

| | | | |
|---|---|---|---|
| purification | | | |
| BBI Activity | 1.2 | 0.03-0.3 | 1.49 |
| KTI activity | 1.29 | 1.5-1.8 | 1.34 |

**EXAMPLE 3: Alternative downstream BBI and KTI purification protocol**

**Description of changes**

**[0122]** Several changes were made to the downstream purification protocol:

**1. Addition of thimerosol to the pools collected from the columns.**

**[0123]** The BBI lots prepared without thimerosal were found to contain a high total aerobic microbial count (CFU/G) that was above the bio-burden specification of ≤100CFU/G. The total aerobic microbial count results (CFU/G) were: KTI, Lot 1: 0; BBI, Lot 2: 15.4; BBI, Lot 3: 61.6; BBI, Lot 4: 15.4. Therefore, thimerosal was added to the pools that were collected from the column to a 0.01% final concentration. All the subsequent lots were found to be within the bio-burden specification. The total yeast and mold counts were zero for all lots.

**2. Improved salt gradients.**

**[0124]** In order to better separate the BBI from KTI, a stepwise salt gradient was performed, using pre-prepared buffers with known conductivity.

**3. Improved filtration process**

**[0125]** The 30,000 MWCO filtration process was adapted to use the KVICK™ holder outside the system instead of the Uniflux10™ system. The use of the KVICK holder enabled cycling of the material through the filters and handling large volumes at a time. The filtration was performed in a contained fashion, to the extent possible.

**[0126]** This change was found to minimize loss of protein and BBI activity. Prior to the change, 40-70 gram of final product with a BBI activity of 0.4-0.6/mg was obtained per purification lot. Afterwards, 85-135 gram of final product with a BBI activity of 0.8-1.4/mg was obtained. Also, the solution used for the ultrafiltration was changed from 0.5 M NaCl to 0.18 M NaCl.

**Detailed protocol**

**[0127]** The downstream purification was performed in a cGMP facility, starting with 800 gr and 350 gr batches of SBTI intermediate. The intermediate was suspended with 40 L buffer A and loaded on the DEAE column (37 L = CV). The quality control (QC) results for the loaded material were 31.47 mgP/ml; KTI: 0.97; BBI: 0.09. The column was washed sequentially with:

1. 1) 4 x CV buffer A (10 mM sodium phosphate pH 6.5, 1.5 mSi (milliSiemens per meter);
2. 2) 2.1 x CV buffer A1 (10 mM sodium phosphate pH 6.5 + 75 mM NaCl, 9.9 mSi);
3. 3) 3 x CV buffer A2 (10 mM sodium phosphate pH 6.5 + 100 mM NaCl, 12 mSi); and
4. 4) 4 x CV buffer A3 (10 mM sodium phosphate pH 6.5 + 180 mM NaCl, 21 mSi).

**[0128]** The BBI pool consisted of fractions F5-F11 and had a volume of 130 L and 1.46 mgP/ml. The KTI pool consisted of fractions F15-F18 and had a volume of 85 L. Thimerosal was added to both pools to final concentration of 0.01%. Stock NaCl solution was added to the BBI pool to a final concentration of 0.5 M NaCl.

**[0129]** Next, the BBI pool was subjected to filtration, using the Kvick holder, with five 30,000 MWCO cassettes. The permeate was retained. The KTI pool was concentrated 5-fold by the Uniflux system with 3 cassettes of 10,000 MWCO to a final volume of 17.5 L. The concentrated pool was diafiltrated against water by the Uniflux10 system with 3 10,000 MWCO cassettes.

**[0130]** To test the efficacy of the above protocol, small-scale column chromatography was performed, and the different fractions were tested, yielding the improved results shown in Figures 4-5, respectively. These results were found to be reproducible over several runs .

**[0131]** A similar protocol was repeated to purify BBI only, except that 3.5 x buffer A having 12.4 mSi was utilized, and the buffer A3 elution was not performed. Additionally, after ultrafiltrating the BBI pool (135 L) with 2 KVICK holders, using 30,000 MWCO filters (2 x 0.55 $m^2$), the permeate was then concentrated 4.5 times, using the same system but with 5,000 MWCO filters. The concentrated retentate was diafiltrated against water in the Uniflux system. Samples of each purification step and the final product were tested by SDS-PAGE, according to the protocol below; results are shown in Figures 6 and 7, respectively.

**[0132]** The SDS-PAGE utilized ready-to-use gels (PhastGels™, 20%, from Amersham Pharmacia) run in a Phast™ system (Pharmacia). The staining solution was 50% methanol, 10% acetic acid, and 0.05% Brilliant Blue. The de-staining solution was 30 % methanol, 10% acetic acid.

**[0133]** A sample from each step was analyzed for protein concentration and BBI activity. The results are summarized in Table 7. For the final product, the quantified imaging showed 84.4% - 84.9% for the BBI band.

**Table 7**: Yield of protein and activity during the BBI purification steps.

| Step | BCA (mgP/ml) | Volume (L) | Total protein (gr) (%) | BBI activity (mg ChymoTrypsin inhibited/1 mgP-ChymoTrypsin Inhibitor) | Total BBI activity, units (%) |
|---|---|---|---|---|---|
| Column Load | | 40 | 1,100 | | |
| BBI pool | 2.1 | 135 | 283 (25.7%) | 0.5 | 141,500 |
| BBI permeate of 30 Kda. | 0.84 | 173 | 145 (13%) | 0.8 | 116,000 (82%) |
| Retentate of 30 KDa | 41.6 | 2.5 | 104 (9.45%) | 0.13 | 13,500 (9.5%) |
| Retentate of 5 KDa after 4.5 x concentration. | 3.84 | 36 | 138.24 (12.5%) | 0.8 | 110,600 (78%) |
| Permeate of 5 KDa | 0.12 | 120 | 14.4 | 0.13 | 1,872 |
| Retentate of 10 Kda after diafiltration | 4.03 | 36 | 145 (13%) | 0.7 | 101,500 (71%) |
| Final after lyophilization "as-is" | 1.07 mgP/mgS | 136 gr solid | 136 gr solid (12.3%) | 0.8/mg solid* | 108,800 (77%) |

* The material contains 10% water, therefore the result of activity is 0.9 on the basis of dry weight.

**[0134]** A flow chart of the improved protocol is shown in Figure 8.

**[0135]** Prior to the improvements described in Examples 1-3, large-scale SBTI purifications yielded 40-70 gram of final product per purification lot, with a BBI activity of 0.4-0.6/mg (while somewhat higher activity was obtained in smaller-scale preparations, this could not be successfully scaled-up). The changes enabled a yield of 85-135 gram of final product with a BBI activity of 0.8-1.4/mg.

**<u>EXAMPLE 4: Downstream BBI and KTI purification protocol using microfiltration</u>**

**[0136]** The protocol in this Example was performed without thimerosal. Starting with the SBTI intermediate, DEAE column chromatography was performed similarly to the last Example but with slightly different parameters, as follows:

3 x CV buffer A (10 mM sodium phosphate pH 6.5, 1.5 mSi)

2.1 x CV buffer A1 (10 mM sodium phosphate pH 6.5 + 70 mM NaCl, 9.9 mSi)

3.5 x CV buffer A2 (10 mM sodium phosphate pH 6.5 + 100 mM NaCl, 12.4 mSi)

3.5 x CV buffer A3 (10 mM sodium phosphate pH 6.5 + 180 mM NaCl, 21 mSi)

**[0137]** Results are shown in Figure 9.

__BBI pool__

**[0138]** The BBI pool, 130 L, was collected and filtered through a Sartobran™ 0.45/0.2 µm filter (0.6 m2).

__KTI pool__

**[0139]** The KTI, 95 L, was collected and filtered through a Sartobran™ 0.45/0.2 µm filter (0.6 $m^2$) and stored at - 20 °C. Final volume = 20 L.

__BBI purification:__

__30,000 MWCO filtration__

**[0140]** The BBI pool was adjusted to 0.18 M NaCl concentration and subjected to filtration using 2 x Kvick™ holders, each with 5 cassettes of 30,000 MWCO. The permeate, after four washes, was collected, 195 L total

__Concentration__

**[0141]** The permeate of the 30,000 MWCO was concentrated 5 times through 2 KVICK holders, each with five 5,000 MWCO cassettes in each. Final volume = 34 L.

__Diafiltration__

**[0142]** The concentrated material was diafiltrated against purified water using a Uniflux 10 system with 5 x 10,000 MWCO filters to a conductivity of less than 1 mSi/cm.

**[0143]** Formulation buffer (0.5 M sodium phosphate buffer, pH 7.6) was added, and the sample was passed through a 0.45/0.2 µm filter directly into Gore® Lyoguard® Freeze-Drying Trays.

## Lyophilization

**[0144]** After lyophilization, 95 gr. of dry material was obtained and collected into glass amber bottles.

**[0145]** All bottles were QC sampled: The water content was found to be 8% by the Karl Fischer method.

**[0146]** Two duplicate samples were tested by SDS-PAGE, and the gel was quantified using an imager. The BBI in both lanes was at 93.5-93.8% purity (Figure 10).

**[0147]** Samples from each BBI purification step were tested for protein concentration, BBI activity and bio-burden. Table 8 summarizes the BBI quantity and quality along the purification process:

**Table 8: BBI purification steps, quantity, characteristics, and bio-burden.**

| Step | BCA (mgP/ml) | Volume (L) | Total protein (gr) | BBI (mg ChymoTrypsin inhibited/1 mgP-ChymoTrypsin Inhibitor) | Total BBI activity | Bio-burden (TAMC) (CFU/ml) |
|---|---|---|---|---|---|---|
| Column Load | NA | 46 | 1,100 | NA | NA | TNTC |
| BBI pool | 1.4 | 130 | 182 | 0.6 | 109,000 | TNTC |
| BBI pool after microfiltration 0.45/0.2 | 1.4 | 130 | 182 | 0.6 | 109,000 | 1 |
| BBI permeate after 30 Kda UF and additional washes | 0.7 | 195 | 136.5 | 0.8 | 109,000 | 10 |
| Retentate of 5 KDa after 5x concentration | 3.6 | 34 | 122.5 | 0.9 | 110,250 | 148 |
| Retentate of 10 Kda after diafiltration | 3.1 | 34 | 105 | 1.0 | 105,000 | TNTC |
| BBI After formulation and microfiltration 0.45/0.2 | 3.0 | 34 | 102 | 0.8 | 81,600 | 0 |
| Final after lyophilization | – | – | 95 gr solid | 0.9 | 85,500 | 0 |

TNTC = too numerous to count

## KTI purification:

## Concentration of KTI

**[0148]** The microfiltrated KTI pool was taken out of cold storage and concentrated using 2 x KVICK holders with 5 5KDa MWCO filters each. Final volume = 17 L

## Diafiltration of KTI

**[0149]** The concentrated material was diafiltrated against water by Uniflux with 5 x 10,000 MWCO filters. Final volume = 20 L

**[0150]** Formulation buffer was added, and the sample was passed through a 0.45/0.2 µm filter directly into Gore lyophilization trays.

## Lyophilization

**[0151]** The lyophilized product was removed and transferred to 4 amber glass bottles.

**[0152]** Samples from each KTI purification step were tested for protein concentration, KTI activity and bio-burden. Table 9 summarizes the KTI quantity and quality along the purification process:

**Table 9: KTI purification steps, quantity, characteristics, and bio-burden.**

| Step | BCA (mgP/ml) | Volume (L) | Total protein (gr) | KTI (mg Trypsin inhibited/1 mgP-Trypsin Inhibitor) | Total KTI activity | Bio-burden (TAMC) (CFU/ml) |
|---|---|---|---|---|---|---|
| Column Load | NA | 46 | 1,100 | NA | NA | TNTC |
| KTI pool | 4.5 | 95 | 427.5 | 1.5 | 641,250 | TNTC |
| KTI pool after microfiltration 0.45/0.2 | 4.3 | 95 | 408.5 | 1.5 | 612,750 | TNTC |
| Retentate of 5 KDa after 5x concentration | 24.7 | 17 | 419.9 | 1.3 | 545,870 | 4.2 x $10^7$/ml |
| Retentate of 10 Kda after diafiltration | 18 | 20 | 360 | 1.5 | 540,000 | 3.4 x $10^7$/ml |
| KTI After formulation and microfiltration 0.45/0.2 | 18 | 20 | 360 | 1.4 | 504,000 | 0.0 |
| Final after lyophilization | _ | _ | 300 gr solid | Not yet | | Not yet |

## Conclusions

**[0153]**

1. 1. The SBTI intermediate has a relatively high bio-burden, which can be removed by the second microfiltration.
2. 2. The final products following microfiltration are within the required specifications.
3. 3. The use of microfiltration has no significant effect on the yield or specific activity of BBI and KTI.

**EXAMPLE 5: Identification of effective emulsifiers for homogenous insulin/fish oil preparations**

**[0154]** Previous formulations of insulin in fish oil were found to slowly precipitate; they were thus unsuitable for large-scale pharmaceutical dosage form preparation. New formulations containing 3.375 g. SBTI per 22.5 g. of fish oil and containing the following emulsifiers were tested: lecithin (trial sequence 1), Polysorbate 80 (Tween-80) (sequence 2), or Gelucire 44/14 (sequence 3), alone or in combination with each other or glycerol monostearate (GMS) (Figure 11). Subsequently, the most promising formulations (indicated with an asterisk) were produced again by melting the Gelucire (which was a waxy solid as ambient temperature), then adding it to the fish oil. After cooling this mixture, the solid components were added in powder form in the following order: EDTA, SBTI, aprotinin, and insulin; and the resulting liquid was mixed and homogenized on a roller mill.

**EXAMPLE 6*: In Vivo* Animal Testing of Various Emulsifier Formulations**

**Materials And Experimental Methods**

***Formulations***

**[0155]** The formulations that were tested are shown below in Table 10. The percentages of emulsifiers given are weight/weight with respect to weight of the liquids present.

**Table 10.** Formulations used in this Example.

| Formulation name | Emulsifiers | Other ingredients |
|---|---|---|
| Experiment 6A | | |
| GMS 2% | GMS 2% only | 75 mg SBTI, 150 mg EDTA, 24 mg aprotinin, 8 mg human insulin, 0.5-0.7 ml of fish oil |
| 2%-2% | 2% GMS, 2% lecithin | Same as above. |

| Formulation name | Emulsifiers | Other ingredients |
|---|---|---|
| Experiment 6A | | |
| 2%-10% | 2% GMS, 10% lecithin | Same as above. |
| 10% lec. | 10% lecithin only | Same as above. |
| Experiment 6B | | |
| A | 5% lecithin, 2% GMS | Same as above. |
| B | 3% lecithin, 12% Gelucire 44/14 | Same as above. |
| C | 6% lecithin, 12% Gelucire 44/14 | Same as above. |
| D | 5% Tween-80, 12% Gelucire 44/14 | Same as above. |
| E | 10% Tween-80, 12% Gelucire 44/14 | Same as above. |
| F | 12% Gelucire 44/14 only | Same as above. |

***Husbandry***

**[0156]** Animals: Only healthy pigs, as certified by a clinical veterinarian, were used for the study. Housing: Solitary when with CVC and grouped at other times. Bedding: Concrete + woodchips. Illumination: 12-12h light cycle. Temperature: 19-25 °C.

***Identification***

**[0157]** Each animal was uniquely identified via ear tags.

***Experimental design***

**[0158]** Animals were deprived of food 24-36 hours prior to testing and during the ensuing monitoring period. Access to water was *ad libitum.*

**[0159]** Animals were anesthetized with 20mg/kg ketamine + 2mg/kg xylazine. Fasting and anesthetized pigs were positioned on their left side before liquid formulations were administered under endoscopic guidance, directly to the duodenum. After injection of the formulation, 1 ml. fish oil was injected, followed by 10 ml. air, to flush the apparatus, thereby ensuring administration of all materials. Pigs were then returned to their pens to allow for full recovery from the anesthetic treatment, which required 10-15 min. Blood samples (0.5 ml. of which were tested) were periodically drawn from the central line catheter (CVC) over the ensuing 240-min monitoring period. Blood glucose concentrations were determined from each sample, at each time point. Piglets were intravenously treated with gentamycin (100 mg/10 kg) after every experiment day to avoid infection. In cases where glucose concentrations dropped below 30 mg/dL, piglets were served commercial pig chow, and glucose concentrations were monitored for an additional 30 minutes thereafter.

**[0160]** A washout period of at least 2 days was enforced between test days.

**Results**

**[0161]** 10 insulin-fish oil formulations with different emulsifiers were tested for *in-vivo* activity on blood glucose levels in 2 separate experiments. Results are shown in Table 11.

**Table 11.** Results of Experiment 6A. The three numbers in each box indicate baseline value, lowest value, and end (20 mg/dL). "Low" indicates a value of less than 20 mg/dL.

| Formulation | Pig 1 | Pig2 | Pig3 | Pig4 | Pig 5 (stoma) | Score* |
|---|---|---|---|---|---|---|
| GMS 2% | 78 → 48 → 65 | 72 → low → **low** | 63 → 23 → 28 | 67 → 32 → 60 | 55 → 21 → 58 | 3 |
| 2%-2% | 82 → Low → 65 | 78 →45 → 76 | 85 →30 → 68 | 78 → 23 → 61 | | 2 |
| 2%-10% | 76 → **Low** → 77 | 76 → **low** → **21** | 76 → low → **low** | 74 → low → 70 | | 4 |
| 10% lec. | 51 → 63 → 71 | 50 → low → **low** | 57 → low → **low** | 61 → low → **low** | | 5 |
| * see Figure 11 legend. | | | | | | |

**[0162]** The results of Experiment 6B, depicted in Figure 12, show that while all the formulations were efficacious, formulations E and F from Table 10 induced the most uniformly sharp drops in glucose levels.

**EXAMPLE 7: Clinical testing of oral protein formulations containing improved SBTI**

**[0163]** A study was performed to determine the safety, pharmacokinetics, and pharmacodynamics of the described oral insulin formulations in healthy volunteers. Subjects received a single dose of one of the following oral insulin tablet formulations on separate visits. Each dose was followed by a 72-hour washout period. Doses were administered in the morning after an 8-hour overnight fast.

**Treatment groups**

**[0164]**

1. 1. 1 capsule of 8-mg Insulin Formulation: 8 mg Insulin, 150 mg EDTA, 75 mg total of a mixture of purified BBI and purified KTI, 150000U Aprotinin, and 12% Gelucire 44/14.
2. 2. 1 capsule of 16-mg Insulin Formulation: 16 mg Insulin, 150 mg EDTA, 75 mg total of a mixture of purified BBI and purified KTI, 150000U recombinant Aprotinin and 12% Gelucire 44/14.
3. 3. 2 capsules of 8-mg Insulin Formulation.

**Screening phase:**

**[0165]** The following evaluations were performed after the subject signed informed consent:

- Medical history
- Physical examination
- Medication history
- ECG
- Vital signs (blood pressure, heart rate). Vital signs were measured in the sitting position after at least 5 minutes of rest.
- Clinical laboratory evaluations (chemistry, hematology)

**Treatment phase**

**[0166]** Subjects entered the clinic on the morning of dosing after an 8-hour overnight fast.

**[0167]** Prior to study drug administration:

- An indwelling catheter was inserted for blood sample collection.
- A glucose test (one drop) was performed 15 minutes (min) prior to study drug administration
- Vital signs were recorded 20 min prior to study drug administration. Vital signs were measured in the sitting position after at least 5 min of rest.

- Blood samples for insulin, plasma glucose and c-peptide analysis were collected 15 min prior to study drug administration.

**Protocol for drug administration:**

**[0168]** The experimental drug was administered orally with at least 300 mL of water. Subjects remained in an upright or sitting position for at least one hour after taking study medication.

- Vital signs (blood pressure, heart rate) were recorded at approximately 2 and 5 hours (hr) post study drug administration. Vital signs were measured in the sitting position after at least 5 min of rest.
- Blood samples for insulin, plasma glucose, and c-peptide analysis were collected every 20 min during the first hr. and then every 15 min up to 5.0 hr post study drug administration.

**[0169]** After completing the evaluations for each period, the subjects were fed and discharged, then asked to return for the next period after a minimum of 72-hour washout period.

**End of study/early discontinuation:**

**[0170]** Prior to discharge from the research unit, subjects underwent the following end-of-study evaluations:

- Vital signs were measured in the sitting position after at least 5 min of rest.
- Clinical laboratory evaluations (hematology, chemistry)

**[0171]** Subjects that discontinued early completed the end-of-study evaluations at the time of discontinuation.

**Results**

**[0172]** The various doses of insulin were well tolerated, with no adverse events reported or observed following any of the treatments. Responses were demonstrated in 7/10 subjects, with maximal blood glucose responses for all treatments observed following a lag of ≥60 min from administration. Significantly lower mean blood glucose $C_{min}$ was observed following the 8+8mg (47.9 ± 11.3 mg/dL, p=0.006) and 16mg (57.3 ± 8.4 mg/dL, p=0.001) treatments, when compared to the 8-mg group (64.6 ± 6.2 mg/dL). Significant reductions in glucose area under the curve (AUC) values were observed following both 8+8mg and 16mg treatments (13.2% and 8.1%, respectively), when compared to 8mg (p=0.003 and 0.008, respectively) (Figure 15 and Table below). Such differences were not seen with formulations containing prior art SBTI preparations.

**[0173]** Summary of Area-Under-the-Curve (AUC) data following insulin administration

| Treatment | Avg AUC (mg/dL*min) | St-dev (mg/dL*min) |
|---|---|---|
| 8 | 22425 | 2153 |
| 16 | 20610 | 1428 |
| 8+8 | 19471 | 2126 |

**EXAMPLE 8: Animal testing of oral protein formulations containing improved SBTI**

**[0174]** Additional animal testing is performed on formulations comprising highly purified SBTI and a protein drug (e.g. insulin or exenatide; exenatide not being covered by the subject-matter of the claims), in a manner similar to one or more of the protocols described herein. In some experiments, liquid formulations are coated with a gelatin and/or enteric-coated capsule, which may be administered orally. In other experiments, liquid formulations are administered directly to the duodenum via a cannula or the like. In certain experiments, animals are allowed to eat prior to or following administration of the formulation, to simulate pre-prandial or post-prandial conditions.

**[0175]** In still other experiments, recombinant SBTI is used as described above. In other experiments, synthetic SBTI is used as described above.

**EXAMPLE 9: Testing of oral exenatide formulations containing improved SBTI in human subjects (exenatide formulations are not covered by the subject-matter of the claims**

**[0176]** The following study is being performed to assess the safety, pharmacokinetics and pharmacodynamics of oral exenatide formulations comprising purified BBI and KTI and optionally 12% Gelucire 44/14 in healthy volunteers and in T2D subjects, with screening and treatment performed similarly to Example 7:

Stage I: Stage I consists of two segments: Segment 1 will assess the safety, tolerability and the PK/PD of escalating doses of oral exenatide in healthy volunteers. In the first segment, the two lower doses of exenatide (150 and 300 µg) will be randomly administered to all healthy, fasting subjects. If deemed safe, further dose escalation (450 and 600 µg exenatide) will be authorized in Segment 2. The highest tolerable dose will then be administered to all healthy patients 60 minutes before a standard meal (Visit 5).

Stage II: This stage will assess the T2DM patient response to escalating doses of exenatide when delivered 60 min before a standard meal. Placebo controls may be included in the study, as may treatment with an active control of Byetta (5μg) subcutaneously delivered 30 min before a standard meal. In addition, all T2DM subjects will be treated with an oral insulin capsule containing 16 mg of insulin and with a combination of oral insulin/oral exenatide, at two independent study visits, 60 minutes before being served a standard meal.

**Interpretation:**

**[0177]** AUC of glucose reductions and insulin excursion will be calculated and compared between the different treatments. The efficacy of the formulation will be shown by insulin excursions in the oral GLP-1-treated group that are greater than those in the non-GLP-1-treated group, and/or smaller glucose excursions in the oral GLP-1-treated group.

**[0178]** In still other experiments, recombinant BBI (optionally with KTI and/or aprotinin) is used as described above in place of purified BBI and KTI. In other experiments, synthetic BBI (optionally with KTI and/or aprotinin) is used as described above in place of purified BBI and KTI.

**EXAMPLE 10: Testing of oral insulin formulations containing improved SBTI in human subjects**

**[0179]** The following study is being performed to assess safety and pharmacodynamics of multiple bedtime doses of oral insulin formulations comprising purified BBI and KTI and optionally 12% Gelucire 44/14, in adult patients with T2DM who are inadequately controlled with diet and metformin, with screening and treatment performed similarly to Example 7:

Primary Objectives: To evaluate the pharmacodynamic effects of the formulation on mean nighttime glucose levels and safety parameters (e.g., hypoglycemia), and to evaluate safety, including incidence of hypoglycemia and cardiovascular events. Primary efficacy endpoints will be determined by:

- The effect of 24 mg insulin on weighted mean nighttime glucose levels based on two nights of (continuous glucose monitoring (CGM)) data*, determined by comparison of:
    1. a) The mean percent change between baseline (run-in period) and 24 mg insulin treatment to
    2. b) The mean percent change between baseline and placebo treatment for the group receiving placebo.

• "CGM data" as used herein is always based entirely on data collected within the first six hours after oral medicinal treatment.

- The effect of 16 mg insulin on weighted mean nighttime glucose levels based on two nights of CGM data determined by comparison of:
    1. a) The mean percent change between baseline (run-in period) and 16 mg insulin treatment to
    2. b) The mean percent change between baseline and Week 4 of placebo treatment for the group receiving placebo.

Secondary Objectives: To evaluate changes in baseline in fasting blood glucose (FBG), morning fasting serum insulin, c-peptide, triglycerides, and HbA1c. Secondary endpoints will be determined by:

- The effect of 24 mg insulin and/or 16 mg insulin on weighted mean nighttime glucose levels will determined by comparison of weighted mean nighttime glucose levels for insulin-treated groups vs. the group receiving placebo.
- The effect of 24 mg insulin and/or 16 mg insulin on changes from baseline to the treatment phase in fasting morning blood glucose, morning fasting serum insulin, morning fasting c-peptide, HbA1c, and triglycerides.
- The effect of 24 mg insulin and/or 16 mg insulin on changes from baseline to the treatment phase in mean glucose assessed by CGM.

**[0180]** In still other experiments, recombinant BBI (optionally with KTI and/or aprotinin) is used as described above in place of purified BBI and KTI. In other experiments, synthetic BBI (optionally with KTI and/or aprotinin) is used as described above in place of purified BBI and KTI.

**EXAMPLE 11: Testing of oral insulin formulations containing improved SBTI in treatment of unstable diabetes**

**[0181]** Subjects with unstable diabetes (for example, subject having a glycated hemoglobin [HgA1c] level of 8-10%) are monitored for several days using a blinded continuous glucose monitor (CGM) to establish a baseline. During several subsequent days, they are administered an insulin-containing formulation described herein, optionally using prior art formulations as a control group, prior to meals. Blinded CGM is performed to determine the efficacy of the formulations.

**[0182]** In still other experiments, recombinant BBI (optionally with KTI and/or aprotinin) is used as described above in place of purified BBI and KTI. In other experiments, synthetic BBI (optionally with KTI and/or aprotinin) is used as described above in place of purified BBI and KTI.

**EXAMPLE 12: Testing of oral insulin formulations containing improved BBI in the absence of other protease inhibitors**

**[0183]** Additional studies are performed, similar to those described above, but using BBI prepared as described herein, in the absence of KTI (e.g. BBI and aprotinin as the only protease inhibitors), and in other experiments, in the absence of any other protease inhibitors. The ability of the improved BBI alone to protect protein drugs in the described formulations in the absence of other protease inhibitors confirms the superiority of BBI prepared as described herein.

**[0184]** In the claims, the word "comprise", and variations thereof such as "comprises", "comprising", and the like indicate that the components listed are included, but not generally to the exclusion of other components.

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PATENTKRAV

1. Oral farmaceutisk sammensætning omfattende en oliebaseret væskeformig formulering, hvor den oliebaserede væskeformige formulering omfatter et terapeutisk protein på op til 100 kilodalton, et chelaterende stof af divalente kationer og en isoleret BBI (Bowman-Birk-hæmmer) og enten hvor;

(i) BBI'en er blevet oprenset til mindst 85 % renhed målt ved natriumdodecylsulfatpolyacrylamid-gelelektroferese (SDS-PAGE), eller

(ii) BBI'en er blevet oprenset til et proteinindhold på mere end 95 % ved BCA (bicinchoninsyre)-analyse, eller

(iii) BBI'en indeholder mindre end 0,1 % højmolekylære kontaminanter;

hvor den væskeformige formulering har en antichymotrypsinaktivitet på mindst 40 mg. chymotrypsin hæmmet pr. ml. af den væskeformige formulering;

hvor det terapeutiske protein er insulin eller en GLP-1 (glucagonlignende peptid-1)-analog.

2. Oral farmaceutisk sammensætning ifølge krav 1, hvor den oliebaserede væskeformige formulering endvidere omfatter en anden trypsinhæmmer end BBI'en.

3. Oral farmaceutisk sammensætning ifølge krav 2, hvor trypsinhæmmeren er KTI (Kunitz-trypsinhæmmer), og eventuelt hvor:

(i) KTI'en er blevet oprenset til mindst 85 % renhed målt ved SDS-PAGE, eller

(ii) KTI'en er blevet oprenset til et proteinindhold på mere end 95 % målt ved BCA-analyse, eller

(iii) KTI'en indeholder mindre end 0,1 % højmolekylære kontaminanter.

4. Oral farmaceutisk sammensætning ifølge krav 1, hvor den væskeformige formulering endvidere omfatter en anti-trypsinaktivitet på mindst 20 mg. trypsin hæmmet pr. ml. af den væskeformige formulering.

5. Oral farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1-4, hvor det terapeutiske protein er insulin.

6. Oral farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1-4, hvor det terapeutiske protein er en GLP-1-analog.

7. Oral farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1-6, hvor den oliebaserede væskeformige formulering endvidere omfatter en polyethylenglycol (PEG)-ester af en monoglycerid, en diglycerid, en triglycerid eller en blanding deraf.

8. Oral farmaceutisk sammensætning ifølge krav 7, hvor den oliebaserede væskeformige formulering endvidere omfatter en fri PEG.

9. Oral farmaceutisk sammensætning ifølge krav 7, hvor PEG-esteren tilvejebringes som en blanding af (a) en monoacylglycerol, en diacylglycerol, en triacylglycerol eller en blanding deraf; og (b) en polyethylenglycol (PEG)-ester af en fedtsyre.

10. Oral farmaceutisk sammensætning ifølge krav 9, hvor:

(i) del (a) af blandingen omfatter $C_8$-$C_{18}$-monoacylglyceroler, -diacylglyceroler og -triacylglyceroler, eller

(ii) del (b) af blandingen omfatter PEG-monoestere og -diestere af en blanding af Cs-Cis-fedtsyrer, eller

(iii) del (a) af blandingen omfatter Cs-Cis-monoacylglyceroler, -diacylglyceroler og -triacylglyceroler; del (b) af blandingen omfatter PEG-32-monoestere og -diestere af en blanding af $C_8$-$C_{18}$-fedtsyrer; den oliebaserede væskeformige formulering endvidere omfatter (c) fri PEG-32; og vægt/vægt-forholdet mellem del (a) af blandingen og summen af del (b) af blandingen og (c) er mellem 10:90 og 30:70, begge inklusive, og eventuelt hvor (a), (b) og (c) tilsammen udgør 8-16 % vægt/vægt, begge inklusive, af den oliebaserede væskeformige formulering.

11. Oral farmaceutisk sammensætning ifølge et hvilket som helst af kravene 7-10, der endvidere omfatter et non-ionisk detergent, og eventuelt hvor det non-ioniske detergent er et polysorbat-baseret detergent.

12. Oral farmaceutisk sammensætning ifølge krav 11, hvor det polysorbat-baserede detergent er polysorbat 80, og eventuelt hvor polysorbat 80'et udgør 3-10 % vægt/vægt, begge inklusive, af den oliebaserede væskeformige formulering.

13. Oral farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1-12, hvor den oliebaserede væskeformige formulering er vandfri.

14. Oral farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1-13, der endvidere omfatter et overtræk, der modstår nedbrydning i mavesækken, og eventuelt hvor overtrækket er en pH-følsom kapsel, eller overtrækket er en blød gelatinekapsel.

15. Oral farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1-14 til anvendelse i en fremgangsmåde til behandling af diabetes og/eller ustabil diabetes, hvilken fremgangsmåde omfatter trinnet med oral indgivelse af væske-oliesammensætningen omfattende det terapeutiske protein til et subjekt.

# DRAWINGS

Drawing

**Flow Diagram of**
**SBTI Intermediate**

**Day 1: Buffer preparations**
Extraction buffer: 25 mM sodium phosphate, 100 mM NaCl, pH 4.5.
Dialysis buffer: 10 mM sodium phosphate, pH 6.5

↓

**Day 2; Soy flour extraction**
Mix 25 Kg flour with 250 L Extraction buffer for 1 hour at RT

↓

**Day 2; Clarification**
Add 7.5 Kg Hyflo, mix and transfer through filter press. Collect supernatant. Wash the filter press with 80 L Extraction buffer

↓

**Day 2; Ammonium Sulfate Precipitation**
Combine extraction of 2 x 25 Kg and add Ammonium Sulfate, 0.209 Kg/L (35% saturated). Mix 2 hr in RT. Store overnight < 15°C

↓

**Day 3: Collect Ammonium Sulfate Precipitate**
Centrifuge by continuous tubular centrifuge ("Sharples") and retain the pellet.

Figure 1A

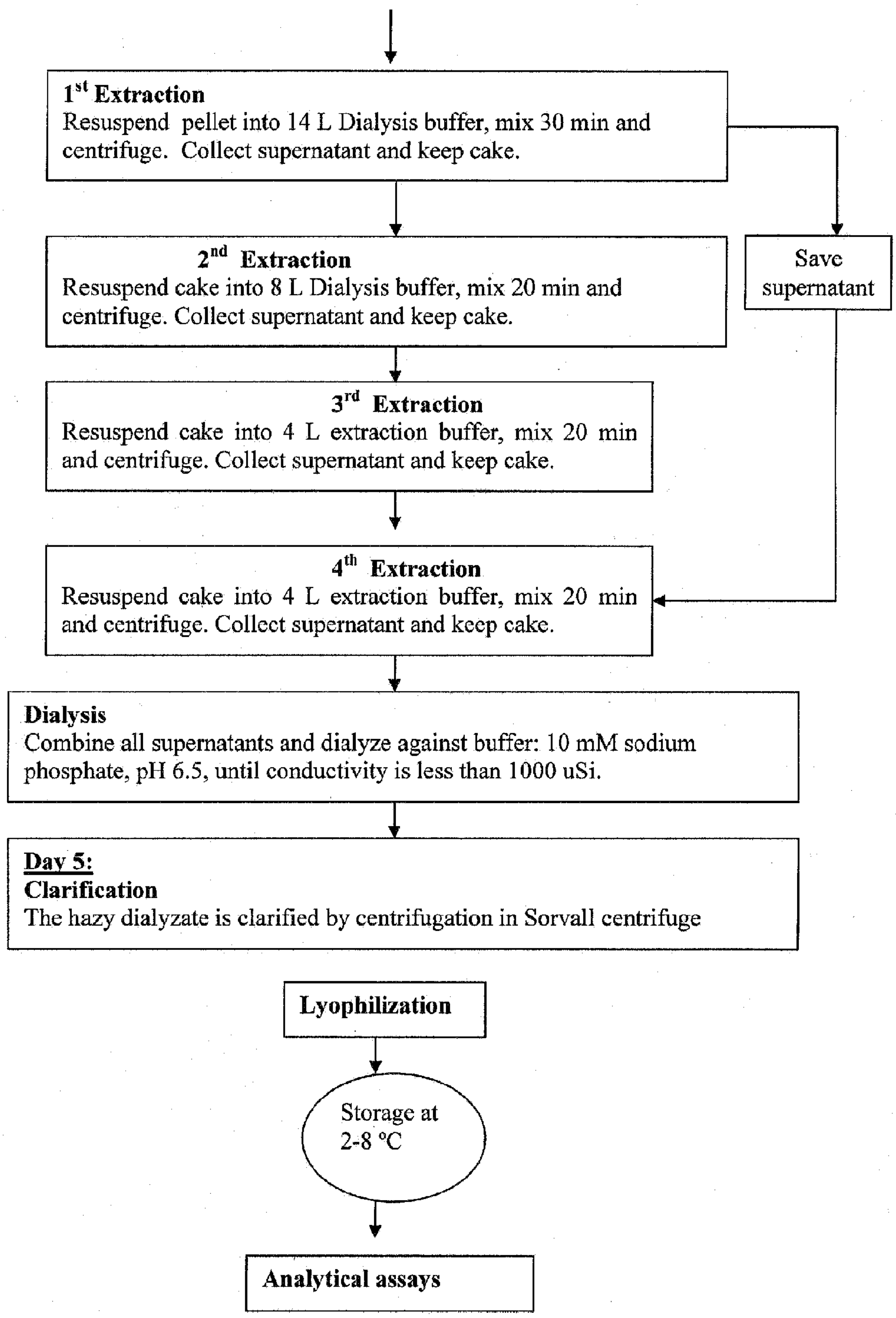



Figure 1A, continued

**Flow Diagram of**
**Downstream purification to produce BBI and KTI**

**DEAE Sepharose Column Chromatography**
1 Kg of suspended SBTI intermediate loaded on 30 L column (Ratio of 1 ml resin for every 30 mg protein). Flow rate: 1 x CV/30 min, Wash with 8 x CV of DEAE Buffer: 10 mM Sodium phosphate, pH 6.5. Elute with NaCl step gradients. Pool the BBI and the KTI separately.

**BBI Purification**
After adding 0.5 M NaCl, filter with UF unit containing 3 x 0.11 $m^2$ filters with 30,000 MWCO. Save permeate.

**Concentration and diafiltration of BBI**
Concentrate 3 fold with UF unit containing 3 x 0.11 $m^2$ filters with 5,000 MWCO. Save retentate and diafiltrate with water until conductivity is less than 1000 uSi.

**Concentration and diafiltration of KTI**
Concentrate 3 fold with UF unit containing 3 x 0.11 $m^2$ filters with 5,000 MWCO. Save retentate and diafiltrate with water until conductivity is less than 1000 uSi.

**Final formulation**
To each protein solution add 1 M sodium phosphate buffer so that the protein percentage will be 90% in solid

**Lyophilization**

**Store at 2-8 °C**

Figure 1B

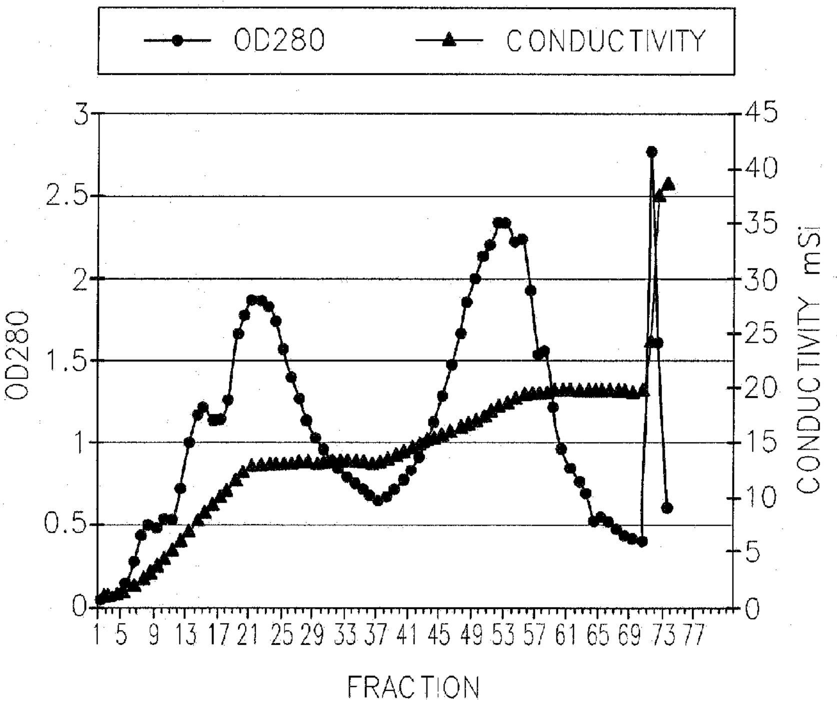



Figure 2

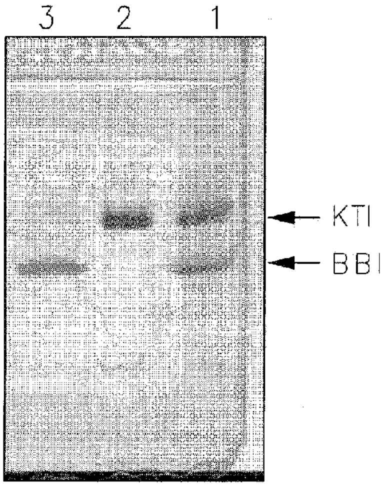



Figure 3

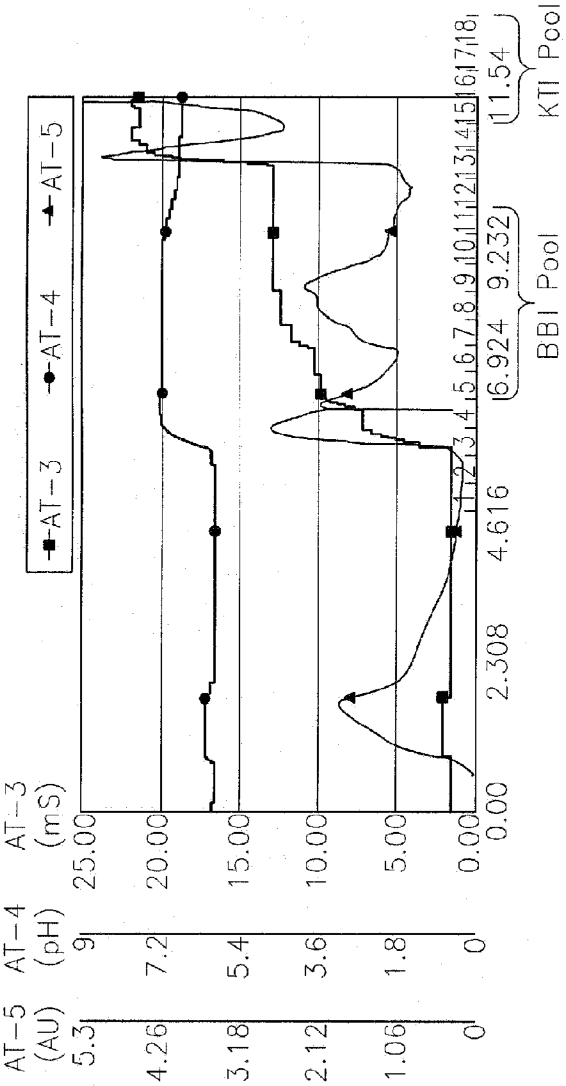



Figure 4

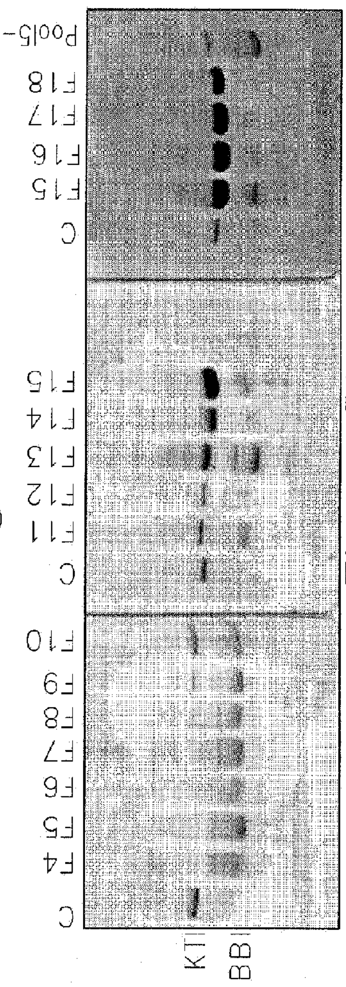



Figure 5A

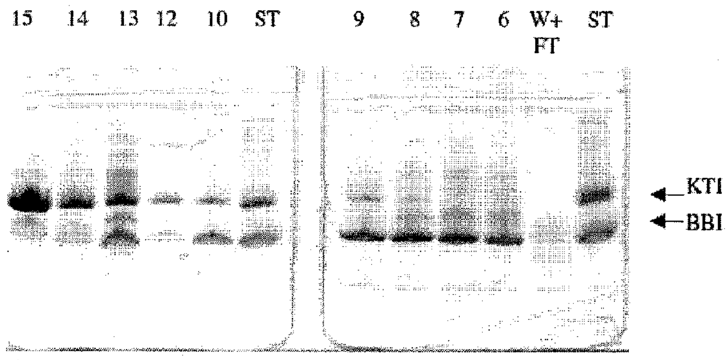



Figure 5B

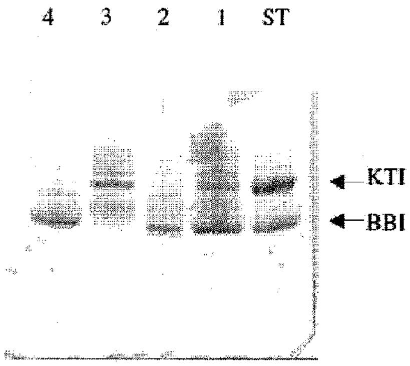
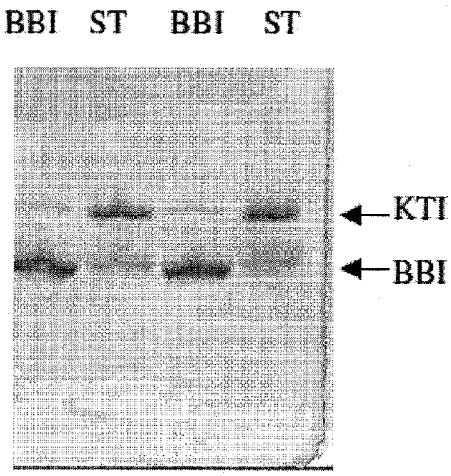



Figure 6




ST: Control, T9003, 1mg/ml.
BBI: 1 mgS/ml

Figure 7

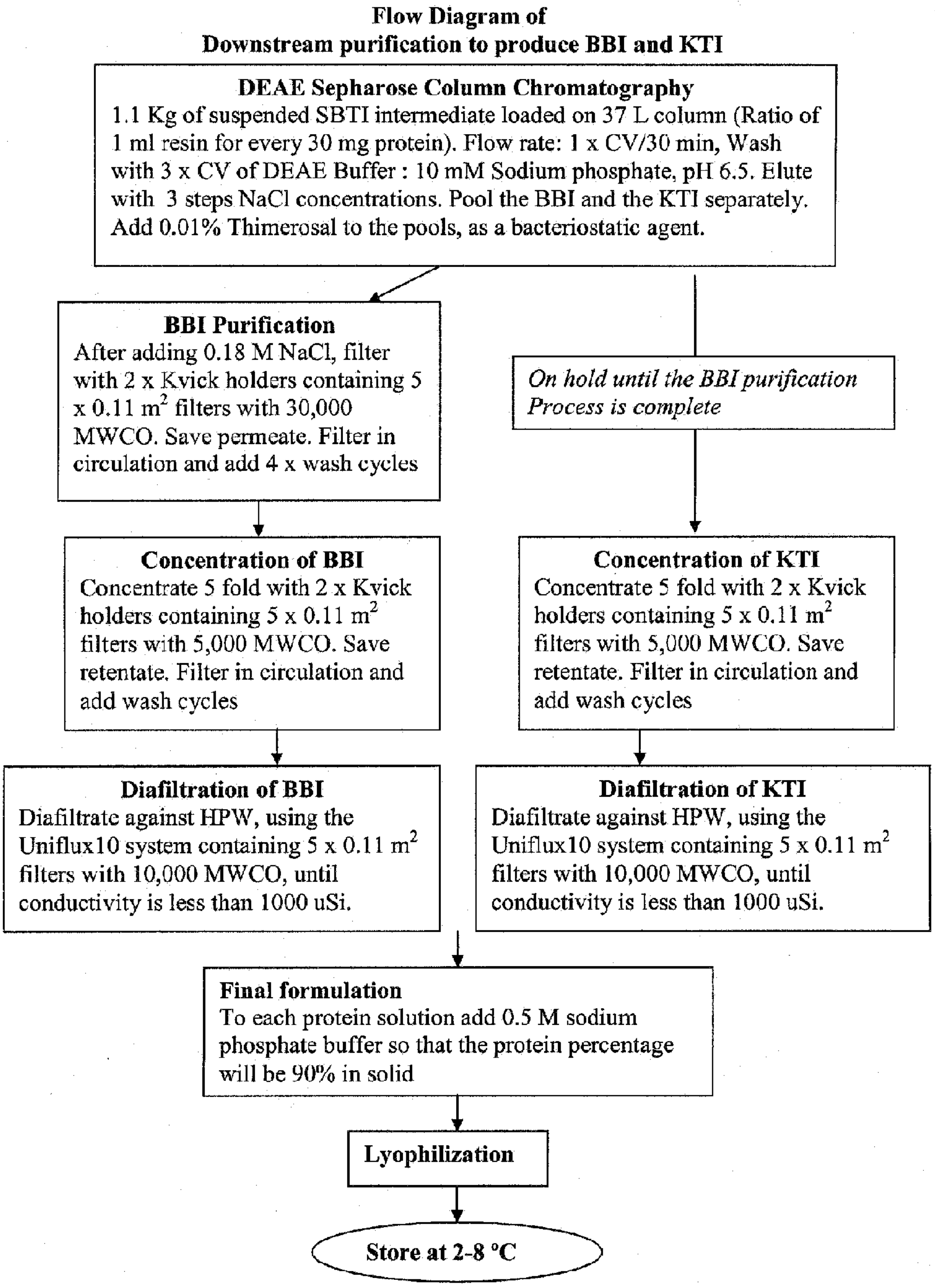

Flow Diagram of
Downstream purification to produce BBI and KTI
DEAE Sepharose Column Chromatography
1.1 Kg of suspended SBTI intermediate loaded on 37 L column (Ratio of 1 ml resin for every 30 mg protein). Flow rate: 1 x CV/30 min, Wash with 3 x CV of DEAE Buffer : 10 mM Sodium phosphate, pH 6.5. Elute with 3 steps NaCl concentrations. Pool the BBI and the KTI separately. Add 0.01% Thimerosal to the pools, as a bacteriostatic agent.
BBI Purification
After adding 0.18 M NaCl, filter with 2 x Kvick holders containing 5 x 0.11 m2 filters with 30,000 MWCO. Save permeate. Filter in circulation and add 4 x wash cycles
On hold until the BBI purification Process is complete
Concentration of BBI
Concentrate 5 fold with 2 x Kvick holders containing 5 x 0.11 m2 filters with 5,000 MWCO. Save retentate. Filter in circulation and add wash cycles
Concentration of KTI
Concentrate 5 fold with 2 x Kvick holders containing 5 x 0.11 m2 filters with 5,000 MWCO. Save retentate. Filter in circulation and add wash cycles
Diafiltration of BBI
Diafiltrate against HPW, using the Uniflux10 system containing 5 x 0.11 m2 filters with 10,000 MWCO, until conductivity is less than 1000 uSi.
Diafiltration of KTI
Diafiltrate against HPW, using the Uniflux10 system containing 5 x 0.11 m2 filters with 10,000 MWCO, until conductivity is less than 1000 uSi.
Final formulation
To each protein solution add 0.5 M sodium phosphate buffer so that the protein percentage will be 90% in solid
Lyophilization
Store at 2-8 °C

Figure 8

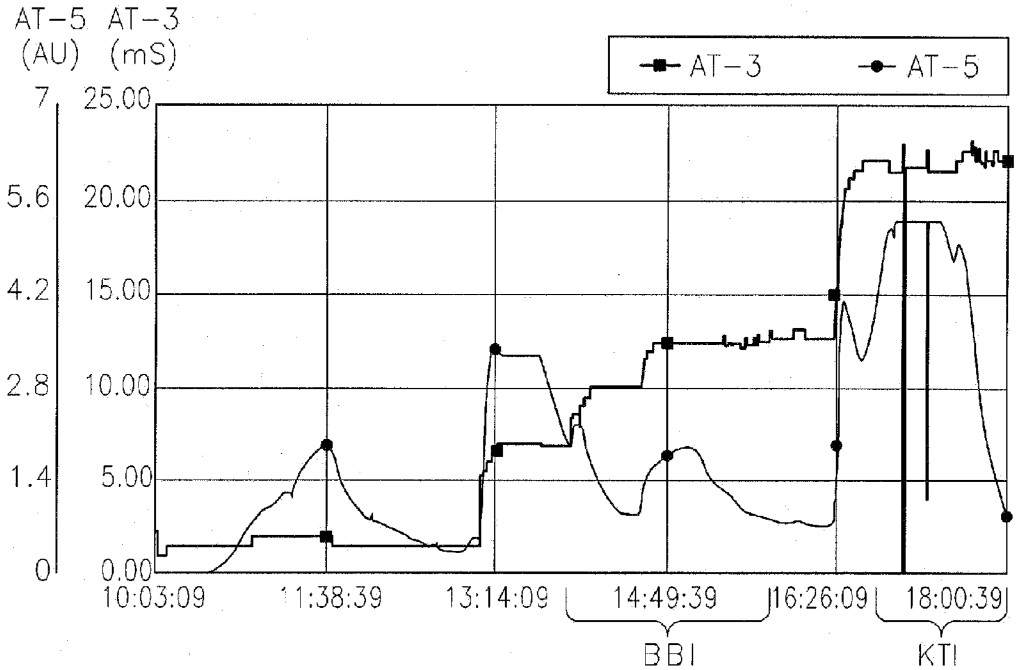



Figure 9

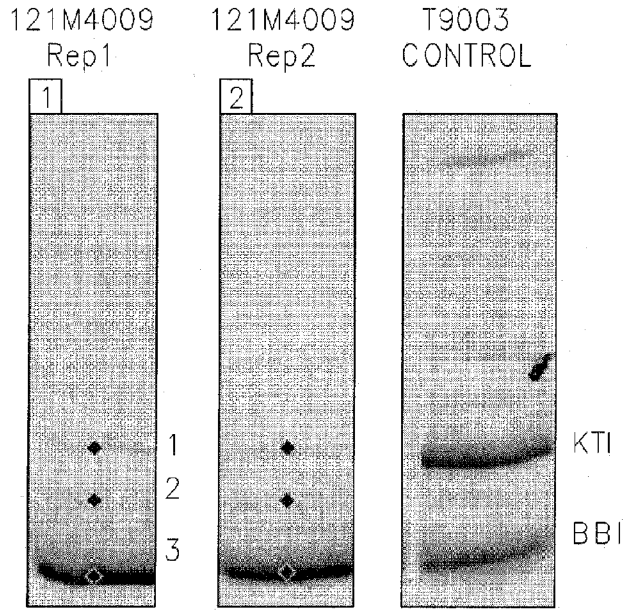



Figure 10

| trial sequence | trial no. | Lecithin | GMS | Tween 80 | Gelucire 44/14 | density | foam buildup | test of the suspension in water | sedimentation | decision feasibility | Effective-ness (1=lowest) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| I Lecithin | a | 5% | | | | | 3 | 1-2 | strong, after 2 hours | aborted 18 hours after preparation due to the sedimentation | |
| | b* | 5% | 2% | | | 1.0658 | 2 | 2 | not visible | sent for testing | t.b.d. |
| | c | 10% | | | | | 4 | n.a. | n.a. | aborted due to foam building | |
| II Tween 80 | a | | | 2% | | | 1 | n.a. | strong, after 1 hour | aborted 2 hours after preparation due to the sedimentation | |
| | b | | | 4% | | | 1 | n.a. | strong, after 1 hour | aborted 2 hours after preparation due to the sedimentation | |
| | c | | | 20% | | | 1 | n.a. | strong, after 1 hour | aborted 2 hours after preparation due to the sedimentation | |
| | d | | | 10% | | | 1 | n.a. | strong, after 1 hour | aborted 2 hours after preparation due to the sedimentation | |
| | e | | 2% | 2% | | | 1 | 2-3 | moderate, after 1 hour | aborted 18 hours after preparation due to the sedimentation | |
| | f | | 2% | 4% | | | 1 | 2-3 | moderate, after 1 hour | aborted 18 hours after preparation due to the sedimentation | |
| | g | | 2% | 20% | | | 1 | n.a. | strong, after 1 hour | aborted 2 hours after preparation due to the sedimentation | |
| | h | | 2% | 10% | | | 1 | 3 | moderate, after 1 hour | aborted 18 hours after preparation due to the sedimentation | |

Figure 11

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| III Gelucire 44/14 | a | | | | 2% | | 1 | n.a. | n.a. | aborted immediately after preparation due to low viscosity | |
| | b | | | | 4% | | 1 | 1-2 | stable after 18 hours | aborted after 18 hours due to lower viscosity than IIIj | |
| | c | | | | 8% | | 1 | 2 | stable after 18 hours | aborted after 18 hours due to lower viscosity than IIIj | |
| | d* | 3 | | | 12% | 1.0443 | 1 | 1-2 | a very thin oil layer (1mm)visible after 18 hours | sent for testing | 2 |
| | e* | 6 | | | 12% | 1.0565 | 1 | 1-2 | a very thin oil layer (1mm)visible after 18 hours | sent for testing | |
| | g* | | | 5 | 12% | 1.0604 | 1 | 2-3 | a very thin oil layer (1mm)visible after 18 hours | sent for testing | 4 |
| | h* | | | 10 | 12% | 1.0599 | 1 | 2-3 | a very thin oil layer (1mm)visible after 18 hours | sent for testing | 4 |
| | j* | | | | 12% | 1.0644 | 1 | 3.5 | stable after 18 hours | sent for testing | 2 |

Continuation of Figure 11

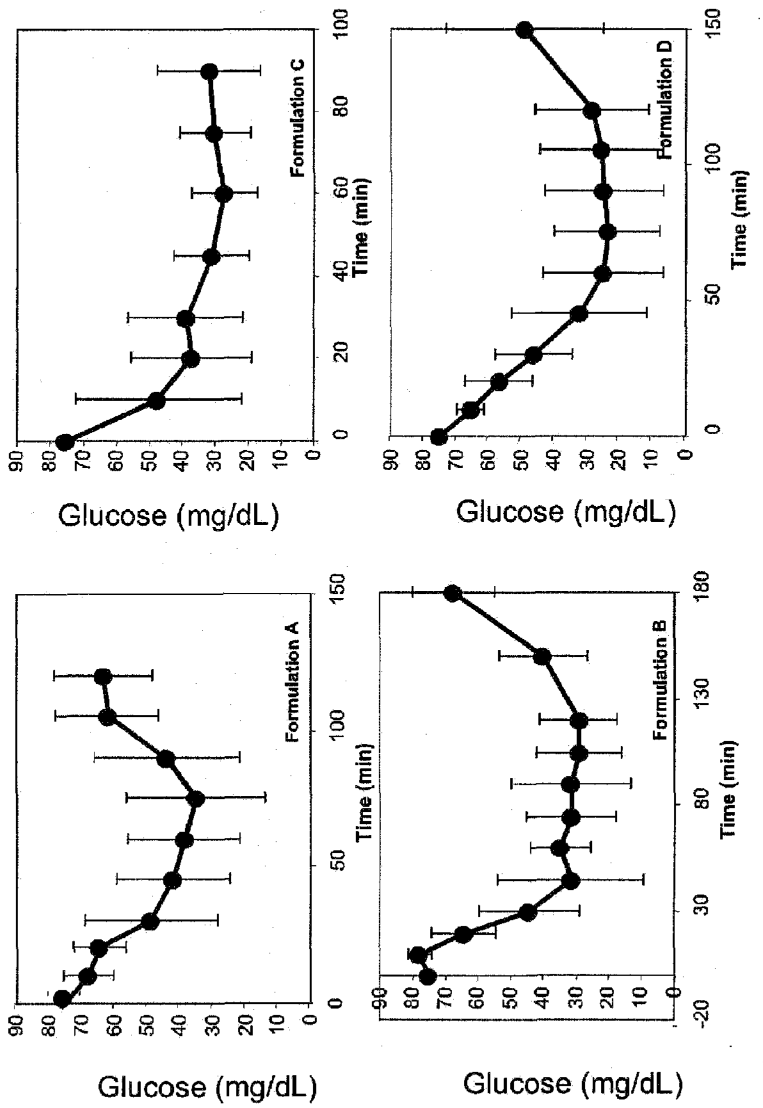

Formulation C
Formulation D
Formulation A
Formulation B
Time (min)
Glucose (mg/dL)

Figure 12A

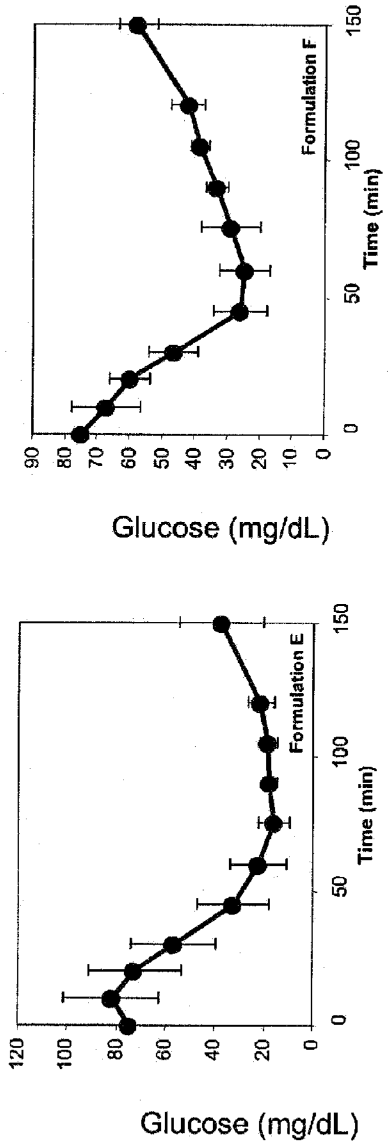

Formulation F
Glucose (mg/dL)
Time (min)
0
10
20
30
40
50
60
70
80
90
0
50
100
150
Formulation E
Glucose (mg/dL)
Time (min)
0
20
40
60
80
100
120
0
50
100
150

Figure 12B

**Figure 13A** **Blood Sugar Record - Name ___** **If glucose under 3.0 mmol, record details on reverse. Date:**

| Time | 0100 | 0200 | 0300 | 0400 | 0500 | 0600 | 0700 | 0800 | 0900 | 1000 | 1100 | 1200 | 1300 | 1400 | 1500 | 1600 | 1700 | 1800 | 1900 | 2000 | 2100 | 2200 | 2300 | 2400 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Blood Sugar | | | | | | | | | | | | | | | | | | | | | | | | |
| Insulin Taken | | | | | | | | | | | | | | | | | | | | | | | | |

**Figure 13B**

**For Every Reading Below 3.0 mmol, Please Record Details On Hypoglycemia Sheet**

| | | | |
|---|---|---|---|
| Date:__________ | Time:__________ | Blood sugar Value: __________ | |
| Sweating | Problems with vision Other__________ | **OR** | None |
| Shaking | Change in behavior __________ | | |
| Heart palpitations | Confusion __________ | | |
| **The reaction was recognized by (Please circle one):** | | | |
| Yourself | Routine test on your meter | Someone else | |
| **Treatment for the reaction needed (Please circle all that apply):** | | | |
| Juice/Food | Help from someone else | Injection of Glucagon | Hospital/Ambulance |

The daily panel shown (A) is repeated seven times so that a week's records is on one page. On the reverse of each sheet (B), a questionnaire is provided for details of each hypoglycemia episode (glucose < 3.0 mmol/l); this is repeated four times for each page.

## Scoring Sheet for Hypoglycemia

Episodes of hypoglycemia associated with reduced awareness Date:

| | | Glucose 2.5 – 2.9Mm | | | | | OR | Glucose < 2.5mM | | | | Per 4 Wks | Per YR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Max Score | Episode | | | | Max Score | Episode | | | | | |
| Occurrence | | 1 | | | | | 2 | | | | | | |
| Symptoms | Autonomic | 0 | | | | | 0 | | | | | | |
| If no autonomic symptoms, choose highest | Visual | 1 | | | | | 2 | | | | | | |
| | Behavioural | 1 | | | | | 2 | | | | | | |
| | Other Neuro | 1 | | | | | 2 | | | | | | |
| | Confusion | 2 | | | | | 4 | | | | | | |
| | None | 4 | | | | | 8 | | | | | | |
| | Seizures | 6 | | | | | 12 | | | | | | |
| Outside help to (choose highest) | Recognize | 6 | | | | | 12 | | | | | | |
| | Treat | 10 | | | | | 20 | | | | | | |
| | Subtotal | | | | | | | | | | | | |
| By history in last year (per episode) | Outside help to recognize | | | 6 | | | | | | | | | |
| | Outside help to treat | | | 10 | | | | | | | | | |
| | Glucagon | | | 40 | | | | | | | | | |
| | Ambulance | | | 80 | | | | | | | | | |
| **TOTAL HYPOGLYCEMIA SCORE** | | | | | | | | | | | | | |

Figure 14

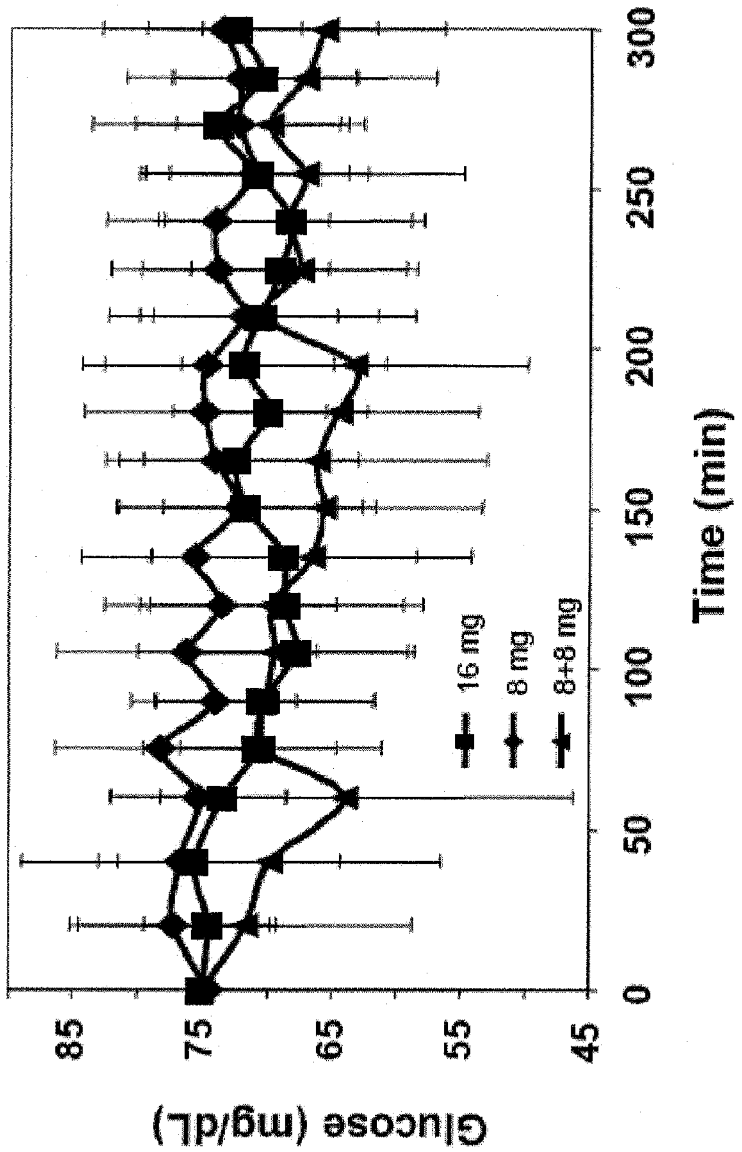

Glucose (mg/dL)
85
75
65
55
45
0
50
100
150
200
250
300
Time (min)
16 mg
8 mg
8+8 mg

Figure 15

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.