Title: PREPARING OF PEPTIDES WITH EXCELLENT SOLUBILITY

Abstract: The formation of inactive, insoluble forms of peptide can be minimized or, alternatively, inactive, insoluble forms of peptide compounds, if present, can be converted into more physiologically active, soluble forms by dissolving peptide samples in aqueous base and then acidifying the aqueous mixture to precipitate the peptide in the presence of at least one of a salt and a co-solvent. Preferably, both a salt and co-solvent are present. By carrying out the precipitation relatively rapidly (at least in a first stage of acidifying in which the pH of the alkaline medium is reduced to a pH in the range of 6 to 7.5, after which acidification is carried out to a final desired pH, e.g., 3 to 6, which can occur more slowly) at relatively low temperature, the dissolution characteristics of the resultant precipitated peptide are even further improved. The process is robust, consistent, and suitable for commercial scale manufacture of peptides.
PREPARING OF PEPTIDES WITH EXCELLENT SOLUBILITY

The present invention relates to methodologies and compositions useful for causing peptides to precipitate in biologically active, soluble forms in which formation of undue beta sheet structure during precipitation is avoided. By way of example, the methodologies can be used to convert insoluble peptide to a more soluble form of the peptide. The methodologies can also be practiced to help ensure that denatured peptide precipitates in a more soluble form in which undue insoluble secondary and tertiary structures are avoided or minimized.

Proteins and other peptides are not only useful in the treatment a wide variety of diseases but also are useful research tools for the discovery of pharmaceutically active compounds. Many proteins and peptides of interest are readily soluble in aqueous solution at physiological pH. For example, the FUZEON® peptide (also known as enfuvirtide or T-20), which is a synthetic, 36-amino-acid peptide, the hybrid peptide T-1249, and derivatives and counterparts of these peptides, have proven beneficial as fusion inhibitors in the treatment of the human immunodeficiency virus (HIV) and the acquired immune deficiency syndrome (AIDS). The FUZEON® peptide and its derivatives are the first inhibitors of HIV to demonstrate consistent, potent activity in persons infected with HIV. Kilby et al. (1998) Nat Med 4:1302 and Kilby et al. (2002) AIDS Res Hum Retroviruses 18:685.

Fusion inhibitors such as the T-20 and T-1249 peptides bind to a region of the glycoprotein 41 envelope of HIV type 1 (HIV-1) that is involved in the fusion of the virus with the membrane of the CD4+ host cell. Wild et al. (1993) AIDS Res. Hum. Retroviruses 9:1051. Fusion inhibitors remain outside the cell and block HIV-1 prior to HIV-1 entering the cell. The FUZEON® peptide and its derivatives minimize drug interactions, side effects and cytotoxicity by potently and selectively inhibiting HIV-1 in vitro.

Another example of a peptide of interest is the Glucagon-Like Peptide 1 (GLP-1). GLP-1 peptide is a 37 amino acid peptide that is secreted by the L-cells of the intestine in response to food ingestion. It has been found to stimulate insulin secretion (insulinotropic action), thereby causing glucose uptake by cells and decreased serum glucose levels (see, e.g., Mojsiov, S. (1992) Int. J. Peptide Protein Research, 40:333-343). However, GLP-1 is poorly active. A subsequent endogenous cleavage between the 6th RAU/02.09.2005
and 7th position produces a more potent biologically active GLP-1(737)OH peptide. Numerous other GLP-1 counterparts are also known. Because of their ability to stimulate insulin secretion, GLP compounds show great promise as agents for the treatment of diabetes, obesity, and related conditions. See also PCT Patent Publication WO 01/55213, hereby incorporated in its entirety by reference.

Proteins and peptides are inherently unstable molecules due to their multiple functional groups, which complicate recombinant reproduction and purification of homogeneous protein preparations having the pharmaceutically desired biological and physicochemical characteristics. Senderoff et al. (1997) J. Pharm. Sci. 87:183. The therapeutic potential of peptides and proteins is dependent on the production and purification of their active forms in commercially viable quantities.

A key characteristic impacting the manufacturability and/or use of peptide products concerns the solubility of these products in aqueous media at physiological pH. As used herein, the term "physiological pH" refers to a pH in the range of from about 6.5 to about 7.5, preferably about 6.9 to about 7.5, more preferably about 7.1 to about 7.4. Such solubility is highly desirable for a variety of reasons. Firstly, most environments of intended use within the body of human or animal patients constitute aqueous media at physiological pH. Secondly, most peptides tend to be more biologically active when soluble in such media.

Biologically active peptide compounds with identical sequences of amino acids (i.e., identical primary structure) nonetheless can exist coiled in at least two different forms (i.e., secondary structures), namely beta sheets and/or alpha helices. A peptide sample often may incorporate both structures to some degree. The solubility characteristics of peptides may significantly depend to a large degree upon the relative amounts of these structures that are present in a peptide sample. With increasing beta sheet structure, the molecular weight of a peptide appears to be multiples of the theoretical peptide weight, and such peptides tend to be soluble in aqueous media only under strongly acidic or strongly basic conditions. Peptides with increasing amounts of beta sheet structure also tend to have more tertiary structure. Additionally, the β-sheet structure tends to be much less biologically active than the α-helical structure. As another drawback, peptides with greater amounts of beta sheet structure may tend to be more difficult to handle, filter, compound, or otherwise process.

The terms "aggregation" and "deaggregation" (deaggregation is sometimes referred to as "disaggregation") are used to describe peptides and their structures. Generally, a peptide including undue amounts of beta sheet structure is referred to as being aggregated, whereas a peptide including desired amounts of alpha helical structure is
referred to as being deaggregated. A peptide can be more or less aggregated or deaggregated from another peptide or other version of the same peptide depending upon the relative amounts of beta sheet and alpha helical structure that is present in the samples being compared.

It should be noted that characterizing a peptide as having one structure and/or the other, or as being more or less aggregated or deaggregated, does not refer to the purity of the peptide. Rather, the two forms are different from each other in a way that polymorphs are different from each other.

It tends to be most desirable to produce peptides that are relatively more deaggregated due to solubility and biological activity concerns. To that end, beta-sheet to alpha-helix coil transition specifically related to peptides has been studied in the art. See, e.g., Kim et al. (1994) J. of Pharm. Sci. 83:1175 and Senderoff et al. (1998) J. Pharm. Sci. 87:183.

Unfortunately, some peptides, such as the T-1249 and GLP-1 peptides and their counterparts, tend to be susceptible to the formation of beta sheet and tertiary structures. Such peptides are especially sensitive to aggregation into beta sheets during precipitation. This frustrates the realization of the pharmaceutical potential of peptide compounds, because this potential is dependent on the production of the active form of these compounds in commercially viable quantities without contamination with significant quantities of byproducts of the inactive form.

Accordingly, it is desired to be able to be able to manufacture a peptide in such way that allows the relative degree to which the resultant peptide is deaggregated or aggregated.

Whether precipitated with or without significant beta sheet structure, precipitated peptide can be difficult to filter. In some instances, the precipitate is too gel-like so that the incipient filter cake holds onto water too tenaciously, making filtering and washing of impurities impractical. It would be highly desirable to avoid this gel-like state and/or convert the gel-like peptide into a more filterable form. With respect to deaggregated peptide, it would be desirable to accomplish this without causing undue aggregation of the precipitated peptide.

It has now been found that the formation of inactive, insoluble forms of peptide can be minimized or, alternatively, inactive, insoluble forms of peptide compounds, if present, can be converted into more physiologically active, soluble forms by dissolving peptide samples in aqueous base and then acidifying the aqueous mixture to precipitate the peptide in the presence of at least one of a salt and a co-solvent. Preferably, both a
salt and co-solvent are present. By carrying out the precipitation relatively rapidly (at least in a first stage of acidifying in which the pH of the alkaline medium is reduced to a pH in the range of 6 to 7.5, after which acidification to a final desired pH, e.g., 3 to 6, can occur more slowly) at relatively low temperature, the dissolution characteristics of the resultant precipitated peptide are even further improved. The process is robust, consistent, and suitable for commercial scale manufacture of peptides.

The method of the present invention can be used to convert insoluble secondary and tertiary peptide structures to a more soluble form. The invention may be practiced with respect to compositions wherein the insoluble peptide structure is the only peptide structure present, or, alternatively, wherein the insoluble peptide structure is present with other structure. Thus, the method can "purify" mixtures comprising both the soluble and insoluble form of a peptide compound by converting the insoluble form to the soluble form. The method is therefore ideally suited for removing small or even trace amounts of the insoluble form from, or minimizing the amount of, the insoluble form in the composition before, for example, administration as a drug or formulation into a drug dosage form.

The methodologies of the invention also allow the filtering characteristics of precipitated peptide to be dramatically improved. Whether precipitated with or without significant beta sheet structure, precipitated peptide can be rendered much easier to filter. With respect to deaggregated peptide, the methodologies accomplish this without causing undue aggregation of the precipitated peptide. As a consequence, filtering is practical and takes less time. As a consequence, process throughput (and therefore yield per unit time) is higher.

In one aspect, the present invention relates to a method of preparing a peptide that is soluble in aqueous solution at a physiological pH. A peptide sample is provided. The peptide sample may or may not include aggregated peptide having a significant amount of beta sheet structure, but the methodology of the invention will help ensure that the beta sheet structure of the resultant precipitated peptide is significantly minimized. The sample is dissolved in an alkaline, aqueous medium. Ingredients comprising a salt and a co-solvent are added to the aqueous medium. After adding at least these ingredients, the aqueous medium is acidified to cause precipitation of the peptide.

In another aspect, the present invention relates to a method of isolating a precipitated peptide. An aqueous medium comprising a precipitated peptide is provided. The precipitated peptide is aged at a first temperature in the range of about 10°C to about 20°C. Then, the precipitated peptide is aged at a second temperature that is from about 2°C to about 15°C higher than the first temperature. Then, the precipitated peptide is
aged at a third temperature that is from about 2°C to about 15°C lower than the second
temperature. The aged precipitated peptide is then filtered

In another aspect, the present invention relates to a method of precipitating a
peptide. An aqueous, alkaline medium comprising a dissolved, denatured peptide is
provided, wherein the medium is derived from ingredients comprising the peptide, a salt,
a co-solvent, and water. The medium is acidified to a first pH in the range of from about
6 to about 8. Desirably, the acidifying step occurs in less than about 30 minutes. The
medium is further acidified to a second pH at which at least about 90% of the peptide
incorporated into the medium is precipitated.

In another aspect, the present invention relates to an aqueous, alkaline, denatured
peptide solution derived from ingredients comprising a peptide sample having beta sheet
structure prior to dissolution, a salt, a co-solvent, and water.

In another aspect, the present invention relates to a method of precipitating a
peptide. An aqueous, alkaline medium comprising a dissolved peptide is provided. The
medium is derived from ingredients comprising the denatured peptide, a salt, a co-
solvent, and water. The medium is acidified under conditions effective to cause
precipitation of the peptide, wherein the acidifying occurs at a temperature in the range
of from about 10°C to about 25°C.

In another aspect, the present invention relates to a method of preparing a peptide
that is soluble in aqueous solution at a physiological pH. A peptide is dissolved in an
aqueous medium derived from ingredients comprising water and a salt, wherein the
peptide has beta sheet structure prior to dissolution. After dissolving the peptide, a co-
solvent is added to the medium. The medium is acidified under conditions effective to
cause precipitation of the peptide.

In another aspect, the present invention relates to a method of making a
precipitated peptide. Information is determined that is indicative of how the presence of
a salt and a co-solvent impact the dissolution characteristics of a peptide when an
aqueous, alkaline mixture derived from ingredients comprising water, the peptide, the
salt, and the co-solvent is acidified under conditions effective to cause precipitation of the
peptide. The information is used to cause precipitation of the peptide.

In another aspect, the present invention relates to a method of isolating a
precipitated peptide. Information is determined that is indicative of how aging a slurry
comprising the precipitated peptide at at least two temperatures impacts the filterability
characteristics of the precipitated peptide. The information is used to age and then filter
the peptide.
The embodiments of the present invention described below are not intended to be exhaustive or to limit the invention to the precise forms disclosed in the following detailed description. Rather, the embodiments are chosen and described so that others skilled in the art can appreciate and understand the principles and practices of the present invention.

The methodologies of the present invention would be very suitable with respect to a wide range of peptides. A preferred class of peptides of the present invention is those that incorporate from about 2 to about 100, preferably from about 4 to about 50, residues of one or more amino acids. Residues of one or more other monomeric, oligomeric, and/or polymeric constituents optionally may also be incorporated into a peptide. Non-peptide bonds also may be present. For instance, the peptides of the invention may be synthesized to incorporate one or more non-peptide bonds. These non-peptide bonds may be between amino acid residues, between an amino acid and a non-amino acid residue, or between two non-amino acid residues. These alternative non-peptide bonds may be formed by utilizing reactions well known to those in the art, and may include, but are not limited to imino, ester, hydrazide, semicarbazide, and azo bonds, to name but a few.

As used herein, the term "monomer" means a relatively low molecular weight material (i.e., generally having a molecular weight less than about 500 Daltons) having one or more polymerizable groups. "Oligomer" means a relatively intermediate sized molecule incorporating two or more monomers and generally having a molecular weight of from about 500 up to about 10,000 Daltons. "Polymer" means a relatively large material comprising a substructure formed two or more monomeric, oligomeric, and/or polymeric constituents and generally having a molecular weight greater than about 10,000 Daltons.

The amino acids from which the peptides are derived may be natural or non-natural. The twenty, common, naturally-occurring amino acids residues and their respective one-letter symbols are as follows: A (alanine); R (arginine); N (asparagine); D (aspartic acid); C (cysteine); Q (glutamine); E (glutamic acid); G (glycine); H (histidine); I (isoleucine); L (leucine); K (lysine); M (methionine); F (phenylalanine); P (proline); S (serine); T (threonine); W (tryptophan); Y (tyrosine); and V (valine). Naturally-occurring, rare amino acids are also contemplated and include, for example, selenocysteine, pyrrollysine.

Non-natural amino acids includes organic compounds having a similar structure and reactivity to that of a naturally-occurring amino acid include, for example, D-amino acids, beta amino acids, gamma amino acids; cyclic amino acid analogs, propargylglycine...
derivatives, 2-amino-4-cyanobutyric acid derivatives, Weinreb amides of a-amino acids, and amino alcohols. Incorporation of such amino acids into a peptide may serve to increase the stability, reactivity and/or solubility of the peptides of the invention.

Representative examples of peptides that may be processed in accordance with the present invention include peptides with fusion inhibiting activity such as enfuvirtide (also known as the T-20 peptide) and the T-1249 peptide; peptides that stimulate insulin secretion such as glucagons-like peptide 1 (GLP-1) and its more potent analog the GLP-1 (7-37)OH peptide; Oxytocin (9 C SP); vasopressin: Felypressin, Pitressin (9 C), Lypressin (9 C), Desmopressin (9 C SP), Terlipressin (12 C); Atosiban (9 C); adrenocorticotropic hormone (ACTH; 24 C); Insulin (51 recombinant or semisynthesis), Glucagon (29 recombinant SP); Secretin (27); calcitonins: human calcitonin(32 C), salmon calcitonin(32 C SP), eel calcitonin(32 C SP), dicarba-eel (elcatonin) (31 C SP); luteinizing hormone-releasing hormone (LH-RH) and analogues: leuprolide(9 C), deslorelin(9 SP), triptorelin(10 SP), goserelin(10 SP), buserelin(9 SP); nafarelin(10 C), cetorelix(10 SP), ganirelix(10 C), parathyroid hormone (PTH) (34 SP); human corticotropin-releasing factor(41 SP), ovine corticotropin-releasing factor(9 C SP); growth hormone releasing factor(9 C SP); somatostatin(9 C SP); lanreotide(9 C SP), octreotide(9 C SP); thyrotropin releasing hormone (TRH) (9 C SP); thyromosin -1(9 C SP); thymopentin (TP-5) (9 C SP); cyclosporin(9 C SP); integrilin(9 C SP); angiotensin-converting enzyme inhibitors: enalapril(9 C SP), lisinopril(9 C SP); fragments of such peptides; counterparts of such peptides, and the like.

Preferably, the principles of the invention are practiced with respect to peptides with fusion inhibiting activity such as enfuvirtide (also known as the T-20 peptide) and the T-1249 peptide; peptides that stimulate insulin secretion such as glucagons-like peptide 1 (GLP-1) and its more potent analog the GLP-1 (7-37)OH peptide; fragments of such peptides; and counterparts of these. More preferably, the principles of the invention are practiced with respect to the T-1249 peptide, GLP-1 peptide, the GLP-1 (7-37) OH peptide, fragments of such peptides, and counterparts of these inasmuch as these peptide materials are more prone to solubility issues, such as may result via aggregation of the peptide material into insoluble beta sheet structures. Most preferably, the principles of the invention are practiced with respect to the T-1249 peptide, fragments thereof, and counterparts thereof.

As used herein, a "counterpart" of a peptide refers to a compound derived from another peptide or peptide counterpart and comprising a backbone incorporating a sequence of 2 or more amino acid residues. Peptide counterparts include but are not limited to peptide analogs, peptide derivatives, follow on compounds, fusion compounds,
and the like. As used herein, a peptide analog generally refers to a peptide having a modified amino acid sequence such as by one or more amino acid substitutions, deletions, inversions, and/or additions relative to another peptide or peptide counterpart. Substitutions preferably may be conservative or highly conservative. A conservative substitution refers to the substitution of an amino acid with another that has generally the same net electronic charge and generally the same size and shape. For instance, amino acids with aliphatic or substituted aliphatic amino acid side chains have approximately the same size when the total number carbon and heteroatoms in their side chains differs by no more than about four. They have approximately the same shape when the number of branches in their side chains differs by no more than about one or two. Amino acids with phenyl or substituted phenyl groups in their side chains are considered to have about the same size and shape. Listed below are five groups of amino acids. Replacing an amino acid in a compound with another amino acid from the same groups generally results in a conservative substitution.

Group I: glycine, alanine, valine, leucine, isoleucine, serine, threonine, cysteine, methionine and non-naturally occurring amino acids with C₃-C₄ aliphatic or C₁-C₄ hydroxyl substituted aliphatic side chains (straight chained or monobranch).

Group II: glutamic acid, aspartic acid and non-naturally occurring amino acids with carboxylic acid substituted C₁-C₄ aliphatic side chains (unbranched or one branch point).

...Group III: lysine, ornithine, arginine and non-naturally occurring amino acids with amine or guanidino substituted C₁-C₄ aliphatic side chains (unbranched or one branch point).

Group IV: glutamine, asparagine and non-naturally occurring amino acids with amide substituted C₁-C₄ aliphatic side chains (unbranched or one branch point).

Group V: phenylalanine, phenylglycine, tyrosine and tryptophan.

A "highly conservative substitution" is the replacement of an amino acid with another amino acid that has the same functional group in the side chain and nearly the same size and shape. Amino acids with aliphatic or substituted aliphatic amino acid side chains have nearly the same size when the total number carbon and heteroatoms in their side chains differs by no more than two. They have nearly the same shape when they have the same number of branches in their side chains. Examples of highly conservative substitutions include valine for leucine, threonine for serine, aspartic acid for glutamic acid and phenylglycine for phenylalanine.
A peptide derivative generally refers to a peptide, a peptide analog, or other peptide counterpart having chemical modification of one or more of its side groups, alpha carbon atoms, terminal amino groups, and/or terminal carboxyl acid group. By way of example, a chemical modification includes, but is not limited to, adding chemical moieties, creating new bonds, and/or removing chemical moieties. Modifications at amino acid side groups include, without limitation, acylation of lysine e-amino groups, N-alkylation of arginine, histidine, or lysine, alkylation of glutamic or aspartic carboxylic acid groups, and deamidation of glutamine or asparagine. Modifications of the terminal amino group include, without limitation, the des-amino, N-lower alkyl, N-di-lower alkyl, and N-acyl (e.g., -CO-lower alkyl) modifications. Modifications of the terminal carboxy group include, without limitation, the amide, lower alkyl amide, dialkyl amide, and lower alkyl ester modifications. Thus, partially or wholly protected peptides constitute peptide derivatives.

Enfuvirtide, also known as T-20, is a peptide that has the 36 amino acid sequence (reading from amino, NH₂ to carboxy, COOH, terminus):

Acetyl -YTLSIHSILIESQNQQEKNEQHELLELDKWASLWNWF-NH₂ (SEQ ID NO. 1)

Representative peptide fragments of enfuvirtide include, but are not limited to, those having amino acid sequences as depicted in Table 1 below or their counterparts. For example, in the table, the amino acid in the 36th position, which is F, may have a carboxylic acid terminus as in the case of the prime metabolite, or it may be modified as the amide in the case of the T-20 peptide itself.

Table 1

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>Seq. ID No.</th>
<th>Corresponding Numbered amino acid sequence of T-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>YTLSIHLSL</td>
<td>2</td>
<td>1-8</td>
</tr>
<tr>
<td>YTLSIHSIHLIESQNQ</td>
<td>3</td>
<td>1-15</td>
</tr>
<tr>
<td>YTLSIHSIHLIESQNQQ</td>
<td>4</td>
<td>1-16</td>
</tr>
<tr>
<td>YTLSIHSIHLIESQNQQEK</td>
<td>5</td>
<td>1-18</td>
</tr>
<tr>
<td>IEESQNQ</td>
<td>6</td>
<td>9-15</td>
</tr>
<tr>
<td>IEESQNQQ</td>
<td>7</td>
<td>9-16</td>
</tr>
</tbody>
</table>
Representative counterparts of enfuvirtide are described in U.S. Pat. Nos. 5,464,933; 5,656,480; 6,281,331; 6,469,136; and 6,015,881; or PCT Publication No. WO 96/19495, all of which are incorporated herein by reference in their respective entireties. The synthesis of peptides having T-20 activity and peptide intermediates used to prepare peptides having T-20 activity also are described in U.S. Pat. Nos. 6,469,136; 6,281,331; 6,015,881; 5,464,933; 5,656,480 and PCT Publication No. WO 96/19495. By custom in the art, a peptide such as T-20 according to Seq. ID No. 1 or the like may be referred to by a designation such as T-20 (1-36), wherein the amino terminus has been assigned the lower number (here the number 1) and the carboxy terminus is assigned the higher number. As a further example, a peptide fragment according to Seq. ID No. 2 may be designated as T-20 (1-8). When not specified, the C-terminal is usually considered to be in the traditional carboxyl form. For purposes of clarity, superscript numerals may be included in a sequence to assist in locating amino acid residues at a particular position. This is shown, for instance, in Seq. ID. No. 35, below. In the nomenclature used herein to designate peptide counterparts in which an amino acid substitution, the substituting amino acid and its position is indicated prior to the parent structure. For example, Val²-
GLP-(7-37)OH designates a GLP-1 analog in which the alanine normally found at position eight of GLP-1(7-37)OH has been replaced with valine.

The T-1249 peptide has the 39 amino acid sequence (reading from acetyl terminus (corresponding to the amino terminus) to the amide terminus (corresponding to the carboxy terminus)

Acetyl -WQEWEQKITALLEQAQIQE KNEYELQKLDKWasLWEWF-NH₂ (SEQ. ID NO. 20)

Representative peptide fragments of T-1249 peptide include, but are not limited to, those having amino acid sequences as depicted in Table 2 below as well as counterparts of these. For example, in the table, the amino acid in the 39th position, which is F, may have a carboxylic acid terminus as in the case of the prime metabolite, or it may be modified as the amide in the case of the T-1249 peptide itself.

Table 2

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>Seq. ID No.</th>
<th>Corresponding Numbered Amino Acid Sequence of T-1249</th>
</tr>
</thead>
<tbody>
<tr>
<td>WQEWEQKITALLEQAQIQE</td>
<td>21</td>
<td>1-20</td>
</tr>
<tr>
<td>KNEYELQKLDKWasLWEWF</td>
<td>22</td>
<td>21-38</td>
</tr>
<tr>
<td>KNEYELQKLDKWasLWEWF</td>
<td>23</td>
<td>21-39</td>
</tr>
<tr>
<td>IQQEKNEYELQKLDKWasL</td>
<td>24</td>
<td>17-35</td>
</tr>
<tr>
<td>WQEWEQKITALLEQAQI</td>
<td>25</td>
<td>1-17</td>
</tr>
<tr>
<td>WQEWEQKITALLEQAQ</td>
<td>26</td>
<td>1-16</td>
</tr>
<tr>
<td>QQEKNEYELQKLDKWasLW</td>
<td>27</td>
<td>18-36</td>
</tr>
<tr>
<td>QQEKNEYELQKLDKWasL</td>
<td>28</td>
<td>18-35</td>
</tr>
<tr>
<td>IQQEKNEYELQKLDKWasLW</td>
<td>29</td>
<td>17-36</td>
</tr>
<tr>
<td>WQEWEQKITALL</td>
<td>30</td>
<td>1-12</td>
</tr>
<tr>
<td>EQAQIQQE KNEYEL</td>
<td>31</td>
<td>13-26</td>
</tr>
</tbody>
</table>
The T-1249 peptide and methods of making the T-1249 peptide and fragments thereof are described in U.S. Pat. No. 6,469,136, incorporated herein by reference in its entirety.

5 Generally, the GLP-1 peptide and its counterparts are peptides that have from about twenty-five to about thirty-seven amino acid residues and that stimulate insulin secretion upon food ingestion. Naturally occurring Glucagon-Like Peptide 1 (GLP-1) itself is a 37 amino acid peptide that is secreted by the L-cells of the intestine in response to food ingestion. It has been found to stimulate insulin secretion (insulinotropic action), thereby causing glucose uptake by cells and decreased serum glucose levels (see, e.g., Mojsos, S. (1992) Int. J. Peptide Protein Research, 40:333-343).

However, GLP-1(1-37) itself is poorly active. A subsequent endogenous cleavage between the 6th and 7th position produces the more potent, naturally occurring, biologically active GLP-1(7-37) OH peptide, which has the following amino acid sequence:


Many other GLP-1 counterparts also are known. For example, GLP-1(7-36)NH2 is the amide form of GLP-1(7-36). Val8-GLP-1 (7-37)OH is a synthetic GLP-1 (7-37)OH analog in which alanine at position 8 has been replaced with valine. Thr16-Lys18-GLP-1(7-37)OH is a synthetic GLP-1(7-37)OH analog in which valine at position sixteen and serine at position eighteen have been replaced with threonine and lysine, respectively. Others include, but are not limited to: GLP-1(7-34), GLP-1(7-35), GLP-1(7-36)NH2, Gln9-GLP-1(7-37), d-Gln9-GLP-1(7-37), Lys18-GLP-1(7-37), Gly18-GLP-1(7-36)NH2, Gly8-GLP-1(7-37)OH, Val8-GLP-1(7-36)NH2, Val8-GLP-1(7-37)OH, Met8-GLP-1(7-36)NH2, Met8-GLP-1(7-37)OH, Ile8-GLP-1(7-36)NH2, Ile8-GLP-1(7-37)OH, Thr8-GLP-1(7-36)NH2, Thr8-GLP-1(7-37)OH, Ser8-GLP-1(7-36)NH2, Ser8-GLP-1(7-37)OH, Asp8-GLP-1(7-36)NH2, Asp8-GLP-1(7-37)OH, Cys8-GLP-1(7-36)NH2, Cys8-GLP-1(7-37)OH,
Thr\textsuperscript{9}-GLP-1(7-37), D-Thr\textsuperscript{9}-GLP-1(7-37), Asn\textsuperscript{9}-GLP-1(7-37), D-Asn\textsuperscript{9}-GLP-1(7-37), Ser22-Arg\textsuperscript{23}-Arg\textsuperscript{24}-Gln\textsuperscript{26}-GLP-1(7-37), Arg\textsuperscript{23}-GLP-1(7-37), Arg\textsuperscript{24}-GLP-1(7-37), Gly\textsuperscript{8}-Gln\textsuperscript{21}-GLP-1(7-37)OH, and the like.

The GLP-1 peptide and its counterparts have also been described in U.S. Pat. No. 5,705,483; U.S. Pat. No. 5,512,549; U.S. Pat. No. 5,188,666; WO 91/11457; WO 98/08871; and WO 01/55213; each of which is incorporated herein by reference in its entirety. Because of their ability to stimulate insulin secretion, GLP compounds show great promise as agents for the treatment of diabetes, obesity, and related conditions.

Significantly, the methodologies of the present invention may be practiced to convert the more insoluble form of a peptide to a more soluble form. The methodology is robust, consistent, and facilitates the large scale, commercial production of peptides. Without wishing to be bound, a rationale may be suggested to explain the conversion of the insoluble peptide form to one that is soluble. It has been reported that the insoluble form of GLP-1 compounds is characterized by the presence of relatively greater amounts of intramolecular and intermolecular beta sheet structure, which results in peptide aggregation and insolubility. In contrast, the more soluble form is characterized by the presence of relatively greater amounts of alpha helices (see Senderoff et. al. (1998) J. Pharm. Sci. 87:183, the entire teachings of which are incorporated herein by reference). The dissolution of insoluble peptide structure in aqueous base is consistent with the breakdown of the intramolecular and intermolecular interactions responsible for beta sheet formation (denatured). Moreover, the isolation of the more soluble form of peptide structure from these solutions is consistent with the reformation of the secondary, alpha helical structure of soluble peptide structure. It is believed that the methodologies of the present invention provide peptide products with significantly reduced aggregation without undue peptide degradation or racemization.

A variety of testing methodologies may be used to qualitatively and/or quantitatively evaluate the deaggregation (solubility) and/or aggregation (insolubility) characteristics of a peptide sample. These methodologies can be used to determine whether a peptide sample should be treated in accordance with the invention. These methodologies may also be used after the treatment to assess the peptide quality. The methodologies may be used in the lab or for quality control for pilot plant or commercial scale production of peptides.

For example, a simple dissolution test can be used to evaluate the solubility characteristics of a peptide sample. The test is conveniently carried out at a temperature in the range of from about 20\degree C to about 27\degree C, preferably about 25\degree C. From about 0.1 to about 0.3 grams of peptide is combined with about 1 to about 3 ml of an aqueous
medium. The medium may be deionized water. In the case of T-20 or T-1249, these peptides are mildly acidic. Consequently, the medium optionally may include some initial amount base, e.g., about 17 mM Na₂CO₃ or the like, to provide a suitable initial pH. Starting at about pH 6.5 to 7.2, preferably about 6.9 to about 7.0, the pH is incrementally increased, e.g., by 0.2 pH units, using 0.1 N to 1 N NaOH until the peptide is fully dissolved or the pH reaches 9. The mixture is rapidly stirred for 5 to 10 minutes between additional increments. Solids adhering to the sides of the testing vessel should be re-suspended. Rapid mixing should be maintained throughout. Results include observations regarding the clarity of the mixture, time required for dissolution, concentration and amount of caustic used, final pH upon dissolution (if this occurs below pH 9), and the like.

For purposes of the invention, peptides such as T-20, T-1249, and GLP-1, as well as counterparts of these, may be deemed to be in a more desired, deaggregated form if dissolution in accordance with this test occurs at a pH of less than about 8, preferably less than about 7.5. If dissolution occurs at a pH of greater than about 7.5, more preferably greater than about 8, the peptide is deemed to be in a less desired aggregated form. This helps to ensure the practicality of handling the peptide during subsequent processing and handling.

Infrared spectroscopy, particularly fourier transform infrared (FTIR) spectroscopy is another useful evaluation tool to qualitatively and quantitatively assess the deaggregated or aggregated status of a peptide sample. Generally, certain absorbance peaks of a spectrum are associated with the presence of beta sheet structure, while other absorbance peaks are associated with the presence of alpha helical structure. The relative area of these respective peaks is indicative of the relative amounts of beta sheet and alpha helix structures of the peptide. Generally, the greater the area of the alpha helix peak(s) relative to the area of the beta sheet structure peaks, the more soluble the sample tends to be at physiological pH. Even the soluble embodiments of peptides may incorporate some beta sheet structure content, but the content is sufficiently low such that the soluble peptide embodiments are soluble in aqueous solution at physiological pH. Thus, although practical implementation of the present invention may not eliminate all beta sheet structure from a peptide, the amount of beta sheet structure may be sufficiently reduced so as not to have an undue impact upon the dissolution and biological activity of the peptide.

The use of FTIR techniques to evaluate the beta sheet and alpha helix structure of peptide materials is further described in WO 01/55213, which is incorporated herein by reference in its entirety. Circular dichroism is another technique that may be used.
A problem with FTIR, circular dichroism and similar testing is that these are not specific to beta sheet structure. However, colorimetric analysis does provide such specificity. Colorimetric analysis is another very useful and relatively inexpensive technique that can be used to qualitatively and quantitatively assess whether and to what degree the structure of a peptide is aggregated. Some colorants, such as Congo Red or the like, have a very high specificity for binding or otherwise interacting with the beta sheet structure form of peptides but not with the alpha helix structure form. Thus, a peptide sample can be added to a mixture containing the colorant and the visually discernible, colorimetric response to the addition of the peptide is an accurate indicator of undue aggregation. When the peptide contains undue amounts of the aggregated form of the peptide, the color change of the solution is marked and relatively rapid. A cloudy supernatant and precipitate is often observed. For instance, when aggregated T-1249 peptide is added to a Congo Red solution, the initially orange red/colored solution rapidly turns a salmon-pink color. Turbidity and precipitation may also be observed. In contrast, when a soluble form of the peptide is added, the color of the solution stays substantially constant, and the peptide readily dissolves.

Thus, colorimetric testing is an excellent qualitative indicator for assessing the solubility characteristics of a peptide. The technique is a highly accurate quantitative tool as well. The absorbance spectra of a colorimetric mixture containing a peptide can be compared to the absorbance spectra of an otherwise identical reference sample having no peptide. If the aggregated β-sheet structure form is present, the sample spectra may tend to be shifted relative to the reference spectra. Additional peaks may also be observed. The differences between the two spectra, for example, can be used to quantify the degree of aggregation. For example if the difference between two corresponding peaks is too great, the sample can be deemed to contain too much of the aggregated form. If the difference is below a certain threshold, the sample can be deemed to be sufficiently soluble to pass the test. This technique is consistent, reliable, and accurate and, consequently, provides an excellent way to monitor the quality of a peptide product during the time of manufacture and/or at the time of intended use.

Molecular weight analysis is also a useful technique to assess beta sheet structure in a peptide. Depending upon the technique and equipment used, the molecular weight may be the weight average molecular weight, number average molecular weight, actual molecular weight, or average molecular weight. Assessment of average molecular weight or weight average molecular weight is preferred. A peptide formed from a known sequence and number of amino acids generally has a theoretical molecular weight that is known with a fair degree of certainty. The theoretical molecular weight can be calculated from the theoretical peptide structure. The molecular weight of a peptide sample can be
determined through any suitable technique such as light scattering analysis. However, the amount of beta sheet structure aggregation of a sample impacts its measured molecular weight. Generally, the measured molecular weight tends to be higher with increasing amounts of beta sheet structure being present.

As an example, the T-1249 peptide has a theoretical molecular weight of about 5000. The measured, apparent molecular weight of actual samples of the peptide may range from 6000 to 100,000 or even more. Generally, we have observed that peptide samples having relatively minor amounts of aggregation, e.g., samples showing a molecular weight of about 10,000 (i.e., about twice the theoretical molecular weight of T-1249) or less, tend to be readily soluble in aqueous solution at physiological pH. In contrast, peptide samples having a molecular weight of more than about 10,000 and often more than 20,000 tend to less soluble. Samples showing a molecular weight on the order of 40,000 or more are highly insoluble and thus believed to be highly aggregated and containing significant amounts of beta sheet structure.

According to a preferred methodology of the present invention for carrying out a deaggregation treatment, a sample comprising the peptide is dissolved in an aqueous, buffered, alkaline solution. In some instances, the peptide is provided as a dried, purified powder, such as in the form of a dried powder purified using HPLC techniques. Optionally, the powder has been lyophilized. Agitation may be used to assist with the dissolution of the peptide in the solution, although agitation should not be so vigorous so as to unduly risk damaging the peptide material.

The aqueous, buffered, alkaline solution is generally derived from ingredients comprising water, at least one salt, and a sufficient amount of at least one base to provide the desired dissolution pH. The peptide and various ingredients constituting the aqueous, buffered, alkaline solution may be combined in any order. In one mode of practice, the solution is prepared from its constituent ingredients and then the peptide is added to the already prepared solution. In another mode of practice, the peptide may be added to an aqueous solution comprising the salt wherein the solution has a pH that is too low for dissolution to occur. A base is then added to this mixture in order to raise the pH to a value at which dissolution will occur. As still another alternative, the salt may be added to the solution before, during, and/or after dissolution. Generally, though, the salt is incorporated into the solution before the pH is lowered in a manner to cause the peptide to precipitate as is described further below.

The concentration of the peptide in the solution may vary over a wide range. As general guidelines, the peptide concentration in the solution may be in the range of from about 3 to about 6 g/l. In a specific mode of practice, preparing a solution containing
about 4 g/l of T-1249 peptide was found to be suitable. As used herein, the concentration of this and other ingredients included in the solution, unless otherwise expressly noted, is determined based upon the volume of the solution at the time the ingredient is added to the solution. Thus, the volume of solution for purposes of determining ingredient concentration would not include co-solvent (described below) if the co-solvent is added later, as is preferred.

A variety of one or more bases may be incorporated into the solution to provide the desired pH. Representative examples of suitable bases include hydroxide bases such as NaOH and bicarbonate and carbonate bases such as sodium or potassium bicarbonate or sodium or potassium carbonate. Sodium hydroxide is preferred, especially 0.5 N to 1 N NaOH. The base is used to adjust the pH to a desired value at which the peptide will dissolve in the solution in a reasonable amount of time. The desired pH value will vary depending upon the nature of the peptide. As general guidelines, if the pH is too low, then dissolution may be partial or may not occur to any significant degree. Even if dissolution were to occur, dissolution might take too long if the pH is too low. On the other hand, if the pH is too high, dissolution may readily occur, but the peptide could be damaged. Balancing these concerns, the pH desirably is as low as might be practical to achieve dissolution in a time frame of from about 1 second to about 3 hours, more preferably less than 2 hours. For many peptides, this corresponds to a dissolution pH in the range of from about 8 to about 11. In a specific mode of practice, dissolving T-1249 peptide in a solution at a pH of about 11 has been found to be suitable.

The salt constituent(s) of the solution improve the dissolution characteristics of the resulting precipitated peptide. Specifically, a soluble peptide that dissolves readily in aqueous solution at lower pH is prepared more consistently when a salt is present at an appropriate concentration. In the absence a salt, the resultant peptide may be difficult to dissolve at physiological pH and, indeed, may only dissolve at unduly higher pH, e.g., 8 or higher, often 9 or higher, and even 11 or higher. Without wishing to be bound, it is believed that the presence of the salt assists in the precipitation of peptide with a more desired, substantially reduced amount of aggregation. In contrast, in the absence of the salt, the peptide may have a tendency to precipitate in a more aggregated, and less desirable, form.

The fact that the salt helps prevent aggregation upon precipitation of a denatured peptide is counter-intuitive to some degree. Generally, salt might be expected to cause aggregation, because the resultant higher ionic strength of the mixture will tend to compress the ionic sphere (also known as the ionic double layer) about each peptide molecule. This allows peptide molecules to come closer together, making it more likely for shorter range, aggregate-inducing forces to come into play. This phenomena is
routinely used to precipitate peptides and is known as salting out. However, the salt of the present invention is used at relatively low concentrations at which the salting out phenomena does not occur. Instead, it is believed that the relatively low concentration of salt helps to solvate the ionic groups on a peptide and thus helps bring it into solution.

A variety of salts would be useful in the practice of the present invention. Examples include sodium carbonate, sodium acetate, ammonium carbonate, ammonium acetate, sodium bicarbonate, ammonium bicarbonate, sodium and potassium versions of these, combinations of these, and the like. Ammonium acetate is most preferred as use of this salt consistently yields precipitated peptide with excellent dissolution characteristics.

The concentration of the salt in the solution may vary over a wide range. Generally, salt is added in an amount that helps protect the secondary peptide structure in the dissolved state and during precipitation. Suitable concentrations may be enough to provide some, little, or generally no buffering action. If too little salt is used, the resultant peptide may show poor dissolution characteristics in aqueous solution at physiological pH. On the other hand, if too much salt is used, then the solubility tends to decrease, and the peptide could precipitate prematurely. Additionally, the peptide may not filter easily after precipitation. Balancing these concerns, using 1 to 200 mM equivalents of salt is one example of a salt concentration range that would be suitable in the practice of the present invention. In a specific mode of practice, using about 5 mM to about 50 mM, more preferably about 10 mM equivalents of salt, especially ammonium acetate, has been found to be suitable.

The dissolution temperature(s) generally refers to the temperature(s) of the aqueous solution in which the peptide is dissolved. Dissolution may occur at any suitable temperature. The desired dissolution temperature will depend to a large degree upon the nature of the peptide being dissolved. If the temperature of the solution, though, is too low, it may be more difficult to achieve dissolution in a reasonable amount of time. If the temperature is too high, the peptide could be damaged and/or the dissolution characteristics of the resulting precipitated peptide may be poor in aqueous solution at physiological pH. Generally, dissolving the peptide in a solution maintained at one or more temperatures in a range from about 10 °C to about 30 °C, preferably about 10 ° to about 25 °C, more preferably about 15 ° to about 20 °C would be preferable. In specific modes of practice, carrying out dissolution at 16 °C, 20 °C, and 25 °C has been suitable.

A co-solvent is preferably incorporated into the solution so that subsequent precipitation of the peptide occurs in the presence of the co-solvent. The co-solvent can be added to the solution before, during, and or after dissolution, but preferably is added
promptly after dissolution of the peptide. The co-solvent refers to one or more additional solvents in which the peptide is soluble at the dissolution pH. Preferably, the peptide is also soluble in the co-solvent at 25°C and physiological pH when the peptide is sufficiently deaggregated that ratio of the measured molecular weight of the peptide to the theoretical molecular weight of the peptide is in the range from about 2:1 to about 1:1. Examples of co-solvents include acetonitrile, methanol, combinations of these, and the like. Acetonitrile is preferred, particularly when the peptide is T-1249.

The presence of the co-solvent favorably impacts the dissolution characteristics of the resultant peptide. Specifically, this approach helps to more consistently provide a peptide product that readily dissolves in aqueous solution at physiological pH. In the absence of a co-solvent, the peptide product may dissolve more slowly and/or only dissolve completely at a pH that is higher than is desired. Without wishing to be bound, it appears that the co-solvent facilitates precipitation of peptide in a substantially more deaggregated form than might otherwise occur if no co-solvent is present.

In preferred embodiments of the invention, a sufficient amount of co-solvent is added to the solution such that the solution contains from about 2 to 50 volume percent, preferably from about 5 to about 30 volume percent, and more preferably from about 10 to about 20 volume percent of the co-solvent. The amount of co-solvent incorporated into the solution, however, can impact the solubility characteristics of the resultant peptide, although the amount of co-solvent needed to obtain more preferred results depend upon factors such as the nature of the peptide, the temperature at which precipitation is carried out, the rate at which precipitation is carried out, and the like.

For instance, when no co-solvent was used, it was found that the resultant T-1249 particles dissolved very slowly with high turbidity even after 14 to 18 hours and even at a pH of 8. A co-solvent improved this performance, although too much or too little provided less than optimum results. With 14% by volume acetonitrile, the peptide product dissolved to some degree at a pH of 6.95 but not completely. The dissolution behavior improved significantly with 18% by volume acetonitrile, as the product dissolved rapidly in 1 hour to a final pH of 6.69 with only very faint turbidity. Yet, when the acetonitrile concentration was increased to 20 to 22 volume percent in additional tests, the dissolution pH was higher, generally around 9.

After dissolution, and desirably after addition of the co-solvent, the pH of the solution optionally further is increased by adding additional base in order to facilitate further deaggregation of the peptide, if desired. For instance, in the case of T-1249, the pH may be increased to pH 11 by adding additional 1 N NaOH. The solution is then desirably promptly filtered. Pressure filtering through a 0.2 micron filter would be
suitable. The filtrate is optionally degassed under vacuum, after which the solution may be aged for a suitable time period before further processing in order to complete the deaggregation process. The desired amount of aging, if any, will depend upon factors such as the nature of the peptide, the dissolution temperature, the nature of the co-solvent, and the like. Generally, aging so that the total time that the peptide is at the elevated pH (including not just aging time, but also filtering time, degassing time, etc.) is in the range of from about 5 minutes to about 6 hours, more preferably about 30 minutes to about 2 hours. After aging, the solution optionally may be filtered again.

After aging, the pH of the solution is reduced, e.g., acidified, under conditions effective to cause the peptide to precipitate. According to the principles of the invention, this is accomplished so that the resultant peptide is readily soluble in aqueous solution at physiological pH. Without wishing to be bound, it is believed that the methodology reduces the tendency of the peptide to precipitate in a form containing undue aggregation of the peptide. The desired final pH will vary depending upon the peptide, but typically will be low enough to cause precipitation of the peptide at a desirable high yield without having the pH be so low as to risk damaging the peptide. As general guidelines, a final pH in the range of from about 3 to about 6, preferably 4 to about 6 could be suitable. As a specific example, a final pH of 5.3 to 5.5 is desirable with respect to the T-1249 peptide.

The pH of the solution preferably is lowered by adding one or more acids to the solution. Examples of acids include HCl, sulfuric acid, acetic acid, oxalic acid, combinations of these, and the like. Acetic acid is preferred. For instance, aqueous, 5% or 10% acetic acid solution have been found to be suitable.

The time period over which the pH is lowered can impact the dissolution characteristics of the resulting precipitated peptide. If this time period is too slow, e.g., the acid is added at too slow a rate, the resultant peptide may dissolve too slowly and/or be insufficiently soluble at physiological pH. It is believed, therefore, that the peptide tends to precipitate in a form with undue amounts of aggregation if pH lowering takes too long. Yet, rapidly lowering the pH all the way to the final pH is not required in all instances. In some modes of practice, peptide product with excellent dissolution properties can still be obtained if the acid is added relatively rapidly to lower the pH only to an intermediate pH. After this initial, relatively rapid addition of acid, acid is added at a second, relatively slower rate to lower the pH of the solution to the final desired pH. Using such a multistage approach to lowering the pH allows the pH reduction to occur without undue peptide aggregation while also minimizing the risk of overshooting the final desired pH. Suitable intermediate pH values would be in the range of from about 6 to about 8, more preferably from about 6.0 to about 7.5. Desirably, the initial rapid
lowering of the pH occurs in a time period of less than about one hour, preferably 30 minutes or less, more preferably 15 minutes or less.

For example, one suitable mode of practice involves lowering the pH of a T-1249 solution initially at a pH of 11. A sufficient amount of acid is added relatively rapidly over a period of 10 minutes to lower the pH to an intermediate value of about 6.0. Then, acid is added more slowly over 10 to 20 minutes to lower the pH to 5.3 to 5.5.

While not wishing to be bound, a rationale to explain the benefits of rapid acidification can be suggested. When the pH is lowered slowly, the charge associated with a molecule is slowly reduced, and the kinetics of aggregation can occur in solution. When pH is lowered quickly, the peptide precipitates so quickly that there is too little time for undue organization to occur on the molecular scale leading to β-sheet formation.

The mixture is desirably mixed well during the course of adding the acid to cause precipitation of the peptide. Yet, the dissolution quality of the resultant peptide can be compromised if agitation is too aggressive. The filtering characteristics of the peptide also can be compromised if the agitation is too aggressive or too mild. It is believed that excessive shear can lead to "striation" at a molecular scale, stretching out protein molecules and thereby sterically facilitating chain-to-chain interaction and β-sheet aggregation.

By way of example, 0.57 liter of a solution at ambient temperature containing about 2.5 grams of peptide was mechanically mixed during acid addition with a mixing blade having 2 blades and a diameter on the order of about 3 inches. Tests were conducted at 100, 200, 400, and 600 rpm. The peptide obtained at 100 rpm filtered poorly. The peptides obtained at the higher mixing rates filtered better and also dissolved readily at relatively low pH. These laboratory results suggest moderate to high agitation during precipitation is preferable. Further tests, though, showed that foaming during precipitation is undesirable. In one test, the mixture was recirculated above the liquid surface by a diaphragm pump for pH measurement in the recirculation loop. This recirculation above the liquid surface coupled with higher agitation generated a significant amount of foam. The resultant peptide showed poor compounding characteristics.

As general guidelines, therefore, it is preferred to agitate the mixture while adding the acid as vigorously as is practical while leaving a sufficient safety margin to avoid foaming the mixture.

The addition of acid to cause precipitation of the peptide may be carried out with the solution at any suitable temperature. Generally, if the temperature is too hot or too
cold, the peptide could degrade or otherwise precipitate with undue aggregation to the undesired structure. As guidelines, carrying out precipitation at a temperature in the range of 10°C to 30°C, preferably 5°C to 25°C, most preferably 16°C to 18°C would be suitable.

After precipitation, the peptide is desirably isolated and dried before being combined with other ingredients, lyophilized, packaged, stored, further processed, and/or otherwise handled. This may be accomplished in any suitable fashion. According to one suitable approach, the peptide is collected via filtering, washed with ample water washes to reduce final salt content to a suitable level, and then dried. It has been found, however, that the precipitated peptide may show gel-like properties. The gel-like precipitate can be difficult to filter. Consequently, prior to filtering, the precipitate preferably is subjected to an aging process with desirable agitation in which the peptide particles are agglomerated to "harden" the particles. This kind of particle agglomeration constituting particle hardening is different from the undesirable aggregation that leads to beta sheet structure on the one hand or the desired a-helix structure on the other. Specifically, aggregation takes place on a molecular scale, whereas agglomeration takes place in a macroscopic or particulate scale. Such aggregation may constitute and/or be similar to quaternary structure, which refers to non-covalent complexes of multiple polypeptide chains with other macromolecules. Agglomerated peptide is more practically handled and isolated.

In a preferred mode of practice, this age-hardening treatment involves aging the peptide with agitation in the course of an innovative cooling/heating/cooling treatment. This improves the filtering characteristics of the peptide without undue damage of the peptide tertiary structure. In a specific mode of practice, the treatment involved aging the particles in aqueous mixture for 5 minutes to 48 hours, preferably 30 minutes to 8 hours, more preferably 30 minutes to 2 hours at a first temperature below ambient temperature preferably being in the range of from more than 0°C to about 20°C, preferably 10°C to 20°C, more preferably about 16°C. Agitation desirably is used to ensure that the particles are well dispersed during the aging. Conveniently, the same kinds of agitation conditions may be used for this aging treatment as were used during precipitation.

Next, the temperature of the mixture is increased by about 2°C to about 30°C, preferably about 5°C to about 15°C to a moderately warmer temperature, wherein the transition to the warmer temperature occurs with agitation over a period of from about 1 minute to about 48 hours, preferably 5 minutes to 8 hours, more preferably 20 minutes to 2 hours. Preferably, the new, moderately warmer temperature is still at ambient or below. In a specific mode of practice, increasing the temperature from 16°C to 21°C in about one hour was found to be suitable. Agitation desirably continues during this transition. The
mixture is then aged at the warmer temperature for a period of from 5 minutes to 8 hours, preferably 20 minutes to 4 hours, more preferably about 3 hours, with agitation.

After this aging step, the temperature of the mixture is lowered by about 2°C to about 30°C, preferably about 5°C to about 15°C to a moderately cooler temperature, wherein the transition to the cooler temperature preferably occurs with agitation over a period of from about 1 minute to about 48 hours, preferably 5 minutes to 8 hours, more preferably 20 minutes to 4 hours. Preferably, the new, moderately cooler temperature is in the range of from above 3°C to about 18°C, more preferably about 10°C. In a specific mode of practice, lowering the temperature from 21°C to 10°C in about two hours was found to be suitable. The mixture is then further aged at the cooler temperature preferably for a period of from about 5 minutes to 48 hours, more preferably about 6 hours.

This aging treatment improves the filtering characteristics of the precipitated particles in that filtering and separating the peptide particles from the filtrate occur more readily without unduly changing the secondary structure of the peptide. Without wishing to be bound, it is believed that the particles agglomerate by the warming, which causes the peptide particles to become soft (or rubbery) and tacky. The resultant agglomerates are hardened by cooling back down to isolation temperatures. In the rubbery state, very minor amounts of aggregation might occur, albeit slowly.

Thus, after this aging, the precipitate is filtered, preferably pressure filtered such as with 1 psig N₂. The filter cake may be washed one or more times with water desirably pre-cooled such as to a temperature in the range of from about 3°C to about 20°C, preferably 5°C to about 15°C, more preferably about 10°C. This helps to lower the salt content of the cake. The filter cake may then be partly or wholly dried, such as by passing nitrogen through the cake with nitrogen at a suitable temperature for a suitable time period, such as 1 minute to 48 hours, preferably 5 minutes to 8 hours, more preferably about 6 hours. Using nitrogen that is at about ambient temperature is convenient and suitable. The cake may be periodically mixed to facilitate drying. Drying optionally may be completed in a separate drying apparatus. Such optional drying preferably occurs under vacuum, e.g., less than 30 mm Hg, at a moderate temperature so as not to degrade the peptide, e.g., at a temperature less than about 30°C, preferably less than about 28°C.

The principles of the present invention will now be further illustrated with respect to the following illustrative examples.
Example 1

The following procedure may be used to subject a peptide sample to a
deggregation treatment in accordance with the present invention.

T1249 peptide (basis: 3 kg) is dissolved in 10 mM aqueous NH4OAc (708 L) at pH
10 and 16 °C using 0.5N NaOH (~16 kg). Acetonitrile (ACN) (134.2 kg) is added to the
mixture to 18 volume% (v/v). The pH is adjusted to 11 with 0.5N NaOH (~5.7 kg). The
mixture is then pressure filtered through 0.2μ filter for about 1 hour. The filtrate is
degassed using vacuum for a period of about 30 min and then is aged for 1/2 hour at pH
11. The total time at pH 11 is about 2 hours.

During the aging step, an appropriate RPM for subsequent precipitation in a
2000 L vessel is determined. Using a 36 inch diameter blade for mixing in a 2000 liter
vessel, the initial agitation rate is set at 40 RPM. The mixture is stirred in this way for 5-
10 min and observed for foam and/or emulsion. This procedure is repeated by increasing
the RPM by 5 units until foam and/or emulsion is observed so long as the mixing rate
does not exceed 75 RPM. The mixing rate for subsequent precipitation is set at an RPM
at 5 units below the RPM at which foam and/or emulsion is observed. If foam and/or
emulsion were to be observed at any time during the precipitation, the rpm would be
reduced in increments of 5 so long as the rpm is not reduced below 40 RPM.

After aging, precipitation is carried out at 16 C without recirculating but with
agitation at the rpm determined above. As necessary, the RPM is increased to maximum
for a minute to knock down the wall cake. Then 12 kg 5% (v/v) aqueous Acetic Acid
(AA) is charged in <10 min to a pH of 6.0. 6 kg 5% (v/v) of aqueous AA is then charged
in 10-20 min at a slower addition rate to a pH of 5.5. The contents are adjusted to a final
pH to 5.3-5.5 quickly (<10 min).

The contents including the precipitated peptide are aged for 1 hour at 16°C with the
same RPM used in the precipitation steps. Then, the contents are heated 21°C in about 1
hour using a maximum bath temperature of 28°C. The contents are aged for 3 hours at
21°C using the same RPM used in the precipitation step. The contents are then cooled to
10°C in about 2 hours (It is recommended not to go below 3°C for a bath temperature.)
and aged for at least 6 h at 10°C and 40 RPM. This aging treatment would reduce the gel-
like characteristic of the initial precipitate and make the precipitate more suitable for
subsequent filtering.

After the aging treatment, the contents are filtered using about 1 psig N₂. The filter
cake is washed with 150 L high purity ("HP") water precooled to 10°C. The cake is blown
down for at least 6 hours with N₂ flow and vacuum at ambient temperature. The filter
cake is periodically mixed/smoothed during filtering. As needed, the product is further
dried in a dryer at <28°C bath and <30 mm Hg vacuum.

Example 2 (Comparative)

This comparative example shows how adding acid too slowly during precipitation
can lead to a peptide product that is still too aggregated. Sample A was a portion of a
highly aggregated batch of T-1249 peptide having an average molecular weight of 98,890
+/-6660. The molar mass was determined using MALS light scattering. Sample A
showed a dissolution pH of 8.983. Sample A was subjected to a deaggregation treatment
in which acid was added very slowly over a period of 169 minutes. The re-worked Sample
A showed a dissolution pH of 7.63 in one analysis and 7.52 in another analysis, showing
normal variation among the samples. These dissolution characteristics are moderately
too high to be suitable. At dissolution, the mixture was faintly turbid and showed a
turbidity of 13 NTU. The molecular weight of the re-worked Sample A using MALS light
scattering was about 27,000.

The procedure used to re-work Sample A was as follows. A 2000 L glass-lined vessel
equipped with a jacket and temperature control was used as the T1249 dissolution vessel.
The vessel was charged with 666 g of Ammonium Acetate and 865 kg High Purity Water
(HPW) and stirred to yield 10mM Ammonium Acetate solution. The dissolution vessel
temperature was adjusted to 16 ± 1 °C. About 80 L of Ammonium Acetate solution was
taken out from the T1249 dissolution vessel and stored in a drum for use as a rinse in a
later step (filtration of solution at a pH of 11). 3.665 kg Sample A was charged to the
precipitation vessel and stirred. 19.55 kg of 0.5N Sodium Hydroxide (NaOH) solution
was slowly charged to the dissolution vessel to dissolve the solids. The pH of the solution
at the end of dissolution was 10.0. 164 Kg of acetonitrile (ACN) was charged to the
precipitation vessel. 6.95 Kg of 0.5N Sodium Hydroxide (NaOH) solution was charged to
the dissolution vessel to raise the pH to 10.92.

The peptide solution was pressure filtered through a 5 μm filter followed by a 0.2
μm filter. The filtrate was collected in a 2000 L glass line precipitation vessel equipped
with a jacket, stirrer (36" diameter retreat curve impeller), temperature control, a pump
to re-circulate the vessel contents from the vessel bottom back to the top of the vessel,
and pH probe in the recirculation loop. The 10mM Ammonium Acetate solution
(approximately 80 L) drummed in the Ammonium Acetate preparation step was charged
to the dissolution vessel as a rinse. The Ammonium Acetate solution was pressure
filtered through a 5 μm filter followed by a 0.2 μm filter to the Precipitation vessel.
The precipitation vessel temperature was adjusted to 16 ± 1 °C. The total time the peptide solution was at a pH of 10.92 was 129 minutes. 25.4 Kg of 5% Acetic Acid solution was slowly charged to the precipitation vessel in 169 minutes at 80 RPM to a final pH of 5.48. A solution sample taken during the acid addition step at a pH of 9.3 and analyzed for the molar mass by the MALS method. The molar mass was 6500 which indicated that the peptide solution was in a de-aggregated state prior to the precipitation. The T1249 slurry was aged at 16 ± 1 °C for 85 minutes at a reduced RPM of 70. Then the slurry was heated to 21 ± 1 °C in 1 hour and aged for 100 minutes at a reduced RPM of 60. The batch was finally cooled to 10 ± 1 °C in 88 minutes and aged for 7 hours at a reduced RPM of 30.

The product slurry was filtered in a Nutsche filter fitted with a 5-10 µm polypropylene filter cloth using ~5 psig nitrogen on the cake. The filtration time for the slurry was 1 hour. The filter cake was washed with 150 Kg of HPW and blown down with nitrogen for 4 hours. The wet cake was dried at 21-28 °C under 25 mm Hg vacuum. The weight of the dry product was 3.0 Kg with a moisture content of 6.2% by Karl Fischer (KF) analysis.

The dry product was tested for dissolution with the following results. The dissolution pH was 7.63 with a turbidity of 23 NTU. The dissolution time was 85 minutes. The solution appeared to be faintly turbid. Dissolution pH for another sample of the dry product was 7.52. Clearly the product failed the dissolution test.

Example 3

Sample B was a portion of a highly aggregated batch of T-1249 peptide having an average molecular weight of 49,480 +/- 5200. The molar mass was determined using MALS light scattering. Sample B showed a dissolution pH of 9.968. Sample B was subjected to a deaggregation treatment in accordance with the procedure set forth in this example. The resultant re-worked Sample B showed an average molecular weight via MALS of 9700. The re-worked Sample B showed a dissolution pH of 6.798. Upon dissolution, the solution was faintly turbid. The turbidity was only 2.0 NTU. A 2000 liter vessel was used containing a 3 foot diameter agitation blade. The vessel was equipped with temperature and pH probes.

The following general sequence of steps may be carried out to re-work Sample B. Specific conditions actually used to re-work Sample B follow the general procedure.

The following initial steps are used to dissolve Sample B in an aqueous medium:
1. Setup a T1249 dissolution vessel equipped with bath temperature and pH probes.

2. Charge 0.701 kg Ammonium Acetate.

3. Charge 909 kg High Purity Water (HPW) to prepare 10mM Ammonium Acetate solution and stir to dissolve the solids.

4. Adjust the temperature to 16 ± 1 °C.

5. Separately store about 80 L Ammonium Acetate solution for use in step-16 below as rinse during the filtration at pH 11.

6. Charge 4 kg of peptide.

7. Charge 0.5N NaOH slowly to adjust the pH to 10 ± 0.2 and dissolve the peptide. Expected charge is 22 kg.

8. Ensure that the solids are completely dissolved.

9. Charge 158 kg Acetonitrile (ACN).

10. Sample the peptide solution and check for % ACN by GC. Target is 18 v/v% ACN (relative to initial ammonium acetate buffer charge in step-3). Adjust the composition as necessary.

The following steps were carried out to achieve peptide deaggregation:

11. Adjust the pH to 11 ± 0.2 with 0.5N NaOH (expect ~6.6 kg charge).

12. Setup the peptide precipitation vessel equipped with a bath. Set the bath temperature at 16 ± 1 °C.

13. Age the peptide solution at pH 11 for 1 h. Ensure that the contents are clear without any solids.

14. Pressurefilter the peptide solution through a 5 μm filter followed by a 0.2 μm filter and collect the filtrate in a precipitation vessel.

15. Charge the 10mM Ammonium Acetate solution drummed in step 5 to the dissolution vessel as a rinse.

16. Pressure filter the rinse solution through a 5 μm filter followed by a 0.2 μm filter to the Precipitation vessel.
17. Degas the filtrate using 50-150 mmHg pressure for 15 to 30 minutes at moderate agitation (tip speed of 2.5-3.0 m/s or 50-60 rpm with the 3 ft diameter agitator blade).

18. Adjust the precipitation vessel temperature to 16 ± 1 °C.

19. Age for additional time at pH 11 so that the total age time at pH 11 is 3 h (start of step 13 to end of step 19).

After deaggregation, the following sequence of steps were carried out to cause precipitation of the peptide in a highly soluble form:

20. Adjust the agitation to a tip speed of 2.5 to 3.0 m/s (55-60 rpm with 3 ft diameter agitator blade) to provide turbulent mixing. If necessary, reduce the agitation to avoid excessive foaming.

21. Charge 16 kg (two thirds of the total charge) of 5% aqueous acetic acid in < 5 min through a spray nozzle located above the liquid surface approximately half way between the vessel wall and the agitator shaft to a pH of ~6.0.

22. Charge 8 kg (one third of the total charge) of 5% aqueous acetic acid in 5-10 min to a pH of ~5.5.

23. Adjust the pH to 5.3-5.5 quickly (<15 min) with 5% aqueous acetic acid.

24. Age the slurry for 1 h at 16 ± 1 °C.

25. Using a maximum jacket temperature of 28 °C, heat the slurry to 21 ± 1 °C in ~1 h.

26. Age the slurry for 3 h at 21 ± 1 °C.

27. Cool to 10 ± 1 °C in <2 h (use a bath temperature of 3 °C at the start).

28. Age for at least 6 h at 10 ± 1 °C at reduced rpm (40 rpm with 3 ft diameter agitator blade or a tip speed of ~2 m/s).

The following sequence of steps may be used to isolate the aged, precipitated peptide:

29. Charge ~500 L deionized water (DIW) to the wash the precipitation vessel and cool the slurry to 10 ± 1 °C.
30. Pressure filter the slurry in a Nutsche filter fitted with an 8-10 μm polypropylene filter cloth using ~1 psig nitrogen on the cake and 500 mmHg vacuum on the filtrate. Leave 1-3 inches of liquid over the cake at the end of filtration.

31. Using slight vacuum (550-600 mmHg) and no pressure, wash the cake with high purity water (2x200 L) precooled to 10 ± 1 °C. Monitor the conductivity of the filtrate. If necessary use additional wash to bring down the conductivity of the effluent to <20 microsiemens.

32. Dry the product at ambient temperature (20-25 °C) with high nitrogen flow (5-10 psig nitrogen pressure on cake) through the cake and vacuum from the cake bottom. Stir the cake every 4-6 hours and sample for KF. The target KF is 6-8%.

33. When the product is dry, package into double poly-lined fiber packs.

The actual procedure used to re-work Sample B was as follows. A 2000 L glass-lined vessel equipped with a jacket and temperature control was used as the T1249 dissolution vessel. The vessel was charged with 805 g of Ammonium Acetate and 1048 kg High Purity Water (HPW) and stirred to yield 10mM Ammonium Acetate solution. The dissolution vessel temperature was adjusted to 16 ± 1 °C. About 80 L of Ammonium Acetate solution was taken out from the T1249 dissolution vessel and stored in a drum for use as a rinse in a later step (filtration of solution at a pH of 11). 4.6 kg Sample B was charged to the precipitation vessel and stirred. 25.2 kg of 0.5N Sodium Hydroxide (NaOH) solution was slowly charged to the dissolution vessel to dissolve the solids. The pH of the solution at the end of dissolution was 10.0. 179.9 Kg of ACN was charged to the precipitation vessel. The ACN concentration was 17.9 v/v%. 7.6 Kg of 0.5N Sodium Hydroxide (NaOH) solution was charged to the dissolution vessel to raise the pH to 10.9. The peptide solution was pressure filtered through a 5 μm filter followed by a 0.2 μm filter using. The filtrate was collected in a 2000 L glass line precipitation vessel equipped with a jacket, stirrer (36" diameter retreat curve impeller), temperature control, a pump to re-circulate the vessel contents from the vessel bottom back to the top of the vessel, and pH probe in the recirculation loop. The 10mM Ammonium Acetate solution (approximately 80 L) drummed in the Ammonium Acetate preparation step was charged to the dissolution vessel as a rinse. The Ammonium Acetate solution was pressure filtered through a 5 μm filter followed by a 0.2 μm filter to the Precipitation vessel. The peptide solution was degassed by applying 50-150 mmHg vacuum for 15-30 minutes.

The precipitation vessel temperature was adjusted to 16 ± 1 °C. The total time the peptide solution was at a pH of 10.92 was 120 minutes. 30.6 Kg of 5% Acetic Acid
solution was charged to the precipitation vessel in 45 minutes in two steps at 60 RPM. The first portion was 90-95% of the total charge and was added in 15 minutes. The second charge was charged in 30 minutes to a final pH of 5.41. The T1249 slurry was aged at 16 ± 1 °C for 60 minutes at a reduced RPM of 55. Then the slurry was heated to 21 ± 1 °C in 1 hour and aged for 180 minutes at a reduced RPM of 55. The batch was finally cooled to 10 ± 1 °C in 110 minutes and aged for 8 hours at a reduced RPM of 40.

The product slurry was filtered in a Nutsche filter fitted with an 5-10 μm polypropylene filter cloth using ~5 psig nitrogen on the cake. The filtration time for the slurry was 55 minutes. The filter cake was washed with 300 Kg of HPW to a conductivity of 12 μS in the effluent. The wet cake was blown down with nitrogen for 4 hours. The wet cake was dried in the Nutsche filter at ambient temperature (20-25 °C) under 20 inches vacuum. The weight of the dry product was 4.22 Kg with a moisture content of 3.71% by Karl Fischer (KF) analysis.

The dry product was tested for dissolution with the following results. The dissolution pH was 6.798 with a turbidity of 2 NTU. The dissolution time was 60 minutes. The solution appeared to be very faintly turbid. Clearly the product passed the dissolution test.

Example 4

The procedure of Example 3 was used to process two additional peptide samples except as noted in the following table. The first sample was the re-worked Sample A prepared in Example 2. The resultant treated sample is referred to herein as re-worked Sample A'. The molar mass of re-worked Sample A' was determined to be about 9300 using MALS light scattering. The re-worked Sample A' showed a dissolution pH of 6.79. Upon dissolution, the solution was clear. The turbidity was only 2.0 NTU.

The second sample, Sample C was a portion of a highly aggregated batch of T-1249 peptide that showed a dissolution pH of 9.235. The resultant re-worked Sample C showed a dissolution pH of 6.73. Upon dissolution, the solution was clear. The turbidity was only 3 NTU.

For convenience, process details of re-working Samples A and B are also provided. As used herein, NTU refers to nethelometric turbidity unit. MALS refers to multi-angle light scattering. GC refers to gas chromatography. QC refers to quality control. LOD refers to loss on drying. KF refers to Karl Fischer. NA means not applicable. ND means not determined. NM means not measured. RT means room temperature.
**Analytical data of re-worked material after treatment:**

<table>
<thead>
<tr>
<th>Re-worked Sample</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample A'</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolution pH</td>
<td>7.52</td>
<td>6.78</td>
<td>6.79</td>
<td>6.73</td>
</tr>
<tr>
<td>Dissolution Appearance / Turbidity (NTU)</td>
<td>VFT / 13</td>
<td>VFT / 2.0</td>
<td>clear / 2.0</td>
<td>Clear / 3</td>
</tr>
<tr>
<td>MALS Ave MW</td>
<td>27,000</td>
<td>9,700</td>
<td>9300</td>
<td></td>
</tr>
<tr>
<td>Congo Red 528 Absorbance (mAU)</td>
<td>71</td>
<td>55</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

**Compounding data for re-worked material after treatment:**

<table>
<thead>
<tr>
<th>Re-worked Sample</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample A'</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compounding results</td>
<td>FAIL</td>
<td>PASS</td>
<td>PASS</td>
<td>PASS</td>
</tr>
<tr>
<td>Solution pH / Appearance</td>
<td>6.86 / slightly turbid</td>
<td>6.4 / Clear few fibers</td>
<td>6.6 / clear very few fibers</td>
<td></td>
</tr>
<tr>
<td>Filtration Tuffryn Time (min)</td>
<td>10% Tuffryn / 3 nylon filters</td>
<td>2.5</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>Filtrate Turbidity (NTU)</td>
<td>11.3</td>
<td>1.6</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Deaggregation data**

<table>
<thead>
<tr>
<th>Re-worked Sample</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample A'</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Acetate charge, g</td>
<td>666</td>
<td>805</td>
<td>490.7</td>
<td>771.1</td>
</tr>
<tr>
<td>Amm Acetate, mM</td>
<td>9.99</td>
<td>9.97</td>
<td>10.03</td>
<td>&lt;10</td>
</tr>
<tr>
<td>HPW, kg</td>
<td>865</td>
<td>1048</td>
<td>635</td>
<td>1090</td>
</tr>
<tr>
<td>T-1249 charged, kg</td>
<td>3.665</td>
<td>4.6</td>
<td>2.77</td>
<td>4.83</td>
</tr>
<tr>
<td>T-1249 concentration, g/L</td>
<td>4.24</td>
<td>4.39</td>
<td>4.36</td>
<td>4.43</td>
</tr>
<tr>
<td>0.5 N sodium hydroxide used, kg</td>
<td>19.55</td>
<td>25.2</td>
<td>15.7</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>Sample A</td>
<td>Sample B</td>
<td>Sample A'</td>
<td>Sample C</td>
</tr>
<tr>
<td>------------------------------</td>
<td>----------</td>
<td>----------</td>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>Recirculation on/off</td>
<td>on</td>
<td>off</td>
<td>off</td>
<td>off</td>
</tr>
<tr>
<td>Recirculation dipleg submerged?</td>
<td>yes</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Foam visible?</td>
<td>Yes</td>
<td>Yes</td>
<td>slight</td>
<td>Slight</td>
</tr>
<tr>
<td>Parameter</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
<td>Value 4</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>5% acetic acid used, kg</td>
<td>25.4</td>
<td>30.6</td>
<td>17.9</td>
<td>30.5</td>
</tr>
<tr>
<td>Time to add 90 - 95% of the acid, min</td>
<td>&gt;120</td>
<td>15</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>Time to add remaining acid, min</td>
<td>30</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Batch temperature during acid addition</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Agitator speed during acid addition, RPM</td>
<td>80</td>
<td>60</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>Tip Speed, m/s</td>
<td>3.8</td>
<td></td>
<td>2.85</td>
<td></td>
</tr>
<tr>
<td>Final pH, QC</td>
<td>5.48</td>
<td>5.41</td>
<td>5.42</td>
<td>5.43</td>
</tr>
<tr>
<td>Hold time pH 9.3, min</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>total acid addition time, min</td>
<td>169</td>
<td>45</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td>Age temperature, °C</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Age time, hr:min</td>
<td>1:25</td>
<td>1:00</td>
<td>1:00</td>
<td>1:00</td>
</tr>
<tr>
<td>Age agitator speed, RPM</td>
<td>70</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>Heat up time, hr:min</td>
<td>1:00</td>
<td>1:00</td>
<td>0:49</td>
<td>1:00</td>
</tr>
<tr>
<td>Heat up target temperature, °C</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Start of Age, Tr °C</td>
<td>20</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Heat up agitator speed, RPM</td>
<td>60</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>Max jacket temperature during heat up, °C</td>
<td>27</td>
<td>28</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>Age temperature, °C</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Age time, hr:min</td>
<td>1:40</td>
<td>3:00</td>
<td>3:00</td>
<td>3:00</td>
</tr>
<tr>
<td>21 C Age agitator speed, RPM</td>
<td>60</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>Tip Speed during 21 C Age, m/s</td>
<td>2.9</td>
<td>2.6</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>total cool down time, hr:min</td>
<td>4:20</td>
<td>1:50</td>
<td>1:55</td>
<td>2:10</td>
</tr>
<tr>
<td>time to cool to 16°C, hr:min</td>
<td>1:23</td>
<td>1:00</td>
<td>0:55</td>
<td>1:00</td>
</tr>
<tr>
<td>Cool down target temperature, °C</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cool down agitator speed, RPM</td>
<td>60</td>
<td>55</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>Age temperature, °C</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age time, hr:min</td>
<td>7:00</td>
<td>8:00</td>
<td>8:00</td>
<td>4:00</td>
</tr>
</tbody>
</table>
### Filtration data:

<table>
<thead>
<tr>
<th>Re-worked Sample</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample A'</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filter type</td>
<td>Nutsche</td>
<td>Nutsche</td>
<td>Nutsche</td>
<td>Nutsche</td>
</tr>
<tr>
<td>Medium #</td>
<td>808</td>
<td>808</td>
<td>808</td>
<td>808</td>
</tr>
<tr>
<td>Medium material</td>
<td>Polypropylene</td>
<td>Polypropylene</td>
<td>Polypropylene</td>
<td>Polypropylene</td>
</tr>
<tr>
<td>Medium pore size, micron</td>
<td>5-10</td>
<td>5-10</td>
<td>5-10</td>
<td>5-10</td>
</tr>
<tr>
<td>Slurry temperature, °C</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Agitator speed, RPM</td>
<td>30</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Main body filtration time, hr:min</td>
<td>1:00</td>
<td>0:55</td>
<td>0:50</td>
<td>0:47</td>
</tr>
<tr>
<td>Filtration pressure, psig</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Water wash used, kg</td>
<td>150</td>
<td>300</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>Water wash temperature, C</td>
<td>21</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Wash time w/ transfer, hr:min</td>
<td>2:00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final wash effluent conductivity, μs</td>
<td>12</td>
<td>9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Wet cake, kg</td>
<td>7.7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Wet cake moisture content, calculated</td>
<td>52.4%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Filtration flux, L/min/m²</td>
<td>73</td>
<td>92</td>
<td>61</td>
<td>65</td>
</tr>
<tr>
<td>Blow down time, hr:min</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>4.5</td>
</tr>
<tr>
<td>Molar Mass by LS of wet cake</td>
<td>27000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Drying data:

<table>
<thead>
<tr>
<th>Re-worked Sample</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample A'</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dryer type</td>
<td>Krauss Mafei</td>
<td>Nutsche</td>
<td>Nutsche</td>
<td>Nutsche</td>
</tr>
<tr>
<td>Jacket temperature, °C</td>
<td>21 - 28.5</td>
<td>RT</td>
<td>RT</td>
<td>RT</td>
</tr>
<tr>
<td>Vacuum</td>
<td>25 mm Hg</td>
<td>20 inches</td>
<td>Full</td>
<td>Full</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td>-----------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>12hr drying sample, LOD</td>
<td>33.04%</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Drying time, hrs</td>
<td>21</td>
<td>10</td>
<td>7</td>
<td>60</td>
</tr>
<tr>
<td>Final KF</td>
<td>6.20%</td>
<td>3.71%</td>
<td>2.36%</td>
<td>2.36%</td>
</tr>
<tr>
<td>Final product temperature, ºC</td>
<td>27</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Dry product, kg</td>
<td>3.0</td>
<td>4.22</td>
<td>2.8</td>
<td>4.356</td>
</tr>
<tr>
<td>Yield % (actual/actual)</td>
<td>81.9</td>
<td>91.7</td>
<td>100.0</td>
<td>90.2</td>
</tr>
<tr>
<td>Purity (HPLC Area%)</td>
<td>93.9</td>
<td>93.8</td>
<td>94.1</td>
<td>93.6</td>
</tr>
<tr>
<td>Molar Mass by LS of dry product</td>
<td>27000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dissolution characteristics of re-worked material after treatment

<table>
<thead>
<tr>
<th>DISSOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolution pH</td>
</tr>
<tr>
<td>Solution Appearance</td>
</tr>
<tr>
<td>Solution (NTU)</td>
</tr>
<tr>
<td>Dissolution time, hr</td>
</tr>
<tr>
<td>Congo Red (mAU)</td>
</tr>
</tbody>
</table>

The following table summarizes additional results from the four tests summarized in the above table:

<table>
<thead>
<tr>
<th>Re-worked Sample</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Sample A'</th>
<th>Sample C</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-1249 charged, kg</td>
<td>3.7</td>
<td>4.6</td>
<td>2.8</td>
<td>4.83</td>
</tr>
<tr>
<td>T-1249 Isolated, kg</td>
<td>3.0</td>
<td>4.22</td>
<td>2.8</td>
<td>4.36</td>
</tr>
<tr>
<td>Yield % (actual/actual)</td>
<td>81.9</td>
<td>91.7</td>
<td>100.0</td>
<td>90.3</td>
</tr>
<tr>
<td>90.3Purity (HPLC Area %)</td>
<td>93.9</td>
<td>93.8</td>
<td>94.1</td>
<td>93.6</td>
</tr>
<tr>
<td>T-1249 NET content (%)</td>
<td>89.7</td>
<td>89.4</td>
<td>89.4</td>
<td>90.8</td>
</tr>
<tr>
<td>Water Content (%)</td>
<td>2.3</td>
<td>2.6</td>
<td>5.44</td>
<td>2.7</td>
</tr>
</tbody>
</table>
Example 5

The following procedure describes a deaggregation process with an aging treatment that improves the filterability of a peptide sample. The process has been shown to agglomerate and "harden" the peptide particles. The precipitation was performed using 50 g of Sample A (Example 2) in a 25 L glass vessel equipped with a stirrer, jacket, and pH probe. The size of the particles was monitored using a Focused Beam Reflectance (FBRM®) instrument from Lasentec, Inc, a unit of Mettler Toledo. The FBRM® uses laser light reflectance to measure the chord length distribution (CLD) of the particles.

Here is a description of the process conditions and results.

The 25 L glass vessel was charged with 11.364 L of deionized water (DIW). The temperature was adjusted to 16 ± 1 °C. 50 g of T1249 solid was charged to the vessel. The solids were dissolved by increasing the pH to 10 with 290 ml of 0.5N NaOH solution. 2500 ml Acetonitrile (ACN) was charged to a concentration of 18 v/v% ACN. The pH was further increased to 11 with 90 ml of 0.5N NaOH solution. The peptide solution was aged for 1 hour and filtered through a 0.2μ filter membrane in 55 minutes. The total time the peptide solution was at a pH of 11 was 140 minutes. 330 ml of 5% Acetic Acid solution was charged to the precipitator at 150 RPM in 13 minutes. The final pH was 5.4.

At this point a sample of the slurry was examined on a microscope. The sample showed entities with no rigid particle structure. The precipitate at the end of precipitation appeared to be soft and gelatin-like material with no rigid particle structure under the microscope. The slurry was aged at 16 ± 1 °C and 120 RPM 125 minutes. During this aging at 16 ± 1 °C there was no significant change in the CLD by the FBRM®. The mean chord length remained relatively constant at 6-10 μm through out this period. The microscope picture also showed only a slight improvement in the appearance of the entities (slightly less gelatine-like appearance). After the aging at 16 ± 1 °C the slurry was heated to 21 ± 1 °C in 30 minutes and aged for 1 hour. The mean chord length increased...
by three fold to approximately 30 μm during the aging at 21 ± 1 °C. The fine particles (1 to 22 μm chord lengths) decreased significantly with a corresponding increase in large particles (22 to 100 μm chord lengths). The particles appeared to be rigid with sharp boundaries under the microscope. The particles also agglomerated during this aging period. The slurry was cooled to 10 ± 1 °C in 1 hour and aged overnight at a reduced RPM of 90. The CLD stabilized during this aging period at 10 ± 1 °C with no break down of the large agglomerates. The slurry was filtered on a 5μ filter membrane using 500 mmHg vacuum in 80 minutes. In similar runs without the aging at 21 ± 1 °C typical filtration times were several hours. Clearly the aging at 21 ± 1 °C significantly improved the filtration characteristics of the precipitate. The wet cake was washed five times using 100 ml DIW in each wash. The conductivity of each wash was measured as 1248 μS (end of 1st wash), 538 μS (end of 2nd wash), 104 μS (end of 3rd wash), 29 μS (end of 4th wash), and 16 μS (end of 5th wash). The product was dried in the filter at ambient temperature for 24 hours using 120 SCCM nitrogen sweep through the cake.

The re-worked Sample A in the 25 L precipitator showed a dissolution pH of 6.832. Upon dissolution in 1 hour, the solution was very faintly turbid. The turbidity was 4.0 NTU.

During aging, particle growth occurs via acetonitrile induced particle interactions. As the solution heated up to 21 C, accelerated growth of the particles was visible. Particle growth continued through the one-hour age at 21°C. Cooling appears to stabilize the particle distribution developed at high temperature.

Other embodiments of this invention will be apparent to those skilled in the art upon consideration of this specification or from practice of the invention disclosed herein. Various omissions, modifications, and changes to the principles and embodiments described herein may be made by one skilled in the art without departing from the true scope and spirit of the invention which is indicated by the following claims.
Claims:

1. A method of preparing a peptide that is soluble in aqueous solution at a physiological pH, comprising the steps of:
   a) providing a peptide sample;
   b) dissolving the sample in an alkaline, aqueous medium;
   c) adding ingredients comprising a salt and a co-solvent to the aqueous medium, wherein at least portions of the salt and co-solvent may be added to the aqueous medium before, after, and/or during steps (a) and/or (b); and
   d) after steps (a), (b), and (c), acidifying the aqueous medium to cause precipitation of the peptide.

2. The method of claim 1, wherein the salt ingredient is added to the alkaline, aqueous medium before the peptide is dissolved in the alkaline, aqueous medium.

3. The method of claim 1, wherein at least a portion of the co-solvent is added to the alkaline, aqueous medium after the peptide is dissolved.

4. The method of claim 1, wherein at least a portion of the salt and co-solvent ingredients are added to the alkaline, aqueous medium before the peptide is dissolved in the medium.

5. The method of claims 1 to 4 wherein the peptide sample comprises a T-20 peptide, a T-1249 peptide or a GLP-1 peptide and counterparts or fragments thereof.

6. The method of claim 5, wherein the peptide sample comprises a T-1249 peptide.

7. The method of claim 5, wherein the peptide sample comprises a T-20 peptide.

8. The method of claim 5, wherein the peptide sample comprises a GLP-1 peptide.

9. The method of claims 1 to 8, wherein the physiological pH is a pH in the range of 6.5 to 7.5.

10. The method of claims 1 to 9, wherein at least a portion of the peptide sample includes a beta sheet structure.

11. The method of claim 10, wherein the peptide comprises an amount of beta sheet structure that is sufficiently high such that the peptide sample is insoluble in aqueous solution at physiological pH.
12. The method of claims 1 to 11, wherein the peptide sample is included in the alkaline, aqueous medium at a concentration in the range of 3 g/l to about 6 g/l.

13. The method of claims 1 to 11, wherein the salt is added to the alkaline, aqueous medium at a concentration in the range of about 1 mM to about 200 mM.

14. The method of claims 1 to 12, wherein the salt is a sodium-, potassium- or ammonium-acetate or carbonate.

15. The method of claim 14, wherein the salt is ammonium acetate.

16. The method of claims 1 to 15, wherein the alkaline aqueous medium has a pH in the range of 9 to 11 during the step of dissolving the peptide sample.

17. The method of claims 1 to 16, wherein the bulk temperature of the alkaline, aqueous solution during the step of dissolving the peptide occurs at a temperature in the range of about 10°C to about 20°C.

18. The method of claims 1 to 17 wherein the co-solvent comprises acetonitrile.

19. The method of claims 1 to 18, wherein from about 10 to about 20 parts by volume of the co-solvent is added to about 100 parts by weight of the alkaline, aqueous medium.

20. The method of claim 1, further comprising the step of, after dissolving the peptide sample in the medium, increasing the pH of the medium.

21. The method of claim 20, wherein the step of increasing the pH of the medium occurs after adding at least a portion of the co-solvent.

22. The method of claim 20 or 21, wherein the pH of the solution is increased to about 11.

23. The method of claims 20 to 22, further comprising, after increasing the pH of the medium, aging the medium.

24. The method of claim 1, wherein the step of acidifying the medium occurs in two or more stages.

25. The method of claim 24, wherein a first stage comprises acidifying the medium to an intermediate pH value and at least one additional stage comprises acidifying the medium to a final pH value, wherein the first stage comprises acidifying the medium at a relatively fast rate as compared to the rate of acidifying in a subsequent stage of acidifying.
26. The method of claim 24 or 25, wherein the acidifying step comprises a first acidifying step comprising lowering the pH of the medium to a value in the range of from about 6 to about 7.5 in a time period of about 30 minutes or less, and a second acidifying step comprising lowering the pH of the medium to a final pH value in the range of from about 3 to about 6.

27. The method of claims 24 to 26, wherein the acidifying step occurs at a temperature in the range of about 10°C to about 25°C.

28. The method of claim 1, further comprising aging the precipitated peptide and then filtering the aged precipitated peptide.

29. The method of claim 28, wherein the step of aging the precipitated peptide comprises aging the peptide at a first temperature in the range of about 10°C to about 20°C, then aging the precipitated peptide at a second temperature that is from about 2°C to about 15°C higher than the first temperature; and then aging the precipitated peptide at a third temperature that is from about 2°C to about 15°C lower than the second temperature.
SEQUENCE LISTING

<110> F. Hoffmann-La Roche AG
<120> Preparing of Peptides with excellent solubility
<130> Case 22975
<150> US 60/640312
<151> 2004-12-30
<160> 34
<170> PatentIn version 3.3
<210> 1
<211> 36
<212> PRT
<213> Artificial
<220>
<221>
<223> synthetic
<400> 1

Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln  
1  5  10  15
Glu Lys Asn Gln Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu  
20  25  30
Trp Asn Trp Phe
35

<210> 2
<211> 8
<212> PRT
<213> Artificial
<220>
<223> synthetic
<400> 2

Tyr Thr Ser Leu Ile His Ser Leu  
1  5

<210> 3
<211> 15
<212> PRT
<213> Artificial
<220>
<223> synthetic
<400> 3

Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln  
1  5  10  15

<210> 4
<211> 16
<212> PRT
Artificial

synthetic

Tyr Thr Ser Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln
1  5  10  15

Glu Lys

Ile Glu Glu Ser Gln Asn Gln
1  5

Ile Glu Glu Ser Gln Asn Gln Gln
1  5

Gln Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser
Leu Trp Asn Trp
20

<210> 9
<211> 21
<212> PRT
<213> artificial

<220> synthetic

<400> 9
Gln Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser
1 5 10 15
Leu Trp Asn Trp Phe
20

<210> 10
<211> 7
<212> PRT
<213> artificial

<220> synthetic

<400> 10
Glu Lys Asn Glu Gln Glu Leu
1 5

<210> 11
<211> 10
<212> PRT
<213> artificial

<220> synthetic

<400> 11
Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Glu Leu
1 5 10

<210> 12
<211> 20
<212> PRT
<213> artificial

<220> synthetic

<400> 12
Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Leu Asp Lys Trp Ala Ser Leu
1 5 10 15
Trp Asn Trp Phe

3/9
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asn Glu Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn</td>
<td>1 5 10 15</td>
</tr>
<tr>
<td>Trp</td>
<td></td>
</tr>
<tr>
<td>Asn Glu Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn</td>
<td>1 5 10 15</td>
</tr>
<tr>
<td>Trp Phe</td>
<td></td>
</tr>
<tr>
<td>Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn</td>
<td>1 5 10</td>
</tr>
<tr>
<td>Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn</td>
<td>1 5 10</td>
</tr>
<tr>
<td>Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn</td>
<td>1 5 10</td>
</tr>
<tr>
<td>Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn</td>
<td>1 5 10</td>
</tr>
<tr>
<td>Leu Glu Leu Asp Lys Trp Ala Ser Leu Trp Asn Phe</td>
<td>1 5 10</td>
</tr>
<tr>
<td></td>
<td>17</td>
</tr>
</tbody>
</table>
<211> 9
<212> PRT
<213> artificial

<220> synthetic

<400> 17
Asp Lys Trp Ala Ser Leu Trp Asn Trp
1
5

<210> 18
<211> 10
<212> PRT
<213> artificial

<220> synthetic

<400> 18
Asp Lys Trp Ala Ser Leu Trp Asn Trp Phe
1
5
10

<210> 19
<211> 19
<212> PRT
<213> artificial

<220> synthetic

<400> 19
Glu Lys Asn Glu Gln Glu Leu Leu Glu Leu Asp Lys Trp Ala Ser Leu
1
5
10
15

Trp Asn Trp

<210> 20
<211> 39
<212> PRT
<213> artificial

<220> synthetic

<400> 20
Trp Gln Glu Trp Glu Gln Lys Ile Thr Ala Leu Leu Glu Gln Ala Gln
1
5
10
15

Ile Gln Gln Glu Lys Asn Glu Tyr Glu Leu Gln Lys Leu Asp Lys Trp
20
25
30

Ala Ser Leu Trp Glu Trp Phe
35

<210> 21
Trp Glu Gln Trp Glu Gln Lys Ile Thr Ala Leu Leu Gln Ala Gln
1 5 10 15
Ile Gln Gln Glu
20

Lys Asn Glu Tyr Glu Leu Gln Lys Leu Asp Lys Trp Ala Ser Leu Trp
1 5 10 15
Glu Trp

Lys Asn Glu Tyr Glu Leu Gln Lys Leu Asp Lys Trp Ala Ser Leu Trp
1 5 10 15
Glu Trp Phe

Ile Gln Gln Glu Lys Asn Glu Tyr Glu Leu Gln Lys Leu Asp Lys Trp
1 5 10 15
Ala Ser Leu
Trp Glu Trp Glu Glu Lys Ile Thr Ala Leu Leu Glu Glu Ala Gin 1 5 10 15

Ile

Trp Glu Trp Glu Glu Lys Ile Thr Ala Leu Leu Glu Glu Ala Gin 1 5 10 15

Gln Gln Lys Asn Glu Tyr Glu Leu Lys Leu Asp Lys Trp Ala 1 5 10 15

Ser Leu Trp

Gln Gln Lys Asn Glu Tyr Glu Leu Gln Lys Leu Asp Lys Trp Ala 1 5 10 15

Ser Leu
<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>Ser</td>
<td>Leu</td>
<td>Trp</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>Gln</td>
<td>Glu</td>
<td>Trp</td>
<td>Glu</td>
<td>Gln</td>
<td>Lys</td>
</tr>
</tbody>
</table>

<p>| | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>Gln</td>
<td>Ala</td>
<td>Gln</td>
<td>Ile</td>
<td>Gln</td>
<td>Gln</td>
<td>Glu</td>
<td>Lys</td>
<td>Asn</td>
</tr>
</tbody>
</table>

<p>| | | | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gln</td>
<td>Lys</td>
<td>Leu</td>
<td>Asp</td>
<td>Lys</td>
<td>Trp</td>
<td>Ala</td>
<td>Ser</td>
<td>Leu</td>
<td>Trp</td>
<td>Glu</td>
<td>Trp</td>
<td>Glu</td>
<td>Trp</td>
</tr>
</tbody>
</table>

<p>| | | | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>Lys</td>
<td>Leu</td>
<td>Asp</td>
<td>Lys</td>
<td>Trp</td>
<td>Ala</td>
<td>Ser</td>
<td>Leu</td>
<td>Trp</td>
<td>Glu</td>
<td>Trp</td>
<td>Glu</td>
<td>Trp</td>
</tr>
</tbody>
</table>
33
Gln Lys Leu Asp Lys Trp Ala Ser Leu Trp Glu Trp Phe
1 5 10

34
Glu Gln Ala Gln Ile Gln Gln Glu Lys Asn Glu Tyr Glu Leu Gln Lys
1 5 10 15
Leu Asp Lys Trp Ala Ser Leu Trp Glu Trp Phe
20 25
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
C07K14/605  C07K14/16  C07K1/30

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 2003/060412 A1 (PROUTY WALTER FRANCIS ET AL) 27 March 2003 (2003-03-27) page 1; claims 1-6,27-30; examples 1,4</td>
<td>1-29</td>
</tr>
<tr>
<td>A</td>
<td>US 5 834 428 A (DRUCKER ET AL) 10 November 1998 (1998-11-10) example 3</td>
<td></td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C.

** Special categories of cited documents:

**A** document defining the general state of the art which is not considered to be of particular relevance

**E** earlier document but published on or after the international filing date

**L** document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

**Y** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

**X** document member of the same patent family

Date of the actual completion of the international search
4 April 2006

Date of mailing of the international search report
12/04/2006

Name and mailing address of the ISA/
European Patent Office, P.B. 5818 Patentians 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2000, Tx. 31 651 epo nl, Fax (+31-70) 340-3018

Authorized officer
Schmidt, Harald
<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td>US 2003060412 A1</td>
<td>27-03-2003</td>
<td>NONE</td>
<td></td>
</tr>
</tbody>
</table>