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(54) Title: PEPTIDES ANTIBODIES DIRECTED THEREAGAINST AND METHODS USING SAME FOR DIAGNOSING AND TREATING AMYLOID-ASSOCIATED DISEASES

(57) Abstract: A peptide comprising at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7 as well as pharmaceutical compositions and kits including same, and methods using same for diagnosing and treating amyloid associated diseases.



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PEPTIDES ANTIBODIES DIRECTED THEREAGAINST AND METHODS
USING SAME FOR DIAGNOSING AND TREATING AMYLOID-ASSOCIATED
DISEASES

5 FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to peptides and antibodies directed thereagainst which can be used to diagnose, prevent, and treat amyloid-associated diseases, such as Type II diabetes mellitus.

10 Amyloid material deposition (also referred to as amyloid plaque formation) is a central feature of a variety of unrelated pathological conditions including Alzheimer's disease, prion-related encephalopathies, type II diabetes mellitus, familial amyloidosis and light-chain amyloidosis.

Amyloid material is composed of a dense network of rigid, nonbranching proteinaceous fibrils of indefinite length that are about 80 to 100 Å in diameter.
15 Amyloid fibrils contain a core structure of polypeptide chains arranged in antiparallel β-pleated sheets lying with their long axes perpendicular to the long axis of the fibril [Both et al. (1997) Nature 385:787-93; Glenner (1980) N. Eng. J. Med. 302:1283-92].

Approximately twenty amyloid fibril proteins have been identified in-vivo
20 and correlated with specific diseases. These proteins share little or no amino acid sequence homology, however the core structure of the amyloid fibrils is essentially the same. This common core structure of amyloid fibrils and the presence of common substances in amyloid deposits suggest that data characterizing a particular form of amyloid material may also be relevant to other forms of amyloid material and
25 thus can be implemented in template design for the development of drugs against amyloid-associated diseases such as type II diabetes mellitus, Alzheimer's dementia or diseases and prion-related encephalopathies.

Furthermore, amyloid deposits do not appear to be inert in vivo, but rather are in a dynamic state of turnover and can even regress if the formation of fibrils is
30 halted [Gillmore et al. (1997) Br. J. Haematol. 99:245-56].

Thus, therapies designed to inhibiting the production of amyloid polypeptides or inhibiting amyloidosis may be useful for treating amyloid associated diseases.

Inhibition of the production of amyloid polypeptides - Direct inhibition of the production of amyloid polypeptides may be accomplished, for example, through the use of antisense oligonucleotides such as against human islet amyloid polypeptide messenger RNA (mRNA). In vitro, the addition of antisense oligonucleotides or the expression of antisense complementary DNA against islet amyloid polypeptide mRNA increased the insulin mRNA and protein content of cells, demonstrating the potential effectiveness of this approach [Kulkarni et al. (1996) J. Endocrinol. 151:341-8; Novials et al. (1998) Pancreas 17:182-6]. However, no experimental results demonstrating the *in vivo* effectiveness of such antisense molecules have been demonstrated.

Inhibition of the formation of amyloid fibrils - Amyloid, including islet amyloid, contains potential stabilizing or protective substances, such as serum amyloid P component, apolipoprotein E, and perlecan. Blocking their binding to developing amyloid fibrils could inhibit amyloidogenesis [Kahn et al. (1999) Diabetes 48:241-53], as could treatment with antibodies specific for certain parts of an amyloidogenic protein [Solomon et al. (1997) Proc. Natl. Acad. Sci. USA 94:4109-12].

The following summarizes current attempts to engineer drugs having the capability of destabilizing amyloid structures.

Destabilizing compounds - Heparin sulfate has been identified as a component of all amyloids and has also been implicated in the earliest stages of inflammation-associated amyloid induction. Kisilevsky and co-workers (Mature Med. 1:143-148, 1995) described the use of low molecular weight anionic sulfonate or sulfate compounds that interfere with the interaction of heparin sulfate with the inflammation-associated amyloid precursor and the β peptide of Alzheimer's disease (AD). Heparin sulfate specifically influences the soluble amyloid precursor (SAA2) to adopt an increased β -sheet structure characteristic of the protein-folding pattern of amyloids. These anionic sulfonate or sulfate compounds were shown to inhibit heparin accelerated A β fibril formation and were able to disassemble preformed fibrils in vitro, as monitored by electron micrography. Moreover, these compounds substantially arrested murine splenic inflammation-associated amyloid progression in vivo in acute and chronic models. However, the most potent compound [i.e., poly-

(vinylsulfonate)] showed acute toxicity. Similar toxicity has been observed with another compound, IDOX (Anthracycline 4'-iodo-4'-deoxy-doxorubicin), which has been observed to induce amyloid resorption in patients with immunoglobulin light chain amyloidosis (AL) [Merlini et al. (1995) Proc. Natl. Acad. Sci. USA].

5 **Destabilizing antibodies** - Anti- β -amyloid monoclonal antibodies have been shown to be effective in disaggregating β -amyloid plaques and preventing β -amyloid plaque formation *in vitro* (U.S. Pat. No. 5,688,561). However, no experimental results demonstrating the *in vivo* effectiveness of such antibodies have been demonstrated.

10 **Destabilizing peptides** - The finding that the addition of synthetic peptides that disrupt the β -pleated sheets (" β -sheet breakers") dissociated fibrils and prevented amyloidosis [Soto et al. (1998) Nat. Med. 4:822-6] is particularly promising from a clinical point of view. In brief, a penta-residue peptide inhibited amyloid beta-protein fibrillogenesis, disassembled preformed fibrils *in vitro* and prevents neuronal
15 death induced by fibrils in cell culture. In addition, the beta-sheet breaker peptide significantly reduced amyloid beta-protein deposition *in vivo* and completely blocked the formation of amyloid fibrils in a rat brain model of amyloidosis.

Small molecules - The potential use of small molecules which bind the amyloid polypeptide, stabilizing the native fold of the protein has been attempted in
20 the case of the transthyretin (TTR) protein [Peterson (1998) Proc. Natl. Acad. Sci. USA 95:12965-12960; Oza (1999) Bioorg. Med. Chem. Lett. 9:1-6]. Thus far, it has been demonstrated that molecules such as thyroxine and flufenamic acid are capable of preventing the conformation change, leading to amyloid formation. However, the use of the compounds in animal models has not been proved yet and might be
25 compromised due to the presence in blood or proteins, other than TTR, capable of binding these ligands.

Antioxidants - Another proposed therapy has been the intake of antioxidants in order to avoid oxidative stress and maintain amyloid proteins in their reduced state (i.e., monomers and dimers). The use of sulfite was shown to lead to more stable
30 monomers of the TTR both *in vitro* and *in vivo* [Altland (1999) Neurogenetics 2:183-188]. However, a complete characterization of the antioxidant effect is still not

available and the interpretation of results concerning possible therapeutic strategies remains difficult.

While reducing the present invention to practice, the present inventors have demonstrated that contrary to the teachings of U.S. Pat. No. 6,359,112 to Kapurniotu, peptide aggregation into amyloid fibrils is governed by aromatic interactions. Such findings enable to efficiently and accurately design peptides, which can be used to diagnose and treat amyloid-associated diseases.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a peptide comprising at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

According to further features in preferred embodiments of the invention described below the amino acid sequence further includes a polar uncharged amino acid selected from the group consisting of serine, threonine, asparagine, glutamine and natural derivatives thereof.

According to still further features in the described preferred embodiments the amino acid sequence further includes at least one positively charged amino acid and at least one negatively charged amino acid.

According to still further features in the described preferred embodiments the at least one positively charged amino acid is selected from the group consisting of lysine, arginine and natural and synthetic derivatives thereof.

According to still further features in the described preferred embodiments the at least one negatively charged amino acid is selected from the group consisting of aspartic acid, glutamic acid and natural and synthetic derivatives thereof.

According to still further features in the described preferred embodiments the amino acid sequence is selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-45.

According to still further features in the described preferred embodiments the peptide is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.

According to another aspect of the present invention there is provided a peptide comprising at least 3 amino acid residues and less than 15 amino acid residues, the

peptide including an amino acid sequence selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-44 wherein the peptide is capable of self-aggregating under physiological conditions.

According to yet another aspect of the present invention there is provided a peptide selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25.

According to still another aspect of the present invention there is provided a peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-44.

According to an additional aspect of the present invention there is provided a peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 10-11 and 21-22.

According to yet an additional aspect of the present invention there is provided a method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual a therapeutically effective amount of a peptide having at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

According to still an additional aspect of the present invention there is provided a method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual therapeutically effective amount of a peptide having at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45.

According to still further features in the described preferred embodiments the peptide is an active ingredient of a pharmaceutical composition which also includes a physiologically acceptable carrier.

According to a further aspect of the present invention there is provided a method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual a therapeutically effective amount of a peptide selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25, wherein the peptide is an active ingredient of a pharmaceutical compositions which also includes a physiologically acceptable carrier.

According to yet a further aspect of the present invention there is provided a method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual a therapeutically effective amount of a peptide selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45.

5 According to still a further aspect of the present invention there is provided a method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual therapeutically effective amount of a peptide having at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence selected from the group consisting of SEQ
10 ID NOs: 8, 10-11, 21-22 and 25, wherein the peptide is an active ingredient of a pharmaceutical composition which also includes a physiologically acceptable carrier.

According to still further features in the described preferred embodiments the peptide is expressed from a nucleic acid construct.

According to still a further aspect of the present invention there is provided a
15 pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide having at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7 and a pharmaceutically acceptable carrier or diluent.

According to still a further aspect of the present invention there is provided a
20 pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25 and a pharmaceutically acceptable carrier or diluent.

According to still a further aspect of the present invention there is provided a
25 pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide having at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25 and a pharmaceutically acceptable carrier or diluent.

30 According to still a further aspect of the present invention there is provided a pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide having at least 3 amino acid residues and

less than 15 amino acid residues, the peptide including an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45 and a pharmaceutically acceptable carrier or diluent.

According to still a further aspect of the present invention there is provided a
5 pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45 and a pharmaceutically acceptable carrier or diluent.

According to still a further aspect of the present invention there is provided a nucleic acid construct comprising a polynucleotide segment encoding a peptide having at least
10 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

According to still a further aspect of the present invention there is provided A nucleic acid construct comprising a polynucleotide segment encoding a peptide selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25.

15 According to still a further aspect of the present invention there is provided a nucleic acid construct comprising a polynucleotide segment encoding a peptide selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45.

According to still a further aspect of the present invention there is provided an antibody or an antibody fragment comprising an antigen recognition region capable of
20 binding a peptide including at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

According to still a further aspect of the present invention there is provided a pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient an antibody or an antibody fragment having an
25 antigen recognition region capable of binding a peptide including at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

According to still a further aspect of the present invention there is provided a method of treating or preventing an amyloid-associated disease in an individual, the
30 method comprising providing to the individual therapeutically effective amount of an antibody or an antibody fragment having an antigen recognition region capable of

binding a peptide including at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

According to still further features in the described preferred embodiments the peptide further comprising at least two serine residues at a C-terminus thereof.

5 According to still further features in the described preferred embodiments the peptide is a linear or cyclic peptide.

According to still further features in the described preferred embodiments the peptide further includes at least one beta-breaker amino acid.

10 According to still further features in the described preferred embodiments the beta-breaker amino acid is proline.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel peptides, compositions and methods, which can be used to diagnose and treat amyloid associated diseases such as type II Diabetes mellitus.

15

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of
20 illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the
25 invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 is a schematic illustration depicting the self-assembly ability and hydrophobicity of a group of peptides from a number of amyloid proteins as deduced
30 using Kyte and Dolittle scale. Note, that no correlation is observed between hydrophobicity and the amyloidogenic potential of the analyzed peptides. The only

apparent indication for potential amyloid fibril formation in this group of peptide is a combination of aromatic nature and minimal length.

FIGs. 2a-c are schematic illustrations of amyloid binding with the inhibitory aromatic reagents: Ro 47-1816/001 (Figure 2a), Thioflavin T (Figure 2b) and CR dye (Figure 2c).

FIGs. 3a-c are schematic illustrations of a primary sequence comparison between human and rodent IAPP and the synthetic peptides of the present invention. Figure 3a is a sequence alignment of human and rodent IAPP. A block indicates a seven amino acid sub-sequence illustrating the major inconsistencies between the sequences. The "basic amyloidogenic unit" is presented by bold letters and underlined. Figure 3b illustrates the chemical structure of the wild type IAPP peptide (SEQ ID NO: 1). Figure 1c illustrates the primary sequences and SEQ ID NOs. of the peptides derived from the basic amyloidogenic unit.

FIGs. 4a-b are graphs illustrating light absorbance at 405 nm as a function of time during fibril formation thus reflecting the aggregation kinetics of IAPP-derived peptides. The following symbols are used: closed squares – N1A, opened circles - G3A, closed circles – wild type, opened triangles - L6A, opened squares – I5A and closed triangles – F2A.

FIG. 5 is a histogram depicting mean particle size of assembled IAPP peptide and derivatives as measured by light scattering. Each column represents the results of 3-5 independent measurements.

FIGs. 6a-n are photomicrographs illustrating Congo Red binding to pre-assembled IAPP peptides. Normal field and polarized field micrographs are shown respectively for each of the following aged peptide suspensions: N1A peptide (Figures 6a-b), F2A peptide (Figures 6c-d), G3A peptide (Figures 6e-f), wild type peptide (Figures 6g-h), I5A peptide (Figures 6i-j) and L6A (Figures 6k-l).

FIGs. 7a-f are electron micrographs of "aged" IAPP peptide and derivatives. N1A peptide (Figure 7a), F2A peptide (Figure 7b), G3A peptide (Figure 7c), wild type peptide (Figure 7d), I5A peptide (Figure 7e) and L6A (Figure 7f). The indicated scale bar represents 100 nm.

FIG. 8a is a nucleic acid sequence alignment of wild type hIAPP and a corresponding sequence modified according to a bacterial codon usage. Modified bases are underlined.

FIG. 8b is a schematic illustration of the pMALc2x-NN vector which is used
5 for cytoplasmic expression of the 48 kDa MBP-IAPP protein. The V8 Ek cleavage site and the (His)₆ tag are fused C-terminally to the *malE* tag vector sequence. A factor *Xa* cleavage site for removal of the MBP tag is indicated.

FIG. 9 is a protein gel GelCode Blue staining depicting bacterial expression and purification of MBP and MBP-IAPP fusion protein. Bacterial cell extracts were
10 generated and proteins were purified on an amylose resin column. Samples including 25 µg protein were loaded in each of Lanes 1-3 whereas 5 µg protein were loaded on each of lanes 4-5. Proteins were resolved on a 12 % SDS-PAGE and visualized with GelCode Blue staining. A molecular weight marker is indicated on the left. Lane 1 – 0.5 mM IPTG-induced soluble extract of MBP. Lane 2 – 0.1 mM IPTG-induced
15 soluble extract of MBP-IAPP. Lane 3 – 0.5 mM IPTG-induced soluble extract of MBP-IAPP. Lane 4 – purified MBP. Lane 5 – purified MBP-IAPP. An arrow marks the MBP-IAPP.

FIGs. 10a-b are a dot-blot image (Figure 10a) and densitometric quantitation thereof (Figure 10b) depicting putative amyloidogenic sequences in hIAPP.

FIG. 11 is a graphic illustration depicting light absorbance at 405 nm as a
20 function of time during fibril formation thus reflecting the aggregation kinetics of IAPP-derived peptides (SEQ ID NOs. 14-19). The following symbols are used: closed squares – FLVHSS, opened circles - FLVHS, closed diamonds – NFLVHSS, opened triangles - NFLVHSSNN, opened squares – FLVH and closed triangles –
25 NFLVH.

FIGs. 12a-f are photomicrographs illustrating Congo Red binding to pre-assembled IAPP peptides. Polarized field micrographs are shown for each of the following one day aged peptide suspensions: NFLVHSSNN peptide (Figures 12a), NFLVHSS (Figure 12b), FLVHSS (Figure 12c), NFLVH (Figure 12d), FLVHS
30 (Figure 12e) and FLVH (Figure 12f).

FIGs. 13a-f are electron micrographs of "aged" IAPP peptides. NFLVHSSNN peptide (Figures 13a), NFLVHSS (Figure 13b), FLVHSS (Figure

13c), NFLVH (Figure 13d), FLVHS (Figure 13e) and FLVH (Figure 13f). The indicated scale bar represents 100 nm.

FIGs. 14a-f are graphs showing secondary structures in the insoluble IAPP aggregates as determined by Fourier transformed infrared spectroscopy. NFLVHSSNN peptide (Figures 14a), NFLVHSS (Figure 14b), FLVHSS (Figure 14c), NFLVH (Figure 14d), FLVHS (Figure 14e) and FLVH (Figure 14f).

FIG. 15 is a chemical structure of a previously reported amyloidogenic peptide fragment of Medin [Haggqvist (1999) Proc. Natl. Acad. Sci. U S A 96:8669-8674].

FIGs. 16a-b are graphs illustrating light absorbance at 405 nm as a function of time during fibril formation thus reflecting the aggregation kinetics of Medin-derived peptides. Figure 16a illustrates a short-term kinetic assay. Figure 16b illustrates a long-term kinetic assay.

FIGs. 17a-f are electron micrographs of "aged" Medin-derived peptides. NFGSVQFA - Figures 17a, NFGSVQ - Figure 17b, NFGSV - Figure 17c, FGSVQ - Figure 17d, GSVQ - Figure 17e and FGSV - Figure 17f. The indicated scale bar represents 100 nm.

FIGs. 18a-f are photomicrographs illustrating Congo Red binding to pre-assembled Medin-derived peptides. Polarized field micrographs are shown for each of the following aged peptide suspensions: NFGSVQFA - Figures 18a, NFGSVQ - Figure 18b, NFGSV - Figure 18c, FGSVQ - Figure 18d, GSVQ - Figure 18e and FGSV - Figure 18f.

FIGs. 19a-c depict the effect of an alanine mutation on the amyloidogenic features of the hexapeptide amyloidogenic fragment of Medin. Figure 19a - is a graph illustrating light absorbance at 405 nm as a function of time during fibril formation thus reflecting the aggregation kinetics of Medin-derived alanine mutant; Figure 19b is an electron micrograph of "aged" Medin-derived alanine mutant, The scale bar represents 100 nm; Figure 19c - is a photomicrograph illustrating Congo Red binding to pre-assembled Medin-derived peptide mutant.

FIGs. 20a-b are the amino acid sequence of human Calcitonin (Figure 20a) and chemical structure of an amyloidogenic peptide fragment of human Calcitonin (Figure 20b). Underlined are residues 17 and 18 which are important to the

oligomerization state and hormonal activity of Calcitonin [Kazantzis (2001) Eur. J. Biochem. 269:780-791].

FIGs. 21a-d are electron micrographs of "aged" Calcitonin-derived peptides. DFNKF - Figure 21a, DFNK - Figure 21b, FNKF - Figure 21c and DFN - Figure 21d.

5 The indicated scale bar represents 100 nm.

FIGs. 22a-d are photomicrographs illustrating Congo Red binding to pre-assembled Calcitonin-derived peptides. Polarized field micrographs are shown for each of the following aged peptide suspensions: DFNKF - Figure 22a, DFNK - Figure 22b, FNKF - Figure 22c and DFN - Figure 22d.

10 FIG. 23 is a graphic illustration showing secondary structures in the insoluble Calcitonin aggregates as determined by Fourier transformed infrared spectroscopy.

FIGs. 24a-c depict the effect an alanine mutation on the amyloidogenic features of the pentapeptide amyloidogenic fragment of Calcitonin. Figure 24a is an electron micrograph of "aged" Calcitonin-derived alanine mutant. The scale bar represents 100 nm; Figure 24b – is a photomicrograph illustrating Congo Red binding to pre-assembled Calcitonin-derived peptide mutant; Figure 24c is a graph showing secondary structures in the mutant peptide as determined by Fourier transformed infrared spectroscopy.

FIG. 25 is an electron micrograph depicting self-assembly of "aged" Lactotransferrin-derived peptide. The scale bar represents 100 nm.

FIG. 26 is an electron micrograph depicting self-assembly of "aged" Serum amyloid A protein-derived peptide. The scale bar represents 100 nm.

FIG. 27 is an electron micrograph depicting self-assembly of "aged" BriL-derived peptide. The scale bar represents 100 nm.

25 FIG. 28 is an electron micrograph depicting self-assembly of "aged" Gelsolin-derived peptide. The scale bar represents 100 nm.

FIG. 29 is an electron micrograph depicting self-assembly of "aged" Serum amyloid P-derived peptide. The scale bar represents 100 nm.

30 FIG. 30 is an electron micrograph depicting self-assembly of "aged" Immunoglobulin light chain-derived peptide. The scale bar represents 100 nm.

FIG. 31 is an electron micrograph depicting self-assembly of "aged" Cystatin C-derived peptide. The scale bar represents 100 nm.

FIG. 32 is an electron micrograph depicting self-assembly of "aged" Transthyretin-derived peptide. The scale bar represents 100 nm.

FIG. 33 is an electron micrograph depicting self-assembly of "aged" Lysozyme-derived peptide. The scale bar represents 100 nm.

5 FIG. 34 is an electron micrograph depicting self-assembly of "aged" Fibrinogen-derived peptide. The scale bar represents 100 nm.

FIG. 35 is an electron micrograph depicting self-assembly of "aged" Insulin-derived peptide. The scale bar represents 100 nm.

10 FIG. 36 is an electron micrograph depicting self-assembly of "aged" Prolactin-derived peptide. The scale bar represents 100 nm.

FIG. 37 is an electron micrograph depicting self-assembly of "aged" Beta 2 microglobulin-derived peptide. The scale bar represents 100 nm.

15 FIG. 38 is a graphic representation of the effect of an inhibitory peptide on IAPP self-assembly. Squares – wild type (wt) IAPP peptide; triangles – wt-IAPP + inhibitory peptide; circles – no peptides.

FIG. 39 is a graphic illustration depicting light absorbance at 405 nm as a function of time during fibril formation thus reflecting the aggregation kinetics of IAPP-derived peptides (SEQ ID NOs. 46-49).

20 FIG. 40 is a histogram representation illustrating turbidity of IAPP analogues following seven day aging.

FIG. 41a-f are electron micrographs of "aged" IAPP analogues. NFGAILSS - Figure 41a; NFGAILSS - Figure 41b; NIGAILSS - Figure 41c; NLGAILSS - Figure 41d; NVGAILSS - Figure 41e and NAGAILSS - Figure 41f. The indicated scale bar represents 100 nm.

25 FIGs. 42a-c illustrate the binding of IAPP- NFGAILSS to analogues of the minimal amyloidogenic sequence SNNXGAILSS (X = any natural amino acid but cysteine). Figure 42a shows short exposure of the bound peptide-array. Figure 42b shows long exposure of the bound peptide-array. Figure 42c shows quantitation of the short exposure (Figure 42a) using densitometry and arbitrary units.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of novel peptides antibodies directed thereagainst, compositions including same and methods of utilizing each for diagnosing or treating amyloid associated diseases such as type II Diabetes mellitus.

5 The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following
10 description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Numerous therapeutic approaches for prevention of amyloid fibril formation
15 or disaggregation of amyloid material have been described in the prior art. However, current therapeutic approaches are limited by cytotoxicity, non-specificity and delivery barriers.

While reducing the present invention to practice and while searching for a novel therapeutic modality to amyloid associated diseases, such as II diabetes
20 mellitus, the present inventor has identified a sequence characteristic of amyloid forming peptides which directs fibril formation. This finding suggests that ordered amyloidogenesis involves a specific pattern of molecular interactions rather than the previously described mechanism involving non-specific hydrophobic interactions [Petkova (2002) Proc. Natl. Acad. Sci. U S A 99:16742-16747].

25 As is further illustrated hereinbelow and in the Examples section which follows, the present inventor attributed a pivotal role for aromatic residues in amyloid formation. The involvement of aromatic residues in the process of amyloid formation is in-line with the well-established role of π -such interactions in molecular recognition and self-assembly [Gillard et al (1997) Chem. Eur. J. 3: 1933-40; Claessens and Stoddart, (1997) J. Phys. Org. Chem. 10: 254-72; Shetty et al (1996) J.
30 Am. Chem. Soc. 118: 1019-27; McGuaghey et al (1998) π -stacking interactions: Alive and well in proteins. J. Biol. Chem. 273, 15458-15463; Sun and Bernstein

(1996) J. Phys. Chem. 100: 13348-66]. π -stacking interactions are non-bonded interactions which are formed between planar aromatic rings. The steric constraints associated with the formation of those ordered stacking structures have a fundamental role in self-assembly processes that lead to the formation of supramolecular structures. Such π -stacking interactions, which are probably entropy driven, play a central role in many biological processes such as stabilization of the double-helix structure of DNA, core-packing and stabilization of the tertiary structure of proteins, host-guest interactions, and porphyrin aggregation in solution [for further review on the possible role of π -stacking interaction in the self-assembly of amyloid fibrils see Gazit (2002) FASEB J. 16:77-83].

Identification of an aromatic sequence which is sufficient for mediating amyloid self-assembly enables for the first time, to generate highly efficient diagnostic, prophylactic and therapeutic peptides which can be utilized to treat or diagnose diseases characterized by amyloid plaque formation.

Thus, according to one aspect of the present invention there is provided a peptide which includes the amino acid sequence set forth in SEQ ID NO: 7 and is capable of self aggregation into fibrils. As is further described hereinbelow, peptides possessing self aggregation capabilities and modificants thereof can be utilized in diagnostic and therapeutic applications.

The sequence set forth in SEQ ID NO: 7 includes at least one aromatic amino acid residue which, as is shown by the results presented in the Examples section, is pivotal to the formation of amyloid fibrils. It will be appreciated that aromaticity rather than hydrophobicity of the aromatic amino acid is the prevailing chemical feature in amyloid self-assembly as illustrated in Examples 36-39 of the Examples section.

The aromatic amino acid can be any naturally occurring or synthetic aromatic residue including, but not limited to, phenylalanine, tyrosine, tryptophan, phenylglycine, or modificants, precursors or functional aromatic portions thereof. Examples of aromatic residues which can be used by the present invention are provided in Table 2 below.

As is demonstrated by the results provided in the Examples section which follows, the present invention facilitates the design of peptides exhibiting varying degrees of self-aggregation kinetics and aggregate structure.

As used herein, the phrase "self-aggregation" refers to the capability of a peptide to form aggregates (e.g. fibrils) in an aqueous solution. The ability of a peptide to self-aggregate and the kinetics and type of such self-aggregation determines a use for the peptide in treating or diagnosing amyloid diseases.

Since aggregation kinetics and aggregate structures are largely determined by the specific residue composition and possibly the length of the peptides generated (see Figure 1), the present invention encompasses both longer peptides (e.g., 10-50 amino acids) which include the sequences set forth in SEQ ID NOs: 4, 8, 10-19, 21-22, 25 or 27-45, or shorter peptides (2-10 amino acid residues) including any of these sequences. Due to their self-aggregating nature these peptides can be used as potent diagnostic reagents.

In order to enhance the rate of amyloid formation, the peptides of the present invention preferably further include at least one polar and uncharged amino acid including but not limited to serine, threonine, asparagine, glutamine or natural or synthetic derivatives thereof (see Table 2).

Additionally, the peptides of the present invention may further include at least one pair of positively charged (e.g., lysine and arginine) and negatively charged (e.g., aspartic acid and glutamic acid) amino acids (e.g., SEQ ID NOs. 27-29). Such amino acid composition may be preferable, since as shown in Examples 21 of the Examples section, it is likely that electrostatic interactions between opposing charges may direct the formation of ordered antiparallel structure.

Since the present inventors have identified the sequence characteristics governing fibril formation, the teachings of the present invention also enable design of peptides which would not aggregate into fibrils and be capable of either preventing or reducing fibril formation or disrupting preformed fibrils and thus can be used as a therapeutic agents.

For example, a peptide encompassed by SEQ ID NO: 9, 10, 11, 17, 19, 25 or 30 can be utilized for therapy since as is shown in the Examples section which follows, such a peptide displays no aggregation (SEQ ID NO: 9) or slow aggregation

kinetics as compared to the wild type peptide (SEQ ID NOs: 9 and 10). It is conceivable that since amyloid formation is a very slow process, these peptide sequences will completely inhibit or significantly delay amyloidosis under physiological conditions.

5 The term "peptide" as used herein encompasses native peptides (either degradation products, synthetically synthesized peptides or recombinant peptides) and peptidomimetics (typically, synthetically synthesized peptides), as well as peptoids and semipeptoids which are peptide analogs, which may have, for example, modifications rendering the peptides more stable while in a body or more capable of
10 penetrating into cells. Such modifications include, but are not limited to N terminus modification, C terminus modification, peptide bond modification, including, but not limited to, CH₂-NH, CH₂-S, CH₂-S=O, O=C-NH, CH₂-O, CH₂-CH₂, S=C-NH, CH=CH or CF=CH, backbone modifications, and residue modification. Methods for preparing peptidomimetic compounds are well known in the art and are specified, for
15 example, in Quantitative Drug Design, C.A. Ramsden Gd., Chapter 17.2, F. Choplin Pergamon Press (1992), which is incorporated by reference as if fully set forth herein. Further details in this respect are provided hereinunder.

 Peptide bonds (-CO-NH-) within the peptide may be substituted, for example, by N-methylated bonds (-N(CH₃)-CO-), ester bonds (-C(R)H-C-O-O-C(R)-N-),
20 ketomethylen bonds (-CO-CH₂-), α -aza bonds (-NH-N(R)-CO-), wherein R is any alkyl, e.g., methyl, carba bonds (-CH₂-NH-), hydroxyethylene bonds (-CH(OH)-CH₂-), thioamide bonds (-CS-NH-), olefinic double bonds (-CH=CH-), retro amide bonds (-NH-CO-), peptide derivatives (-N(R)-CH₂-CO-), wherein R is the "normal" side chain, naturally presented on the carbon atom.

25 These modifications can occur at any of the bonds along the peptide chain and even at several (2-3) at the same time.

 Natural aromatic amino acids, Trp, Tyr and Phe, may be substituted for synthetic non-natural acid such as Phenylglycine, TIC, naphthylelanine (Nol), ring-methylated derivatives of Phe, halogenated derivatives of Phe or o-methyl-Tyr.

30 In addition to the above, the peptides of the present invention may also include one or more modified amino acids or one or more non-amino acid monomers (e.g. fatty acids, complex carbohydrates etc).

As used herein in the specification and in the claims section below the term "amino acid" or "amino acids" is understood to include the 20 naturally occurring amino acids; those amino acids often modified post-translationally *in vivo*, including, for example, hydroxyproline, phosphoserine and phosphothreonine; and other unusual amino acids including, but not limited to, 2-aminoadipic acid, hydroxylysine, isodesmosine, nor-valine, nor-leucine and ornithine. Furthermore, the term "amino acid" includes both D- and L-amino acids.

Tables 1 and 2 below list naturally occurring amino acids (Table 1) and non-conventional or modified amino acids (Table 2) which can be used with the present invention.

Table 1

| Amino Acid | Three-Letter Abbreviation | One-letter Symbol |
|-------------------------|----------------------------------|--------------------------|
| alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid | Asp | D |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glutamic Acid | Glu | E |
| glycine | Gly | G |
| Histidine | His | H |
| isoleucine | Ile | I |
| leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| tryptophan | Trp | W |
| tyrosine | Tyr | Y |
| Valine | Val | V |
| Any amino acid as above | Xaa | X |

Table 2

| Non-conventional amino acid | Code | Non-conventional amino acid | Code |
|---|-------------|------------------------------------|-------------|
| α -aminobutyric acid | Abu | L-N-methylalanine | Nmala |
| α -amino- α -methylbutyrate | Mgab | L-N-methylarginine | Nmarg |
| aminocyclopropane- | Cpro | L-N-methylasparagine | Nmasn |
| carboxylate | | L-N-methylaspartic acid | Nmasp |
| aminoisobutyric acid | Aib | L-N-methylcysteine | Nmcys |
| aminonorbornyl- | Norb | L-N-methylglutamine | Nmgin |
| carboxylate | | L-N-methylglutamic acid | Nmglu |
| cyclohexylalanine | Chexa | L-N-methylhistidine | Nmhis |
| cyclopentylalanine | Cpen | L-N-methylisoleucine | Nmile |
| D-alanine | Dal | L-N-methylleucine | Nmleu |
| D-arginine | Darg | L-N-methyllysine | Nmlys |

| | | | |
|----------------------------------|---------|---|--------|
| D-aspartic acid | Dasp | L-N-methylmethionine | Nmmet |
| D-cysteine | Dcys | L-N-methylnorleucine | Nmle |
| D-glutamine | Dgln | L-N-methylnorvaline | Nmnva |
| D-glutamic acid | Dglu | L-N-methylornithine | Nmorn |
| D-histidine | Dhis | L-N-methylphenylalanine | Nmphe |
| D-isoleucine | Dile | L-N-methylproline | Nmpro |
| D-leucine | Dleu | L-N-methylserine | Nmser |
| D-lysine | Dlys | L-N-methylthreonine | Nmthr |
| D-methionine | Dmet | L-N-methyltryptophan | Nmtrp |
| D-ornithine | Dorn | L-N-methyltyrosine | Nmtyr |
| D-phenylalanine | Dphe | L-N-methylvaline | Nmval |
| D-proline | Dpro | L-N-methylethylglycine | Nmetg |
| D-serine | Dser | L-N-methyl-t-butylglycine | Nmtbug |
| D-threonine | Dthr | L-norleucine | Nle |
| D-tryptophan | Dtrp | L-norvaline | Nva |
| D-tyrosine | Dtyr | α -methyl-aminoisobutyrate | Maib |
| D-valine | Dval | α -methyl- γ -aminobutyrate | Mgab |
| D- α -methylalanine | Dmala | α -methylcyclohexylalanine | Mchexa |
| D- α -methylarginine | Dmarg | α -methylcyclopentylalanine | Mcpen |
| D- α -methylasparagine | Dmasn | α -methyl- α -naphthylalanine | Manap |
| D- α -methylaspartate | Dmasp | α -methylpenicillamine | Mpen |
| D- α -methylcysteine | Dmcys | N-(4-aminobutyl)glycine | Nglu |
| D- α -methylglutamine | Dmgln | N-(2-aminoethyl)glycine | Naeg |
| D- α -methylhistidine | Dmhis | N-(3-aminopropyl)glycine | Norn |
| D- α -methylisoleucine | Dmile | N-amino- α -methylbutyrate | Nmaabu |
| D- α -methylleucine | Dmleu | α -naphthylalanine | Anap |
| D- α -methyllysine | Dmlys | N-benzylglycine | Nphe |
| D- α -methylmethionine | Dmmet | N-(2-carbamylethyl)glycine | Ngln |
| D- α -methylornithine | Dmorn | N-(carbamylmethyl)glycine | Nasn |
| D- α -methylphenylalanine | Dmphe | N-(2-carboxyethyl)glycine | Nglu |
| D- α -methylproline | Dmpro | N-(carboxymethyl)glycine | Nasp |
| D- α -methylserine | Dmser | N-cyclobutylglycine | Ncbut |
| D- α -methylthreonine | Dmthr | N-cycloheptylglycine | Nchep |
| D- α -methyltryptophan | Dmtrp | N-cyclohexylglycine | Nchex |
| D- α -methyltyrosine | Dmtyr | N-cyclodecylglycine | Ncdec |
| D- α -methylvaline | Dmval | N-cyclododecylglycine | Ncdod |
| D- α -methylalanine | Dnmala | N-cyclooctylglycine | Ncoct |
| D- α -methylarginine | Dnmarg | N-cyclopropylglycine | Ncpro |
| D- α -methylasparagine | Dnmasn | N-cycloundecylglycine | Ncund |
| D- α -methylaspartate | Dnmasp | N-(2,2-diphenylethyl)glycine | Nbhm |
| D- α -methylcysteine | Dnmcys | N-(3,3-diphenylpropyl)glycine | Nbhe |
| D-N-methylleucine | Dnmleu | N-(3-indolyl)ethyl glycine | Nhtrp |
| D-N-methyllysine | Dnmlys | N-methyl- γ -aminobutyrate | Nmgabu |
| N-methylcyclohexylalanine | Nmchexa | D-N-methylmethionine | Dnmmet |
| D-N-methylornithine | Dnmorn | N-methylcyclopentylalanine | Nmcpen |
| N-methylglycine | Nala | D-N-methylphenylalanine | Dnmphe |
| N-methylaminoisobutyrate | Nmaib | D-N-methylproline | Dnmpro |
| N-(1-methylpropyl)glycine | Nile | D-N-methylserine | Dnmser |
| N-(2-methylpropyl)glycine | Nile | D-N-methylserine | Dnmser |

| | | | |
|--|------------|---|--------|
| N-(2-methylpropyl)glycine | Nleu | D-N-methylthreonine | Dnmthr |
| D-N-methyltryptophan | Dnmtrp | N-(1-methylethyl)glycine | Nva |
| D-N-methyltyrosine | Dnmtyr | N-methyl- α -naphthylalanine | Nmanap |
| D-N-methylvaline | Dnmval | N-methylpenicillamine | Nmpen |
| γ -aminobutyric acid | Gabu | N-(<i>p</i> -hydroxyphenyl)glycine | Nhtyr |
| L- <i>t</i> -butylglycine | Tbug | N-(thiomethyl)glycine | Ncys |
| L-ethylglycine | Etg | penicillamine | Pen |
| L-homophenylalanine | Hphe | L- α -methylalanine | Mala |
| L- α -methylarginine | Marg | L- α -methylasparagine | Masn |
| L- α -methylaspartate | Masp | L- α -methyl- <i>t</i> -butylglycine | Mtbug |
| L- α -methylcysteine | Mcys | L-methylethylglycine | Metg |
| L- α -methylglutamine | Mgln | L- α -methylglutamate | Mglu |
| L- α -methylhistidine | Mhis | L- α -methylhomophenylalanine | Mhphe |
| L- α -methylisoleucine | Mile | N-(2-methylthioethyl)glycine | Nmet |
| D-N-methylglutamine | Dnmgln | N-(3-guanidinopropyl)glycine | Narg |
| D-N-methylglutamate | Dnmglu | N-(1-hydroxyethyl)glycine | Nthr |
| D-N-methylhistidine | Dnmhis | N-(hydroxyethyl)glycine | Nser |
| D-N-methylisoleucine | Dnmile | N-(imidazolylethyl)glycine | Nhis |
| D-N-methylleucine | Dnmleu | N-(3-indolylethyl)glycine | Nhtrp |
| D-N-methyllysine | Dnmlys | N-methyl- γ -aminobutyrate | Nmgabu |
| N-methylcyclohexylalanine | Nmchexa | D-N-methylmethionine | Dnmmt |
| D-N-methylornithine | Dnmorn | N-methylcyclopentylalanine | Nmcpn |
| N-methylglycine | Nala | D-N-methylphenylalanine | Dnmph |
| N-methylaminoisobutyrate | Nmaib | D-N-methylproline | Dnmpro |
| N-(1-methylpropyl)glycine | Nile | D-N-methylserine | Dnmser |
| N-(2-methylpropyl)glycine | Nleu | D-N-methylthreonine | Dnmthr |
| D-N-methyltryptophan | Dnmtrp | N-(1-methylethyl)glycine | Nval |
| D-N-methyltyrosine | Dnmtyr | N-methyl- α -naphthylalanine | Nmanap |
| D-N-methylvaline | Dnmval | N-methylpenicillamine | Nmpen |
| γ -aminobutyric acid | Gabu | N-(<i>p</i> -hydroxyphenyl)glycine | Nhtyr |
| L- <i>t</i> -butylglycine | Tbug | N-(thiomethyl)glycine | Ncys |
| L-ethylglycine | Etg | penicillamine | Pen |
| L-homophenylalanine | Hphe | L- α -methylalanine | Mala |
| L- α -methylarginine | Marg | L- α -methylasparagine | Masn |
| L- α -methylaspartate | Masp | L- α -methyl- <i>t</i> -butylglycine | Mtbug |
| L- α -methylcysteine | Mcys | L-methylethylglycine | Metg |
| L- α -methylglutamine | Mgln | L- α -methylglutamate | Mglu |
| L- α -methylhistidine | Mhis | L- α -methylhomophenylalanine | Mhphe |
| L- α -methylisoleucine | Mile | N-(2-methylthioethyl)glycine | Nmet |
| L- α -methylleucine | Mleu | L- α -methyllysine | Mlys |
| L- α -methylmethionine | Mmet | L- α -methylnorleucine | Mnle |
| L- α -methylnorvaline | Mnva | L- α -methylornithine | Morn |
| L- α -methylphenylalanine | Mphe | L- α -methylproline | Mpro |
| L- α -methylserine | mser | L- α -methylthreonine | Mthr |
| L- α -methylvaline | Mtrp | L- α -methyltyrosine | Mtyr |
| L- α -methylleucine | Mval Nnbhm | L-N-methylhomophenylalanine | Nmhph |
| N-(N-(2,2-diphenylethyl)carbamylmethyl-glycine | Nnbhm | N-(N-(3,3-diphenylpropyl)carbamylmethyl(1)glycine | Nnbhe |
| 1-carboxy-1-(2,2-diphenylethylamino)cyclopropane | Nmbc | | |

Since the present peptides are preferably utilized in therapeutics or diagnostics which require the peptides to be in soluble form, the peptides of the present invention preferably include one or more non-natural or natural polar amino acids, including but not limited to serine and threonine which are capable of increasing peptide solubility due to their hydroxyl-containing side chain.

For therapeutic application, the peptides of the present invention preferably further include at least one beta-sheet breaker amino acid residue such as proline (e.g., SEQ ID NO. 45, see background section) which is characterized by a limited phi angle of about -60 to +25 rather than the typical beta sheet phi angle of about -120 to -140 degrees, thereby disrupting the beta sheet structure of the amyloid fibril.

The peptides of the present invention are preferably utilized in a linear form, although it will be appreciated that in cases where cyclization does not severely interfere with peptide characteristics, cyclic forms of the peptide can also be utilized.

Cyclic peptides can either be synthesized in a cyclic form or configured so as to assume a cyclic form under desired conditions (e.g., physiological conditions).

Thus, the present invention provides conclusive data as to the identity of the structural determinant of amyloid peptides, which directs fibril assembly.

As such, the present invention enables design of a range of peptide sequences, which can be utilized for prevention/treatment or diagnosis of amyloidosis.

It will be appreciated that the present inventor could identify the consensus aromatic sequence of the present invention (SEQ ID NO: 7) in numerous amyloid related proteins (see Examples 6-35 of the Examples section). Thus, the present invention enables accurate identification of amyloidogenic fragments in essentially all amyloidogenic proteins.

Furthermore, the fact that small aromatic molecules, such as Ro 47-1816/001 [Kuner et al. (2000) J. Biol. Chem. 275:1673-8, see Figure 2a] and 3-p-toluoyl-2-[4'-(3-diethylaminopropoxy)-phenyl]-benzofuran [Twyman (1999) Tetrahedron Letters 40:9383-9384] have been demonstrated effective in inhibiting the polymerization of the beta polypeptide of Alzheimer's disease [Findeis et al. (2000) Biochem. Biophys. Acta 1503:76-84], while amyloid specific dyes such as Congo-Red (Figure 2b) and thioflavin T (Figure 2c), which contain aromatic elements are generic amyloid

formation inhibitors, substantiate the recognition motif of the present invention as sufficient for amyloid self-assembly.

The availability of the peptides of the present invention allows for the generation of antibodies directed thereagainst, which may be used to dissociate or prevent the formation of amyloid plaques (U.S. Pat. No. 5,688,561).

The term "antibody" refers to intact antibody molecules as well as functional fragments thereof, such as Fab, F(ab')₂, and Fv that are capable of binding to macrophages. These functional antibody fragments are defined as follows: (i) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (ii) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (iii) (Fab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds; (iv) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (v) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of making these fragments are known in the art. (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

Methods of generating antibodies (i.e., monoclonal and polyclonal) are well known in the art. Antibodies may be generated via any one of several methods known in the art, which methods can employ induction of in vivo production of antibody molecules, screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed [Orlandi D.R. et al. (1989) Proc. Natl. Acad. Sci. 86:3833-3837, Winter G. et al. (1991) Nature 349:293-299] or generation of monoclonal antibody molecules by continuous cell lines in culture. These include but are not limited to, the

hybridoma technique, the human B-cell hybridoma technique, and the Epstein-Bar-Virus (EBV)-hybridoma technique [Kohler G., et al. (1975) *Nature* 256:495-497, Kozbor D., et al. (1985) *J. Immunol. Methods* 81:31-42, Cote R.J. et al. (1983) *Proc. Natl. Acad. Sci.* 80:2026-2030, Cole S.P. et al. (1984) *Mol. Cell. Biol.* 62:109-120].

5 Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment.

 Antibody fragments can be obtained by pepsin or papain digestion of whole
10 antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an
15 enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R., *Biochem. J.*, 73: 119-126, 1959. Other methods of cleaving antibodies, such as separation of
20 heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

 Fv fragments comprise an association of V_H and V_L chains. This association may be noncovalent, as described in Inbar et al., *Proc. Nat'l Acad. Sci. USA* 69:2659-
25 62, 1972. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an
30 oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V

domains. Methods for producing sFvs are described, for example, by Whitlow and Filpula, *Methods*, 2: 97-105, 1991; Bird et al., *Science* 242:423-426, 1988; Pack et al., *Bio/Technology* 11:1271-77, 1993; and Ladner et al., U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

5 Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for
10 example, Larrick and Fry, *Methods*, 2: 106-10, 1991.

For human applications, the antibodies of the present invention are preferably humanized. Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies)
15 which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some
20 instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or
25 substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will include at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596
30 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature* 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., *J. Immunol.*, 147(1):86-95 (1991)]. Similarly, human can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10, 779-783 (1992); Lonberg et al., *Nature* 368 856-859 (1994); Morrison, *Nature* 368 812-13 (1994); Fishwild et al., *Nature Biotechnology* 14, 845-51 (1996); Neuberger, *Nature Biotechnology* 14, 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13 65-93 (1995).

As is mentioned hereinabove, one specific use for the peptides of the present invention is prevention or treatment of diseases associated with amyloid plaque formation.

Thus, according to yet another aspect of the present invention, there is provided a method of treating an amyloid-associated disease in an individual. Preferred individual subjects according to the present invention are mammals such as canines, felines, ovines, porcines, equines, bovines, humans and the like.

The term "treating" refers to reducing or preventing amyloid plaque formation, or substantially decreasing plaque occurrence in the affected tissue. The phrase "amyloid plaque" refers to fibrillar amyloid as well as aggregated but not fibrillar amyloid, hereinafter "protofibrillar amyloid", which may be pathogenic as well. For example, an aggregated but not necessarily fibrillar form of IAPP was found to be toxic in culture. As shown by Anaguiano and co-workers [(2002) *Biochemistry* 41:11338-43] protofibrillar IAPP, like protofibrillar α -synuclein, which is implicated in Parkinson's disease pathogenesis, permeabilized synthetic vesicles by a pore-like mechanism. The formation of the of the IAPP amyloid pore was temporally correlated to the formation of early IAPP oligomers and disappearance thereof to the appearance of amyloid fibrils. These results suggest that protofibrillar IAPP may be critical to type II diabetes mellitus as other protofibrillar proteins are critical to the development of Alzheimer's and Parkinson's diseases.

Amyloid-associated diseases treated according to the present invention include, but are not limited to, type II diabetes mellitus, Alzheimer's disease (AD), early onset Alzheimer's disease, late onset Alzheimer's disease, presymptomatic Alzheimer's disease, Parkinson's disease, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, medullary carcinoma, aortic medical carcinoma, Insulin injection amyloidosis, prion-systematic amyloidosis, chronic inflammation amyloidosis, Huntington's disease, senile systemic amyloidosis, pituitary gland amyloidosis, Hereditary renal amyloidosis, familial British dementia, Finnish hereditary amyloidosis, familial non-neuropathic amyloidosis [Gazit (2002) *Curr. Med. Chem.* 9:1667-1675] and prion diseases including scrapie of sheep and goats and bovine spongiform encephalopathy (BSE) of cattle [Wilesmith and Wells (1991) *Curr Top Microbiol Immunol* 172: 21-38] and human prion diseases including (i)

kuru, (ii) Creutzfeldt-Jakob Disease (CJD), (iii) Gerstmann-Straussler-Sheinker Disease (GSS), and (iv) fatal familial insomnia (FFI) [Gajdusek (1977) Science 197: 943-960; Medori, Tritschler et al. (1992) N Engl J Med 326: 444-449].

The method includes providing to the individual a therapeutically effective amount of the peptide of the present invention. The peptide can be provided using any one of a variety of delivery methods. Delivery methods and suitable formulations are described hereinbelow with respect to pharmaceutical compositions.

It will be appreciated that when utilized for treatment of amyloid diseases, the peptide of the present invention includes an amino acid sequence suitable for preventing fibril formation, reducing fibril formation, or disaggregating formed aggregates by competitive destabilization of the preformed aggregate. For example, SEQ ID NO: 45 can be utilized for treatment of amyloid diseases, particularly type II diabetes mellitus since as shown in Example 35 of the Examples section which follows, such a sequence interferes with IAPP self-assembly as demonstrated by the decreased ability of the amyloidogenic peptide to bind thioflavin T in the presence of an inhibitory peptide.

Alternatively, the peptides set forth in SEQ ID NOs: 10 or 11 can be used as
5 potent inhibitors of type II diabetes since as shown in the Examples section which follows, substitution of either leucine or isoleucine in the peptide elicits very slow kinetics of aggregation. Since amyloid formation in vivo is a very slow process, it is conceivable that under physiological conditions no fibrilization will occur upon the substitution of isoleucine or leucine to alanine in the context of the full length IAPP.

10 Alternatively, self-aggregating peptides such as those set forth in SEQ ID NOs. 17, 19 and 28-30, can be used as potent inhibitors of amyloid fibrilization, since such peptides can form heteromolecular complexes which are not as ordered as the homomolecular assemblies formed by amyloid fragments.

It will be appreciated that since one of the main obstacles in using short
15 peptide fragments in therapy is their proteolytic degradation by stereospecific cellular proteases, the peptides of the present invention are preferably synthesized from D-isomers of natural amino acids [i.e., inverso peptide analogues, Tjernberg (1997) J. Biol. Chem. 272:12601-5, Gazit (2002) Curr. Med. Chem. 9:1667-1675].

Additionally, the peptides of the present invention include retro, inverso and retro-inverso analogues thereof. It will be appreciated that complete or extended partial retro-inverso analogues of hormones have generally been found to retain or enhance biological activity. Retro-inversion has also found application in the area of rational design of enzyme inhibitors (see U.S. Pat. No. 6,261,569).

As used herein a "retro peptide" refers to peptides which are made up of L-amino acid residues which are assembled in opposite direction to the native peptide sequence.

Retro-inverso modification of naturally occurring polypeptides involves the synthetic assembly of amino acids with α -carbon stereochemistry opposite to that of the corresponding L-amino acids, i.e., D- or D-allo-amino acids in inverse order to the native peptide sequence. A retro inverso analogue, thus, has reversed termini and reversed direction of peptide bonds, while essentially maintaining the topology of the side chains as in the native peptide sequence.

Additionally, since one of the main issues in amyloid fibril formation is the transition of the amyloid polypeptide from the native form to stacked β -strand structure, inhibitory peptides preferably include N-methylated amino acids which constrain peptide-backbone due to steric effects [Kapurniotu (2002) 315:339-350]. For example, aminoisobutyric acid (Aib or methyl alanine) is known to stabilize an α -helical structure in short natural peptides. Furthermore, the N-methylation also affects the intermolecular NH to CO H-bonding ability, thus suppressing the formation of multiplayer β -strands, which are stabilized by H-bonding interactions.

It will be further appreciated that addition of organic groups such as a cholyl groups to the N-terminal or C-terminal of the peptides of the present invention is preferred since it was shown to improve potency and bioavailability (e.g., crossing the blood brain barrier in the case of neurodegenerative diseases) of therapeutic peptides [Findeis (1999) Biochemistry 38:6791-6800]. Furthermore, introducing a charged amino acid to the recognition motif, may result in electrostatic repulsion which inhibits further growth of the amyloid fibrils [Lowe (2001) J. Mol. Biol. 40:7882-7889].

As mentioned hereinabove, the antibodies of the present invention may also be used to treat amyloid-associated diseases.

The peptides and/or antibodies of the present invention can be provided to an individual *per se*, or as part of a pharmaceutical composition where it is mixed with a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one
5 or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the peptide or antibody
10 preparation, which is accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An
15 adjuvant is included under these phrases. One of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter et al. (1979).

Herein the term "excipient" refers to an inert substance added to a
20 pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in
25 "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal,
30 direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer a preparation in a local rather than systemic manner, for example, via injection of the preparation directly into a specific region of a patient's body.

Pharmaceutical compositions of the present invention may be manufactured by
5 processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically
10 acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's
15 solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well
20 known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable
25 auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers
30 such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or
5 pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active
10 ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in
15 dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray
20 presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a
25 powder mix of the compound and a suitable powder base such as lactose or starch.

The preparations described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be
30 suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The preparation of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal models and such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage

can be chosen by the individual physician in view of the patient's condition. [See e.g., Fingl, et al., (1975) "The Pharmacological Basis of Therapeutics", Ch. 1 p.1].

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

It will be appreciated that the peptides or antibodies of the present invention can also be expressed from a nucleic acid construct administered to the individual employing any suitable mode of administration, described hereinabove (i.e., in-vivo gene therapy). Alternatively, the nucleic acid construct is introduced into a suitable cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the individual (i.e., ex-vivo gene therapy).

To enable cellular expression of the peptides or antibodies of the present

invention, the nucleic acid construct of the present invention further includes at least one cis acting regulatory element. As used herein, the phrase "cis acting regulatory element" refers to a polynucleotide sequence, preferably a promoter, which binds a trans acting regulator and regulates the transcription of a coding sequence located downstream thereto.

Any available promoter can be used by the present methodology. In a preferred embodiment of the present invention, the promoter utilized by the nucleic acid construct of the present invention is active in the specific cell population transformed. Examples of cell type-specific and/or tissue-specific promoters include promoters such as albumin that is liver specific [Pinkert et al., (1987) *Genes Dev.* 1:268-277], lymphoid specific promoters [Calame et al., (1988) *Adv. Immunol.* 43:235-275]; in particular promoters of T-cell receptors [Winoto et al., (1989) *EMBO J.* 8:729-733] and immunoglobulins; [Banerji et al. (1983) *Cell* 33:729-740], neuron-specific promoters such as the neurofilament promoter [Byrne et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477], pancreas-specific promoters [Edlunch et al. (1985) *Science* 230:912-916] or mammary gland-specific promoters such as the milk whey promoter (U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). The nucleic acid construct of the present invention can further include an enhancer, which can be adjacent or distant to the promoter sequence and can function in up regulating the transcription therefrom.

The constructs of the present methodology preferably further include an appropriate selectable marker and/or an origin of replication. Preferably, the construct utilized is a shuttle vector, which can propagate both in *E. coli* (wherein the construct comprises an appropriate selectable marker and origin of replication) and be compatible for propagation in cells, or integration in a gene and a tissue of choice. The construct according to the present invention can be, for example, a plasmid, a bacmid, a phagemid, a cosmid, a phage, a virus or an artificial chromosome.

Currently preferred in vivo nucleic acid transfer techniques include transfection with viral or non-viral constructs, such as adenovirus, lentivirus, Herpes simplex I virus, or adeno-associated virus (AAV) and lipid-based systems. Useful lipids for lipid-mediated transfer of the gene are, for example, DOTMA, DOPE, and DC-Chol [Tonkinson et al., *Cancer Investigation*, 14(1): 54-65 (1996)]. The most

preferred constructs for use in gene therapy are viruses, most preferably adenoviruses, AAV, lentiviruses, or retroviruses. A viral construct such as a retroviral construct includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means such as alternate
5 splicing, nuclear RNA export, or post-translational modification of messenger. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and positive and negative strand primer binding sites appropriate to the virus used, unless it is already present in the viral construct. In addition, such a construct typically includes a signal sequence for secretion of the peptide or antibody
10 from a host cell in which it is placed. Preferably the signal sequence for this purpose is a mammalian signal sequence. Optionally, the construct may also include a signal that directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA
15 synthesis, and a 3' LTR or a portion thereof. Other vectors can be used that are non-viral, such as cationic lipids, polylysine, and dendrimers.

Because of the self-aggregating nature of the peptides of the present invention it is conceivable that such peptides can also be used as potent detectors of amyloid
fibrils/plaques in biological samples. This is of a special significance to amyloid-
20 associated diseases such as Alzheimer's disease wherein unequivocal diagnosis can only be made after postmortem examination of brain tissues for the hallmark neurofibrillary tangles (NFT) and neuritic plaques.

Thus, according to yet another aspect of the present invention there is provided a method of detecting a presence or an absence of an amyloid fibril in a
25 biological sample.

The method is effected by incubating the biological sample with a peptide of the present invention capable of co-aggregating with the amyloid fibril and detecting
the peptide, to thereby detect the presence or the absence of amyloid fibril in the biological sample. A variety of peptide reagents, which are capable of recognizing
30 conformational ensembles are known in the art some of which are reviewed in Bursavich (2002) J. Med. Chem. 45(3): 541-58 and in Baltzer Chem Rev. 101(10):3153-63.

The biological sample utilized for detection can be any body sample such as blood (serum or plasma), sputum, ascites fluids, pleural effusions, urine, biopsy specimens, isolated cells and/or cell membrane preparation. Methods of obtaining tissue biopsies and body fluids from mammals are well known in the art.

5 The peptide of the present invention is contacted with the biological sample under conditions suitable for aggregate formation (i.e., buffer, temperature, incubation time etc.); suitable conditions are described in Example 2 of the Examples section. Measures are taken not to allow pre-aggregation of peptides prior to incubation with the biological sample. To this end freshly prepared peptide stocks are preferably
10 used.

Protein complexes within a biological sample can be detected via any one of several methods known in the art, which methods can employ biochemical and/or optical detection schemes.

To facilitate complex detection, the peptides of the present invention are
15 highlighted preferably by a tag or an antibody. It will be appreciated that highlighting can be effected prior to, concomitant with or following aggregate formation, depending on the highlighting method. As used herein the term "tag" refers to a molecule, which exhibits a quantifiable activity or characteristic. A tag can be a fluorescent molecule including chemical fluorescers such as fluorescein or
20 polypeptide fluorescers such as the green fluorescent protein (GFP) or related proteins (www.clontech.com). In such case, the tag can be quantified via its fluorescence, which is generated upon the application of a suitable excitatory light. Alternatively, a tag can be an epitope tag, a fairly unique polypeptide sequence to which a specific antibody can bind without substantially cross reacting with other cellular epitopes.
25 Such epitope tags include a Myc tag, a Flag tag, a His tag, a leucine tag, an IgG tag, a streptavidin tag and the like.

Alternatively, aggregate detection can be effected by the antibodies of the present invention.

Thus, this aspect of the present invention provides a method of assaying or
30 screening biological samples, such as body tissue or fluid suspected of including an amyloid fibril.

It will be appreciated that such a detection method can also be utilized in an assay for uncovering potential drugs useful in prevention or disaggregation of amyloid deposits. For example, the present invention may be used for high throughput screening of test compounds. Typically, the co-aggregating peptides of the present invention are radiolabeled, to reduce assay volume. A competition assay is then effected by monitoring displacement of the label by a test compound [Han (1996) J. Am. Chem. Soc. 118:4506-7 and Esler (1996) Chem. 271:8545-8].

It will be appreciated that the peptides of the present invention may also be used as potent detectors of amyloid deposits in-vivo. A designed peptide capable of binding amyloid deposits, labeled non-radioactively or with a radio-isotope, as is well known in the art can be administered to an individual to diagnose the onset or presence of amyloid-related disease, discussed hereinabove. The binding of such a labeled peptide after administration to amyloid or amyloid-like deposits can be detected by in vivo imaging techniques known in the art.

The peptides of the present invention can be included in a diagnostic or therapeutic kit. For example, peptide sets of specific disease related proteins or antibodies directed thereagainst can be packaged in a one or more containers with appropriate buffers and preservatives and used for diagnosis or for directing therapeutic treatment.

Thus, the peptides can be each mixed in a single container or placed in individual containers. Preferably, the containers include a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic.

In addition, other additives such as stabilizers, buffers, blockers and the like may also be added.

The peptides of such kits can also be attached to a solid support, such as beads, array substrate (e.g., chips) and the like and used for diagnostic purposes.

Peptides included in kits or immobilized to substrates may be conjugated to a detectable label such as described hereinabove.

The kit can also include instructions for determining if the tested subject is suffering from, or is at risk of developing, a condition, disorder, or disease associated with amyloid polypeptide of interest.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization"

Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Alanine scan of the hIAPP basic amyloidogenic unit – rational and peptide synthesis

Pancreatic amyloid is found in more than 95 % of type II diabetes patients. Pancreatic amyloid is formed by the aggregation of a 37 amino acid long islet amyloid polypeptide (IAPP, GenBank Accession No. gi:4557655), the cytotoxicity thereof being directly associated with the development of the disease. IAPP amyloid formation follows a nucleation-dependent polymerization process, which proceeds through conformational transition of soluble IAPP into aggregated β -sheets. Recently it has been shown that a hexapeptide (22-27) (NFGAIL, SEQ ID NO: 111) of IAPP, also termed as the "basic amyloidogenic unit" is sufficient for the formation of β -sheet-containing amyloid fibrils [Konstantinos et al. (2000) J. Mol. Biol. 295:1055-1071].

To gain further insight into the specific role of the residues that compose "the basic amyloidogenic unit", a systematic alanine scan was performed. Amino-acids were replaced with alanine in order to specifically change the molecular interface of the peptides, without significantly changing their hydrophobicity or tendency to form β -sheet structures. alanine-scan was performed in the context of the block that is unique to human IAPP (Figure 3a). This block includes two serine residues that follow the NFGAIL motif in the full-length polypeptide. These eight amino-acid

peptide sequences were used since the shorter peptides are hydrophobic and as such less soluble. Figure 3b shows a schematic representation of the chemical structure of the wild-type peptide while Figure 3c indicates the amino-acid substitutions in the different mutant peptides that were generated.

5 **Methods and Reagents** - Peptide synthesis was performed by PeptidoGenic Research & Co. Inc (Livermore, CA USA). The sequence identity of the peptides was confirmed by ion spray mass-spectrometry using a Perkin Elmer Sciex API I spectrometer. The purity of the peptides was confirmed by reverse phase high-pressure liquid chromatography (RP-HPLC) on a C₁₈ column, using a linear gradient
10 of 10 to 70% acetonitrile in water and 0.1% trifluoroacetic acid (TFA).

EXAMPLE 2

Kinetics of aggregation of IAPP peptide fragment and mutant derivatives as monitored by turbidity measurements

15 To study self-assembly of the IAPP peptide derived fragments, aggregation and insolubilization kinetics were monitored using turbidity measurements at 405 nm.

Kinetic aggregation assay – Fresh peptide stock solutions were prepared by dissolving lyophilized form of the peptides in DMSO, a disaggregating solvent, at a
20 concentration of 100 mM. To avoid any pre-aggregation, fresh stock solutions were prepared prior to each and every experiment. Peptide stock solutions were diluted into assay buffer and plated in 96-well plates as follows: 2 µl of peptides stock solutions were added to 98 µl of 10 mM Tris pH 7.2, resulting in a 2 mM final concentration of the peptide in the presence of 2% DMSO. Turbidity data was
25 measured at 405 nm. A buffer solution including 2 % DMSO was used as a blank. Turbidity was measured at room temperature over several time points.

Results - As shown in Figure 4a, wild-type peptide fragment (SEQ ID NO: 1) showed an aggregation kinetic profile that was very similar to those previously reported for non-seeded hIAPP hexapeptide [Tenidis et al. (2000) J. Mol. Biol
30 295:1055-71]. Such a profile is strongly indicative of a nucleation-dependent polymerization mechanism [Jarrett and Lansbury (1992) Biochemistry 31:6865-70]. Following a lag-time of 20 minutes, wild type peptide self-assembled into insoluble

fibrils. Peptide G3A (SEQ ID NO: 4) showed essentially the same profile as that of wild type peptide. The N1A peptide (SEQ ID NO: 2) mediated higher kinetics of aggregation, albeit with different kinetic profile as compared to that of wild-type peptide. Interestingly, the aggregation of N1A seemed to be less nucleation-dependent. Substitution of the isoleucine or leucine to alanine (peptides I5A, SEQ ID NO: 5 and L6A, SEQ ID NO: 6 respectively) reduced the kinetics of aggregation but did not abolish it completely. Substitution of the phenylalanine residue to alanine (peptide F2A, SEQ ID NO:3) led to a total loss of peptide ability to aggregate. The F2A peptide was completely soluble in the aqueous assay buffer.

Altogether, kinetic aggregation studies of the amyloidogenic fragments suggested a major role to the phenylalanine residue in the process of amyloid formation by the IAPP active fragment.

EXAMPLE 3

Measurement of aggregate mean particle size

While the turbidity assay provided an important estimate regarding the aggregation potential and kinetics of the various peptides, it did not provide information about the size of the actual aggregates formed. It will be appreciated that although the apparent hydrodynamic diameter of amyloid structures varies due to irregularity of the amyloid structure, it may still provide a clear indication about the order of magnitude of the structure formed and present a quantitative criterion for comparing the structures formed by the various peptides.

Therefore, the average size of the aggregates, formed by the various peptides, was determined using dynamic light scattering (DLS) experiments.

Method - Freshly prepared peptide stock solutions at a concentration of 10 mM were diluted in 10 mM Tris buffer pH 7.2 and further filtrated through a 0.2 μ m filter to a final concentration of 100 μ M peptide and 1% DMSO. Particle size measurement was conducted with a laser-powered ALV-NIBS/HPPS non-invasive backscattering instrument. Autocorrelation data was fitted using the ALV-NIBS/HPPS software to derive average apparent hydrodynamic diameters.

Results - The average apparent hydrodynamic diameters of the structures that were formed by the various peptides are presented in Figure 5.

Altogether, the apparent hydrodynamic diameter of the structures formed by the various peptides seemed to be consistent with the results obtained by the turbidity assay. As with the turbidity assay, the wild-type peptide and G3A peptide formed particles of very similar hydrodynamic diameters. Smaller structures were observed with the derivative peptides: N1A, I5A and L6A. Thus, in accordance with the turbidity assay, the DLS experiments clearly illustrate that no large particles were formed by the F2A peptide under the indicated experimental conditions.

EXAMPLE 4

10 *Examination of amyloidogenic performance of wild type peptide and derivatives through Congo Red (CR) binding assay*

Congo red (CR) staining combined with polarization microscopy was utilized to test amyloidogenicity of the peptides of the present invention. Amyloid fibrils in general, and fibrillar IAPP in particular, bind CR and exhibit gold/green birefringence under polarized light [Cooper (1974) Lab. Invest. 31:232-8; Lansbury (1992) Biochemistry 31:6865-70].

Method and reagents – Peptide solutions incubated in a 10 mM Tris buffer (pH 7) for four days were dried on a glass microscope slide. Staining was effected by the addition of 1 mM CR in 10 mM Tris buffer pH 7.2 followed by a 1 minute incubation. To remove excess CR, slides were rinsed with double-distilled water and dried. Saturated CR solutions solubilized in 80% ethanol (v/v) were used for poorly aggregating peptides. In such cases, staining was effected without rinsing. Birefringence was determined using a WILD Makroskop m420 (x70) equipped with a polarizing stage.

25 **Results** - Wild type, N1A and G3A peptides bound CR and exhibited the characteristic green/gold birefringence (see Figures 6g, 6a and 6e for normal field and Figures 6h, 6b and 6f for polarized light microscopy, respectively). Peptides I5A and L6A, bound CR and exhibited rare but characteristic birefringence (Figures 6i and 6k for normal field and Figures 6j and 6l for polarized light, respectively). Peptide F2A (NAGAIL) showed no capability of binding CR (Figure 6c for normal field and Figure 6d for polarized light). Dried buffer solution stained with CR was used as a negative control (see Figures 6m and 6n for normal and polarized light, respectively).

Interestingly, no significant difference in binding was observed for the negative control and the F2A peptide.

To substantiate the inability of F2A peptide to form fibrils, a peptide solution incubated for 14 days was used in the binding assay. Although some degree of aggregation was visually observed following two weeks of peptide "aging", CR staining showed no amyloid structure (results not shown). Under the same conditions wild-type peptide incubation resulted in significant CR birefringence.

EXAMPLE 5

Ultrastructural analysis of the fibrillogenic peptide and mutants

The fibrillogenic potential of the various peptides was assessed by electron microscopy analysis.

Method – Peptide solutions (2 mM peptide in 10 mM Tris buffer pH 7.2), were incubated overnight at room temperature. Fibrils formation was assessed using 10 µl sample placed on 200-mesh copper grids, covered with carbon-stabilized formvar film (SPI Supplies, West Chester PA). Following 20-30 seconds of incubation, excess fluid was removed and the grids were negatively stained with 2% uranyl acetate in water. Samples were viewed in a JEOL 1200EX electron microscope operating at 80 kV.

Results – To further characterize the structures formed by the various peptides, negative staining electron microscopy analysis was effected. In accordance with previous results, filamentous structures were observed for all peptides (Figures 7a-f) but F2A which generated amorphous fibrils (Figure 7b). Frequency of appearance of fibrils formed by the I5A and L6A peptides (Figures 7e and 7f, respectively) was lower in comparison to that of wild type (Figure 7d), N1A, and G3A peptides (Figures 7a and 7c, respectively). Although the EM fields shown for peptides F2A, I5A and L6A, were rarely observed, the results presented by these images support the quantitative results presented in the previous sections and thus provide qualitative analysis of fibril morphology.

The tangled net-like structures that were observed for the wild-type, N1A, and G3A peptides could be explained by the fast kinetics of formation of these fibrils (see Example 2). More distinct structures and longer fibrils, albeit less frequent, were

observed with peptides I5A and L6A. These longer fibrils may be a result of a slower kinetics, which allow for a more ordered fibril organization.

Taken together, the qualitative results of the electron microscopy and CR analyses strongly suggest that the phenylalanine residue in the hexaamyloid peptide is crucial for its amyloidogenic potential.

EXAMPLE 6

Mapping recognition domains in the hIAPP basic amyloidogenic unit – rational and MBP-IAPP fusion protein synthesis

To systematically map and compare potential recognition domains, the ability of hIAPP (GenBank Accession No. gi:4557655) to interact with an array of 28 membrane-spotted overlapping peptides that span the entire sequence of hIAPP (i.e., hIAPP₁₋₁₀, hIAPP₂₋₁₁..., hIAPP₂₈₋₃₇) was addressed [Mazor (2002) J. Mol. Biol. 322:1013-24].

Materials and Experimental Procedures

Bacterial strains - E. coli strain TG-1 (Amersham Pharmacia, Sweden) was used for molecular cloning and plasmid propagation. The bacterial strain BL21(DE3) (Novagen, USA) was used for protein overexpression.

Engineering synthetic IAPP and MBP-IAPP fusion proteins – A synthetic DNA sequence of human IAPP modified to include a bacterial codon usage (SEQ ID NO: 58) was generated by annealing 8 overlapping primers (SEQ ID NOs. 50-57). PCR was effected through 30 cycles of 1 minute at 95 °C, one minute at 55 °C, and one minute at 72 °C. The annealing product was ligated and amplified using primers IAPP1 (SEQ ID NO: 50) and IAPP8 (SEQ ID NO: 57). An MBP-IAPP (MBP GenBank Accession No. gi:2654021) fusion sequence was then constructed using the IAPP synthetic template, which was amplified using primer YAR2 (SEQ ID NO: 60) and primer YAR1 (SEQ ID NO. 59), thereby introducing a V8 Ek cleavage site and a (His)₆ tag at the N-terminus of IAPP. The two primers included a *Not* I and an *Nco* I cloning sites, respectively. The resultant PCR product was digested with *Nco* I and *Not* I and ligated into the pMALc2x-NN expression vector. The pMALc2x-NN expression vector was constructed by cloning the polylinker site of pMALc-NN¹⁹ into pMALc2x (New England Biolabs, USA) [BACH (2001) J. Mol. Biol. 312:79-93].

Protein expression and purification – E. coli BL21 cells transformed with expression plasmid pMALc2x-IAPP encoding MBP-IAPP under the strong Ptac promoter were grown in 200 ml of LB medium supplemented with 100 µg/ml ampicillin and 1% (W/V) glucose. Once reaching an optical density of $A_{600} = 0.8$, protein expression was induced with 0.1 or 0.5 mM IPTG at 30°C for 3 hours (h).

Cell extracts were prepared in 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 200 mM NaCl and a protease inhibitors cocktail (Sigma) using a freeze-thaw followed by a brief sonication as previously described [Gazit (1999) J. Biol. Chem. 274:2652-2657]. Protein extracts were clarified by centrifugation at 20,000 g and stored at 4 °C. MBP-IAPP fusion protein was purified by passing the extract over an amylose resin column (New England Biolabs, USA) and recovered by elution with 20 mM maltose in the same buffer. Purified MBP-IAPP was stored at 4 °C. Protein concentration was determined using the Pierce Coomassie plus reagent (Pierce, USA) with BSA as a standard. MBP and MBP-IAPP protein fractions were analyzed on SDS/12 % polyacrylamide gels, which were stained with GelCode Blue (Pierce, USA).

To study whether the disulfide bond in the MBP-IAPP are oxidized, purified MBP and MBP-IAPP proteins were reacted with 5 equivalents of N-iodoacetyl-N'-(8-sulfo-1-naphthyl) ethylenediamine (IAEDANS) (Sigma, Rehovot, Israel) for overnight at room temperature in the dark. Free dye was separated from labeled protein by gel filtration chromatography on a QuickSpin G-25 Sephadex column. MBP and MBP-IAPP fluorescence was then determined. Only small fluorescence labeling was detected (on average less than 0.1 probe molecules per protein molecules) and there was no significant difference between the labeling of MBP and MBP-IAPP, which suggested that the disulfide bridge in the expressed IAPP molecules was predominantly oxidized.

Results

Expression and purification of recombinant MBP-IAPP - Since previous attempts to express the intact hIAPP in bacteria were unsuccessful, the protein was expressed as an MBP fusion, which protected hIAPP from undesirable aggregation during expression [Bach (2001) J. Mol. Biol. 312:79-93]. Synthesis of the fusion protein was effected using a bacterial codon usage as shown in Figure 8a. The resulting fusion sequence was cloned into pMALc2x-NN as shown in Figure 8b and

introduced into E. coli BL21(DE3). Growth conditions, cell extract preparation and protein purification were effected as described hereinabove. IPTG induction resulted in the accumulation of high levels of MBP-IAPP in the soluble fraction with less than 5% of the MBP-IAPP fusion protein was found in the insoluble fraction of the cell extract (data not shown). Aliquots from typical purification steps of MBP and MBP-IAPP are shown in Figure 9. As shown, the 48 kDa MBP-IAPP accumulated to 25% of the total soluble protein as calculated by densitometric scanning of GelCode Blue-stained SDS/Polyacrylamide gels. When induced at 30°C in a shake flask ($A_{600} = 2.0$), MBP-IAPP accumulated as soluble protein in the cytoplasm at a level of about 150 mg/l of cell culture. Despite losses during purification, MBP-IAPP was purified to near-homogeneity at a yield of 80 mg/l of cells. For future application and convenient homogeneity purification of IAPP, in addition to the factor Xa cleavage site for removal of the MBP tag, an additional His-Tag was also included (Figure 8b). The His-Tag could be removed by Ek V8 cleavage at the N-terminal Lys residue of the IAPP sequence, resulting in the release of wild type IAPP.

EXAMPLE 7

Identification of molecular recognition sequences in the hIAPP polypeptide

IAPP peptide array construction - Decamers corresponding to consecutive overlapping sequences of hIAPP₁₋₃₇ SEQ ID NOs. 61-88) were synthesized on a cellulose membrane matrix using the SPOT technique (Jerini AG, Berlin, Germany). The peptides were covalently bound to a Whatman 50 cellulose support (Whatman, Maidstone, England) via the C-terminal amino-acids. N-terminal acetylation was used for peptide scanning because of higher stability to peptide degradation, and better representation of the native recognition motif.

Peptides Synthesis - Peptide synthesis was effected using solid-phase synthesis methods performed by Peptron, Inc. (Taejeon, Korea). Correct identity of the peptides was confirmed by ion spray mass-spectrometry using a HP 1100 series LC/MSD [Hewlett-Packard Company, Palo Alto, CA]. The purity of the peptides was confirmed by reverse phase high-pressure liquid chromatography (RP-HPLC) on a C₁₈ column, using a 30 minute linear gradient of 0 to 100% acetonitrile in water and 0.1% trifluoroacetic acid (TFA) at flow rate of 1 ml/min.

Binding studies – The cellulose peptide array was initially blocked with 5 % (V/V) non fat milk in Tris buffered saline (TBS, 20 mM Tris pH 7.5 , 150 mM NaCl). Thereafter, cellulose membrane was incubated in the presence of 10 µg/ml MBP-IAPP₁₋₃₇ at 4°C for 12 h in the same blocking buffer. The cellulose membrane was then washed repeatedly with 0.05 % Tween 20 in TBS. MBP-IAPP₁₋₃₇ bound to the cellulose membrane was detected with an anti MBP monoclonal antibody (Sigma, Israel). HRP-conjugated goat anti mouse antibodies (Jackson Laboratories, USA) were used as a secondary antibody. Immunoblots were developed using the Renaissance western blot Chemiluminescence Reagent (NEN, USA) according to Manufacturer's instructions and signal was quantified using densitometry. Regeneration of the cellulose membrane for reuse was carried out by sequential washing with Regeneration buffer I including 62.5 mM Tris, 2% SDS, 100 mM 2-mercaptoethanol, pH 6.7, and Regeneration buffer II including 8 M urea, 1% SDS, 0.1% 2-mercaptoethanol. Efficiency of the washing steps was monitored by contacting the membrane with the chemiluminescence reagent, as described.

Results

Identification of binding sequences in the IAPP polypeptide - To identify structural motifs in the IAPP molecule that mediates the intermolecular recognition between hIAPP molecules, 28 possible overlapping decamers corresponding to amino acids 1-10 up to 28-37 of the hIAPP₁₋₃₇ molecule were synthesized on a cellulose membrane matrix. Cellulose membrane-bound peptides were incubated with MBP-hIAPP₁₋₃₇ overnight. Following washing of the cellulose membrane in a high-salt buffer, immunoblots on the cellulose membrane were analyzed and binding was quantified by densitometry (Figure 10b). It will be appreciated that the measured binding is semiquantitative, since peptide coupling efficiency during synthesis can vary.

As shown in Figures 10a-b, a number of peptide segments exhibited binding to MBP-IAPP; An amino acid sequence localized to the center of the IAPP polypeptide (i.e., hIAPP₇₋₁₆ to hIAPP₁₂₋₂₁) displayed the most prominent binding to MBP-hIAPP₁₋₃₇. Another binding region was identified at the C-terminal part of IAPP (hIAPP₁₉₋₂₈ to hIAPP₂₁₋₃₀), although binding in this case was considerably less prominent; A third binding spot was located to the N-terminal part of IAPP (hIAPP₂₋₁₁), however, no

typical distribution around a central motif was evident in this case, suggesting that this result may be false. Even after overexposure of the blot (data not shown), no binding near this peptide (to either hIAPP₁₋₁₀ or hIAPP₃₋₁₂) was detected. Furthermore given the close proximity of the 2-11 region to the disulfide bridge may not allow the process of fibrillization under physiological conditions.

To rule out involvement of MBP itself in binding the arrayed peptides, the peptide coupled cellulose membrane was incubated with MBP alone and analyzed by immunoblotting. No binding was identified after development of the membrane (not shown).

These results identified in addition to the previously defined binding motif of hIAPP [i.e., basic amyloidogenic unit, hIAPP₂₀₋₂₉, Westermark (1990) Proc. Natl. Acad. Sci. 13:5036-40; Tenidis (2000) J. Mol. Biol. 295:1055-1071; Azriel and Gazit (2001) J. Biol. Chem. 276:34156-34161], a major central domain of molecular recognition within hIAPP. The profile of the binding distribution of the peptide array (Figure 10b), suggests that NFVLH (SEQ ID No. 17) may serve as the core recognition motif.

EXAMPLE 8

Characterization of aggregation kinetics of hIAPP peptide fragments as monitored by turbidity measurements

Binding analysis of the recombinant MBP-hIAPP fusion protein to the hIAPP peptide array (Example 7), identified a putative self-assembly domain within the central part of the hIAPP protein.

In order to identify the minimal structural motif that is capable of forming amyloid fibrils, a series of peptides encompassed within the putative self-assembly domain were tested for aggregation as monitored using turbidity measurements at 405 nm.

Table 3 below, illustrates the examined peptides.

Table 3

| <i>HLAPP peptide fragments (hIAPP coordinates)</i> | <i>SEQ ID NO:</i> | <i>Peptide sequence</i> |
|---|--------------------------|--------------------------------|
| 14-22 | 14 | NFLVHSSNN |
| 14-20 | 15 | NFLVHSS |
| 15-20 | 16 | FLVHSS |

| | | |
|-------|----|-------|
| 14-18 | 17 | NFLVH |
| 15-19 | 18 | FLVHS |
| 15-18 | 19 | FLVH |

Materials and Experimental Procedures

Kinetic Aggregation Assay – freshly prepared peptide stock solutions were generated by dissolving the lyophilized form of the peptides in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml. To avoid any pre-aggregation, fresh stock solutions were prepared for each experiment. Peptide stock solutions were diluted into the assay buffer in enzyme-linked immunosorbent assay (ELISA) plate wells as follows: 8 μ L of peptide stock solutions were added to 92 μ L of 10 mM Tris, pH 7.2 (hence the final concentration of the peptide was 8 mg/ml in the presence of 8% DMSO). Turbidity data were collected at 405 nm. Buffer solution containing the same amount of DMSO as the tested samples was used as blank, which was subtracted from the results. Turbidity was measured continuously at room temperature using THERMOMax ELISA plate reader (Molecular Devices, Sunnyvale CA).

Results

Turbidity assay was performed in-order to determine the ability of the various peptides (Table 3) to aggregate in an aqueous medium. Fresh stock solutions of the different peptide fragments were made in DMSO, and then diluted into a Tris buffer solution and turbidity, as a hallmark of protein aggregation, was monitored for two hours. As shown in Figure 11, the peptides NFLVHSS, FLVHSS and FLVHS exhibited high turbidity. It will be appreciated that the lag-time, as was previously reported for amyloid formation by the NFGAIL short peptide [Tenidis (2000) Supra], is very short or lacking at all and thus could not be detected under these experimental conditions, however the aggregation kinetic profiles were similar to those obtained for the hexapeptide hIAPP₂₂₋₂₇ (NFGAIL). On the other hand, the peptide NFLVHSSNN exhibited very low turbidity, while NFLVH and FLVH have shown almost no turbidity at all. Even after significantly longer incubation no significant turbidity was observed with the latter two peptides. The lack of amyloid fibrils formation may be due to electrostatic repulsion of the partially charged histidine residues.

EXAMPLE 9***Examination of hIAPP peptide amyloidogenic through Congo Red (CR) binding assay***

Congo red (CR) staining combined with polarization microscopy was utilized
5 to test amyloidogenicity of the peptides of the present invention. Amyloid fibrils bind CR and exhibit gold/green birefringence under polarized light [Puchtler (1966) J. Histochem. Cytochem. 10:355-364].

Materials and Experimental Procedures

Congo Red Staining and Birefringence – A 10 μ L suspension of 8 mg/ml
10 peptide solution in 10 mM Tris buffer, pH 7.2 aged for at least one day was allowed to dry overnight on a glass microscope slide. Staining was performed by the addition of a 10 μ L suspension of saturated Congo Red (CR) and NaCl in 80% ethanol (v/v) solution as previously described [Puchtler (1966) Supra]. The solution was filtered via 0.45 μ m filter. The slide was then dried for few hours. Birefringence was
15 determined with a SZX-12 Stereoscope (Olympus, Hamburg, Germany) equipped with cross polarizers.

Results

Congo Red Staining and Birefringence- In order to determine any possible amyloid nature of the aggregates formed at the turbidity assay (see Example 8), a
20 CR birefringence assay was performed. Peptide fragments were tested for amyloidogenicity by staining with CR and examination under a light microscope equipped with cross-polarizers. Consistent with the kinetic assay results, and as shown in Figures 120b-c and 12e, the peptides NFLVHSS, FLVHSS and FLVHS exhibited a typical birefringence. On the other hand, peptides NFLVHSSNN, NFLVH
25 and FLVH exhibited very weak birefringence or no birefringence at all (Figures 12a, 12d and 12f). Peptide NFLVHSSNN exhibited a weaker characteristic birefringence (Figure 12a). The peptide NFLVH exhibited a powerful smear of birefringence at the edges of the sample (Figure 12d). The peptide FLVH exhibited no birefringence (Figure 12f). In order to test whether the FLVH peptide did not form amyloid fibrils
30 due to a long lag-time, a sample of five days aged peptide solution was examined. The same peptide was also tested in aqueous solution and at very high concentrations (10 mg/ml), however no Birefringence was detected in all cases indicating the peptide

did not form amyloid (data not shown).

EXAMPLE 10

Ultrastructural analysis of the fibrillogenic hIAPP peptides

5 The fibrillogenic potential of the various peptides was assessed by electron microscopy analysis.

Materials and Experimental Procedures

10 **Transmission Electron Microscopy** – A 10 μ L sample of 8 mg/ml peptide solution in 10 mM Tris buffer, pH 7.2 aged for at least one day was placed on 400-mesh copper grids (SPI supplies, West Chester PA) covered by carbon-stabilized Formvar film. Following 1 minute, excess fluid was removed, and the grid was then negatively stained with 2% uranyl acetate in water for another two minutes. Samples were viewed in a JEOL 1200EX electron microscope operating at 80 kV.

Results

15 To further characterize the structures formed by the various peptides, negative staining electron microscopy analysis was effected. In accordance with previous results, all peptide fragments exhibited fibrillar structures except the FLVH peptide in which only amorphous aggregates were found (Figures 13a-f). NFLVHSSNN peptide exhibited long thin coiling filaments similar to those formed by the full-length peptide as described above (Figure 13a). Peptides NFLVHSS, FLVHSS, FLVHS exhibited large broad ribbon-like fibrils as described for the NFGAIL fragment [Tenidis (2000) Supra., Figures 13c-e, respectively]. The fibrils formed by NFLVH peptide were thin and short and could be considered as protofilaments rather than filaments. Their appearance was at much lower frequency, and the EM picture does not represent the general fields but rather rare events (Figure 13d). As shown in Figure 13f, the FLVH peptide mediated the formation of amorphous aggregates.

20
25

EXAMPLE 11

Secondary structure analysis of hIAPP peptide fragments

30 Fourier transform infrared spectroscopy (FT-IR) was effected to determine the secondary structure of the hIAPP amyloidogenic peptide fibrils and the non-fibrillar peptides.

Materials and Experimental Procedures

Fourier Transform Infrared Spectroscopy - Infrared spectra were recorded using a Nicolet Nexus 470 FT-IR spectrometer with a DTGS detector. Samples of aged peptide solutions, taken from turbidity assay, were suspended on a CaF₂ windows (Sigma)-plate and dried by vacuum. The peptide deposits were resuspended with double-distilled water and subsequently dried to form thin films. The resuspension procedure was repeated twice to ensure maximal hydrogen to deuterium exchange. The measurements were taken using a 4 cm⁻¹ resolution and 2000 scans averaging. The transmittance minima values were determined by the OMNIC analysis program (Nicolet).

Results

FT-IR studies – As shown in Figure 14a-f, all the fibrillar peptides exhibited FT-IR spectra with a well-defined minimum bands typical for β -sheet structure around 1620-1640 cm⁻¹. On the other hand the spectrum of the tetrapeptide FLVH that has no appearance for fibrils according to the other methods, is typical for a random coil structure. The NFLVHSSNN peptide spectrum exhibited a transmittance minimum at 1621 cm⁻¹ indicating a large β -sheet content, as well as minima at 1640 cm⁻¹ and 1665 suggesting presence of non- β structures. Another minor minimum was observed at 1688 cm⁻¹ indicative for anti-parallel β -sheet (Figure 14a). The NFLVHSS peptide spectrum exhibited major minimum band at 1929 cm⁻¹ 1675 cm⁻¹. this spectrum is classical for an anti-parallel β -sheet structure (Figure 14b). A similar spectrum was observed for the peptide FLVHS with a major minimum at 1625 cm⁻¹ and a minor minimum at 1676 cm⁻¹ (Figure 14e). The spectrum of FLVHSS peptide showed also a major minimum at 1626 cm⁻¹. The spectrum had also some minor minima around 1637-1676 cm⁻¹ but those were shaped more like noise than signal (Figure 14c). The spectrum of NFLVH peptide showed a minimum at 1636 cm⁻¹ which was also indicative of β -sheet, however, in comparison with the other spectra, this band was shifted which could indicate presence of non- β structures, as well as observed minima at 1654 cm⁻¹ and 1669 cm⁻¹ (Figure 14d). By contrast, the FLVH peptide spectrum exhibited no minimum at 1620-1640 cm⁻¹, but showed multiple minima around 1646-1675 cm⁻¹ typical to random coil structure (Figure 14f).

To study whether the FLVH tetrapeptide could not form amyloid fibrils at all

or the undetectable fibrils formation was a result of a slow kinetics, a solution of the peptide at the same experimental conditions was incubated for two months and the existence of fibrils was tested. However, no evidence for amyloid fibril formation was detected using EM microscopy, CR staining, or FT-IR spectroscopy. These results may suggest that tetrapeptides are incapable of forming fibrils due to energetic consideration. That is, the energetic contribution of the stacking of a strand composed of three peptide bonds is lower than the entropic cost of oligomerization.

Taken together, the ultrastructural observations are consistent with the findings as determined by the turbidity and Congo red birefringence assays. All together the experimental data identified a novel pentapeptide element within the hIAPP peptide, the FLVHS peptides, which has strong amyloid forming capability. Interestingly, an NFLVH peptide found in the same central domain of the hIAPP polypeptide was found to be amyloidogenic however, the ability thereof to form fibrils was somehow inferior.

EXAMPLE 12

Identification of the minimal amyloidogenic peptide fragment of Medin

Background

Medin (GenBank Accession No. gi:5174557) is the main constitute of aortic medial amyloid deposits [Häggqvist (1999) Proc. Natl. Acad. Sci. USA. 96:8674-8669]. Previous studies found aortic medial amyloid in 97% of the subjects above the age of 50 [Mucchiano (1992) Am. J. Pathol. 140:811-877]. However, the pathological role of those amyloid deposits is still unknown. It was suggested that these amyloid play a role in the diminished elasticity of aortic vessels that is related to old age [Mucchiano (1992) Supra; Häggqvist (1999) Supra]. While the study clearly identified a tryptic peptide NFGSVQFV as the medin amyloidogenic peptide, the minimal sequence of the peptide that is still amyloidogenic and the molecular determinants that mediate the amyloid formation process were not determined. Such information is critical for true understanding of the fibrillization process in the specific case of Medin but also as a paradigm for the process of amyloid fibrils formation in general.

The minimal active fragment of Medin was determined using functional and structural analyses of truncated analogues derived from the published octapeptide [Häggqvist (1999) Supra].

Materials and Experimental procedures

5 Peptide synthesis is described in Example 7.

Table 4 below illustrates the studied peptides.

Table 4

| <i>Peptide sequence</i> | <i>SEQ ID NO:</i> |
|--------------------------------|--------------------------|
| NH ₂ -NFGSVQVF-COOH | 20 |
| NH ₂ -NFGSVQ -COOH | 21 |
| NH ₂ -NFGSV -COOH | 22 |
| NH ₂ - FGSVQ -COOH | 23 |
| NH ₂ - GSVQ -COOH | 24 |
| NH ₂ - FGSV -COOH | 25 |
| NH ₂ -NAGSVQ -COOH | 26 |

Results

10 In order to get further insights into the structural elements of Medin that retain the molecular information needed to mediate a process of molecular recognition and self-assembly, the ability of short peptide fragments and analogues of Medin to form amyloid fibrils in vitro was studied. Figure 15a shows a schematic representation of the chemical structure of the largest peptide fragment studied.

15

EXAMPLE 13

Kinetics of aggregation of Medin-derived peptide fragments

Turbidity assay was effected as described in Example 8.

20 In order to get first insights regarding the aggregation potential of the various Medin derived peptides, turbidity assay was performed. Freshly made stocks of the amyloidogenic octapeptide and truncated analogues thereof were prepared in DMSO. The peptides were than diluted to aqueous solution and the turbidity was monitored by following the absorbance at 405 nm as a function of time. As shown in Figure 16a, the NFGSV pentapeptide exhibited the highest degree of aggregation within
 25 minutes of incubation. Physical examination of the solution indicated that the peptide formed a gel structure. The kinetics of aggregation of the NFGSVQV octapeptide was too fast to be measured since turbidity was already observed immediately with the dilution into aqueous solution (Figures 16a-b). Similar fast

kinetics were also observed with the GSVQ tetrapeptide. The truncated NFGSVQ, FGSVQ, and FGSV peptides showed a gradual increase in turbidity over ~30 minutes (Figure 16b) which was followed by a slight decrease, which could be explained by sedimentation of large aggregates. Altogether, such kinetics and turbidity values were similar to those previously observed with amyloidogenic peptides of similar size (Azriel and Gazit, 2001).

EXAMPLE 14

Ultrastructural analysis of Medin-derived peptide fragments

Electron microscopy analysis was effected as described in Example 10.

The fibrillization potential of Medin-derived peptide fragments was effected by electron microscopy (EM) using negative staining. Stock solutions of the peptide fragments were suspended and aged for 4 days. Fibrillar structures were clearly seen in solutions that contained both the NFGSVQFA octapeptide (Figure 17a) and the truncated NFGSVQ (Figure 17b). In both cases the structures were similar to those observed with much longer polypeptides, such the IAPP and the β -amyloid ($A\beta$) polypeptides. The shorter gel-forming NFGSV pentapeptide did not form a typical amyloid structure but a network of fibrous structures (Figure 17c). It should be noted that fibrous networks were recently observed upon the gelation of the glutathione peptide [Lyon and Atkins, (2001) J. Am. Chem. Soc. 123:4408-4413]. No typical fibrils could be detected in solutions that contained the FGSVQ pentapeptide, the GSVQ tetrapeptide, or the FGSV tetrapeptide in spite of extensive search. While in the case of the FGSVQ peptide (Figure 17d) somewhat fibrillar and ordered structure could be seen, although significantly different than those formed by typical amyloidogenic peptide), in the case of the GSVQ and the FGSV peptides, no fibrillar structures could be found (Figures 17e and 17f, respectively).

EXAMPLE 15

Examination of amyloidogenic performance of Medin-derived peptides through Congo Red (CR) binding assay

CR staining was effected as described in Example 9.

A CR staining was effected to determine whether the structures formed by the various Medin-derived peptides show a typical birefringence. As shown in Figure 18b, the NFGSVQ hexapeptide bound CR and exhibited a characteristic bright and strong green-gold birefringence. The NFGSVQFV octapeptide also exhibited significant birefringence (Figure 18a), although less typical than that observed with the hexapeptide. The gel-forming NFGSV peptide deposits exhibited very low degree of birefringence (Figure 18c). The FGSVQ and FGSV peptide showed no birefringence upon staining with CR (Figures 18d and 18f, respectively). There was clearly no significant difference between those two peptides and a negative control (i.e., buffer solution with no peptide) Interestingly, unexpected high level of birefringence was observed with the GSVQ tetrapeptide (Figure 18e), while the morphology of the structures formed therefrom (Figure 18e) was clearly different from that of amyloid fibrils, indicating that these structures may have a significant degree of order that is reflected in strong birefringence.

EXAMPLE 16

The effect of phenylalanine substitution on the self-assembly of Medin

To elucidate a possible role for the phenylalanine residue in the process of amyloid fibrils formation by the minimal amyloid-forming hexapeptide, the phenylalanine amino acids was replaced with an alanine. The alanine-substituted peptide was prepared and examined in the same way as described for the various fragments of Medin. As shown in Figure 19a, a significantly lower turbidity was observed with the alanine-substituted peptide as compared to the wild-type hexapeptide. When aged solution of the NAGSVQ peptide was visualized by EM, no clear fibrillar structures could be detected (Figure 19b). This is in complete contrast to the high abundance fibrillar structures seen with the wild-type peptide (Figure 17b). Furthermore, the structures that were visualized did not show any degree of order as observed with the NFGSV and FGSVQ peptides as described above, Figures 17c-d, but were very similar to the completely non-fibrillar structures as were observed with the FGSV tetrapeptide (Figure 17e). Interestingly, some degree of birefringence could still be detected (Figure 19c) with the alanine-substituted peptide (as was observed with the GSVQ peptide, Figure 18e). These results raise further doubts regarding the

use of CR staining as a sole indicator of amyloid formation [Khurana (2001) J. Biol. Chem. 276:22715-22721].

Altogether these results show that the truncated fragment of Medin which is capable of forming amyloid fibrils is the hexapeptide NFGSVQ (SEQ ID NO: 21),
5 although a shorter pentapeptide fragment, NFGSV (SEQ ID NO: 22), exhibited a network of fibrous structures which were not typical of amyloids. The amyloid forming NFGSVQ hexapeptide is noticeably similar to the minimal amyloidogenic fragment of the islet amyloid polypeptide (IAPP, see Examples 1-5). Taken together, the results are consistent with the assumed role of stacking interactions in the self-
10 assembly processes that lead to the formation of amyloid fibrils and the suggested correlation between amyloid fibrils and β -helix structures.

EXAMPLE 17

Identification of the minimal amyloidogenic peptide fragment of human

Calcitonin

Human Calcitonin (hCT, GenBank Accession No. gi:179880) is a 32 amino acid long polypeptide hormone that is being produced by the C-cells of the thyroid and is involve in calcium homeostasis [Austin and Health (1981) N. Engl. J. Med. 304:269-278; Copp (1970) Annu. Rev. Physiol. 32:61-86; Zaidi (2002) Bone 30:655-
20 663]. Amyloid fibrils composed of hCT were found to be associated with medullary carcinoma of the thyroid [Kedar (1976) Isr. J. Sci. 12:1137; Berger (1988) Arch. A. Pathol. Anat. Histopathol. 412:543-551; Arvinte (1993) J. Biol. Chem. 268:6415-6422]. Interestingly, synthetic hCT was found to form amyloid fibrils *in vitro* with similar morphology to the deposits found in the thyroid [Kedar (1976) Supra; Berger
25 (1988) Supra; Arvinte (1993) Supra; Benvenga (1994) J. Endocrinol. Invest. 17:119-122; Bauer (1994) Biochemistry 33:12276-12282; Kanaori (1995) Biochemistry 34:12138-43; Kamihara (2000) Protein Sci. 9:867-877]. The *in vitro* process of amyloid formation is affected by the pH of the medium [23]. Electron microscopy experiments have revealed that the fibrils formed by hCT are approximately 80Å in
30 diameter and up to several micrometers in length. The fibrils are often associated with one another and in vitro amyloid formation is affected by the pH of the medium [Kamihara (2000) Supra.].

Calcitonin has been used as a drug for various diseases including Paget's disease and osteoporosis. However, the tendency of hCT to associate and form amyloid fibrils in aqueous solutions at physiological pH is a significant limit for its efficient use as a drug [Austin (1981) Supra; Copp (1970) Supra; Zaidi (2002) Supra].

5 Salmon CT [Zaidi (2002) Supra], the clinically used alternative to hCT, causes immunogenic reaction in treated patients due to low sequence homology. Therefore, understanding the mechanism of amyloid formation by hCT and controlling this process is highly important not only in the context of amyloid formation mechanism but also as a step toward improved therapeutic use of Calcitonin.

Circular dichroism (CD) studies have shown that in water monomeric hCT has little ordered secondary structure at room temperature [Arvinde (1993) Supra]. However, studies of hCT fibrils using circular dichroism, fluorescence, and infrared spectroscopy revealed that fibrillated hCT molecules have both α -helical and β -sheet secondary structure components [Bauer (1994) Supra]. NMR spectroscopy studies have shown that in various structure promoting solvents like TFE/H₂O, hCT adopts an amphiphilic α -helical conformation, predominantly in the residue range 8-22 [Meadows (1991) Biochemistry 30:1247-1254; Motta (1991) Biochemistry 30:10444-10450]. In DMSO/H₂O, a short double-stranded antiparallel β -sheet form in the central region made by residues 16-21 [Motta (1991) Biochemistry 30:2364-71].

Based on this structural data and the proposed role of aromatic residues in the process of amyloid formation, the present inventor has identified a short peptide fragment, which is sufficient for mediating Calcitonin self-assembly [Reches (2002) J. Biol. Chem. 277:35475-80].

10 ***The studied peptides*** – Based on the previously reported susceptibility of amyloid formation to acidic pH [Kanaori (1995) Supra], it was suggested that negatively-charged amino-acids, which undergo protonation at low pH, may play a key role in the process of amyloid formation. The only negatively-charged amino-acid in hCT is Asp¹⁵ (Figure 20a). Furthermore, a critical role for residues Lys¹⁸ and Phe¹⁹

15 in the oligomerization state and bioactivity of hCT was recently shown [Kazantzis (269) Eur. J. Biochem. 269:780-91]. Together with the occurrence of two phenylalanine residues in the region focused the structural analysis of the amyloidogenic determinants in hCT to amino acids 15-19. Figure 20b shows a

schematic representation of the chemical structure of the longest peptide and Table 5 below, indicates the various peptide fragments that were used in the study.

Table 5

| <i>Amino acid coordinates on hCT</i> | <i>Peptide sequence</i> | <i>SEQ ID NO:</i> |
|--------------------------------------|-----------------------------|-------------------|
| 15-19 | NH ₂ -DFNKF-COOH | 27 |
| 16-19 | NH ₂ -FNKF-COOH | 28 |
| 15-18 | NH ₂ -DFNK -COOH | 29 |
| 15-17 | NH ₂ -DFN -COOH | 30 |
| F>A 15-19 | NH ₂ -DANKF-COOH | 31 |

5

EXAMPLE 18

Ultrastructural analysis of Calcitonin-derived peptide fragments

Electron microscopy analysis was effected as described in Example 10.

The fibrillization potential of Calcitonin-derived peptide fragments was effected by electron microscopy (EM) using negative staining. Stock solutions of the peptide fragments were suspended in 0.02M NaCl, 0.01M Tris pH 7.2, aged for 2 days and negatively stained. Fibrillar structures, similar to those formed by the full-length polypeptide [Arvinte (1993) Supra; Benvenaga (1994) Supra; Bauer (1994) Supra; Kanaori (1995) Supra; Kamihara (2000) Supra], were clearly seen with high frequency in solutions that contained the DFNKF pentapeptide (Figure 21a). The shorter DFNK tetrapeptide also formed fibrillar structures (Figure 21b). However, the structures formed were less ordered as compared to those formed by the DFNKF pentapeptide. The amount of fibrillar structures formed by DFNK was also lower as compared to the DFNKF pentapeptide. No clear fibrils could be detected using solutions that contained the FNKF tetrapeptide and the DFN tripeptide, in spite of extensive search. In the case of the FNKF tetrapeptide only amorphous aggregates could be found (Figure 21c). The DFN tripeptide formed more ordered structures (Figure 21d) that resembled the structure formed by gel-forming tripeptide [Lyon (2001) Supra]. To study whether the FNKF tetrapeptide and the DFN tripeptide peptide cannot form fibrils whatsoever or the observation is a result of slow kinetics, a solution of the peptides at the same experimental conditions was incubated for two weeks. Also in this case no clear fibrillar structures could be detected (data not shown).

EXAMPLE 19***Examination of amyloidogenic performance of Calcitonin-derived peptides through Congo Red (CR) binding assay***

CR staining was effected as described in Example 9.

5 A CR staining was effected to determine whether the structures formed by the various hCT-derived peptides show a typical birefringence. As shown in Figures 22a-d, all the studies peptides showed some degree of birefringence. However, the green birefringence, which was observed with the DFNKF-pentapeptide was clear and strong (Figure 22a). The level of birefringence that was observed with the other
10 peptides was lower but significant since no birefringence could be detected using control solutions which did not contain the peptides. The lower level of birefringence of the DFNK tetrapeptide (Figure 22b) was consistent with the lower extent of fibrillization as observed using EM (Figure 21b). It will be appreciated, though, that the birefringence observed with the FNKF tetrapeptide and the DFN
15 tripeptide might represent some degree of ordered structures [Lyon (2001) Supra].

EXAMPLE 20***Secondary structure of the aggregated hCT-derived peptides***

FT-IR spectroscopy was effected as described in Example 11.

20 Amyloid deposits are characteristic of fibrils rich with β -pleated sheet structures. To get a quantitative information regarding the secondary structures that were formed by the various peptide fragments FT-IR spectroscopy was used. Aged peptide solutions were dried on CaF_2 plates forming thin films as described in Example 11. As shown in Figure 23, the DFNKF pentapeptide exhibited a double
25 minima (at 1639 cm^{-1} and 1669 cm^{-1}) an amide I FT-IR spectrum that is consistent with anti-parallel β -sheet structure and is remarkably similar to the spectrum of the amyloid-forming hexapeptide fragment of the islet amyloid polypeptide [Tenidis (2000) Supra]. The amide I spectrum observed with the DFNK tetrapeptide (Figure 23) was less typical of a β -sheet structure. While it exhibited a minimum at 1666 cm^{-1} that may reflect an anti-parallel β -sheet it lacked the typical minimum around
30 $1620\text{-}1640\text{ cm}^{-1}$ that is typically observed with β -sheet structures. The FNKF tetrapeptide exhibited a FT-IR spectrum that is typical of a non-ordered structure

(Figure 23) and is similar to spectra of the short non-amyloidogenic fragments of the islet amyloid polypeptide [Tenidis (2000) Supra]. The DFN tripeptide exhibited a double minima (at 1642 cm^{-1} and 1673 cm^{-1} , Figure 23) amide I FT-IR spectrum that is consistent with a mixture of β -sheet and random structures. This may further indicate that the structures observed by EM visualization may represent some degree of ordered structure composed of predominantly β -sheet structural elements.

EXAMPLE 21

The effect of phenylalanine substitution on the self-assembly of Calcitonin-derived peptides

In order to get insight into a possible role for the phenylalanine residues in the process of Calcitonin self-assembly, the phenylalanine amino acids were replaced with alanine in the context of the pentapeptide (SEQ ID NO: 31). When aged solution of the DANKF pentapeptide was visualized by EM, no clear fibrillar structures could be detected (Figure 24a). Structures that were visualized exhibited some degree of order (as compared to the amorphous aggregates seen with the FNKF tetrapeptide), however, no green-gold birefringence could be observed (Figure 24b). The FT-IR spectrum of the DANKA pentapeptide was similar to that of the FNKF tetrapeptide and other short non-amyloidogenic peptide, typical of non-ordered structures [Tenidis (2000) Supra]. Taken together, the effect of the phenylalanine to alanine substitution is very similar to the effect of such a change in the context of a short amyloid-forming fragment of the islet amyloid polypeptide [Azriel (2001) Supra].

Altogether, the ability of an hCT-derived pentapeptide (SEQ ID NO: 27) to form well-ordered amyloid fibrils was demonstrated. The typical fibrillar structure as seen by electron microscopy visualization (Figure 21a), the very strong green birefringence upon staining with CR (Figure 22a), and the typical anti-parallel β -sheet structure (Figure 23a), all indicate that the DFNKF pentapeptide is a very potent amyloid forming agent. Other pentapeptides capable of self-assembling were shown in hereinabove. Yet, in terms of the degree of birefringence and electron microscopy morphology, the hCT fragment seems to be the pentapeptide with the highest amyloidogenic potential similar to the potent amyloidogenic fragment of the β -amyloid ($A\beta$) polypeptide, KLVFFAE [Balbach (2000) Biochemistry 39:13748-59].

It is possible that electrostatic interactions between the opposing charges on the lysine and aspartic acids direct the formation of ordered antiparallel structure. Interestingly, the DFNK polypeptide exhibited a significantly lower amyloidogenic potential as compared to the DFNKF peptide. It is possible that a pentapeptide is a lower limit for
5 potent amyloid former. This is consistent with recent results that demonstrate that two pentapeptides of IAPP, NFLVH and FLVHS, can form amyloid fibrils, but their common denominator, the tetrapeptide FLVH, could not form such fibrils (see Examples 1-5).

10

EXAMPLE 22

Identification of an amyloidogenic peptide from Lactotransferrin

Amyloid fibril formation by lactotransferrin (GenBank Accession No. gi:24895280) is associated familial subepithelial corneal amyloid formation [Sacchetti and Kelly (2002) Nat Rev Drug Discov 1:267-75]. Based on the
15 proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic features of a Lactotransferrin-derived peptide, LFNQTG (SEQ ID NO: 32) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Lactotransferrin-derived peptide to
20 form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 25, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that LFNQTG of Lactotransferrin is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide
25 sequences.

EXAMPLE 23

Identification of an amyloidogenic peptide from Serum amyloid A protein

Fragments of Serum amyloid A proteins (GenBank Accession No. gi:134167)
30 were found in amyloid-state in cases of Chronic inflammation amyloidosis (Westermarck et al. (1992) Biochem. Biophys. Res. Commun. 182: 27-33). Based on the proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic

features of a Serum amyloid A protein-derived peptide, SFFSFL (SEQ ID NO: 33) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Serum amyloid A protein-derived peptide to form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 26, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that SFFSFL of serum amyloid A protein is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide sequences.

EXAMPLE 24

Identification of an amyloidogenic peptide from BriL

The human BRI gene is located on chromosome 13. The amyloid fibrils of the BriL gene product (GenBank Accession No. gi:12643343) are associated with neuronal dysfunction and dementia (Vidal et al (1999) Nature 399, 776-781). Based on the proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic features of a BriL-derived peptide, FENKF (SEQ ID NO: 34) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the BriL-derived peptide to form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 27, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that FENKF of BriL is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide sequences.

EXAMPLE 25

Identification of an amyloidogenic peptide from Gelsolin

Fragments of Gelsolin proteins (GenBank Accession No. gi:4504165) were found in amyloid-state in cases of Finnish hereditary amyloidosis [Maury and Nurmiaho-Lassila (1992) Biochem. Biophys. Res. Commun. 183: 227-31]. Based on

the proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic features of a Gelsolin-derived peptide, SFNNG (SEQ ID NO: 35) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Gelsolin-derived peptide to form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 28, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that SFNNG of BriL is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide sequences.

10

EXAMPLE 26

Identification of an amyloidogenic peptide from Serum amyloid P

Amyloid fibril formation by beta-amyloid is promoted by interaction with serum amyloid-P (GenBank Accession No. gi:2144884). Based on the proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic features of a Serum amyloid P-derived peptide, LQNFTL (SEQ ID NO: 36) were studied.

15

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Serum amyloid P-derived peptide to form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 29, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that LQNFTL of Serum amyloid P is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide sequences.

20

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EXAMPLE 27

Identification of an amyloidogenic peptide from Immunoglobulin light chain

Amyloid fibrils formation by Immunoglobulin light chain (GenBank Accession No. gi:625508) is associated with primary systemic amyloidosis [Sacchettini and Kelly (2002) Nat Rev Drug Discov 1:267-75]. Based on the proposed role of aromatic

30

residues in amyloid self-assembly, the amyloidogenic features of an Immunoglobulin light chain-derived peptide, TLIFGG (SEQ ID NO: 37) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the immunoglobulins light chain-derived peptide to form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 30, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that TLIFGG of the immunoglobulin light chain is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide sequences.

EXAMPLE 28

Identification of an amyloidogenic peptide from Cystatin C

Amyloid fibril formation by Cystatin C (GenBank Accession No. gi:4490944) is associated with hereditary cerebral amyloid angiopathy [Sacchettini and Kelly (2002) Nat Rev Drug Discov 1:267-75]. Based on the proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic features of a Cystatin C-derived peptide, RALDFA (SEQ ID NO: 38) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Cystatin C-derived peptide to form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 31, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that RALDFA of the Cystatin C is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide sequences.

EXAMPLE 29

Identification of an amyloidogenic peptide from Transthyretin

Amyloid fibril formation by Transthyretin (GenBank Accession No. gi:72095) is associated with familial amyloid polyneuropathy (Sacchettini and Kelly (2002) Nat Rev Drug Discov 1:267-75). Based on the proposed role of aromatic residues in

amyloid self-assembly, the amyloidogenic features of an Transthyretin-derived peptide, GLVFVS (SEQ ID NO: 39) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Transthyretin-derived peptide to
5 form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 32, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that GLVFVS of Transthyretin is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide
10 sequences.

EXAMPLE 30

Identification of an amyloidogenic peptide from Lysozyme

Amyloid fibril formation by Lysozyme (GenBank Accession No. gi:299033) is
15 associated with familial non-neuropathic amyloidosis [Sacchettini and Kelly (2002) Nat Rev Drug Discov 1:267-75]. Based on the proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic features of a Lysozyme-derived peptide, GTFQIN (SEQ ID NO: 40) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Lysozyme-derived peptide to form
20 fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 33, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that GTFQIN of Lysozyme is important for the polypeptide self-assembly. These results further substantiate the
25 ability of the present invention to predict amyloidogenic peptide sequences.

EXAMPLE 31

Identification of an amyloidogenic peptide from Fibrinogen

Amyloid fibril formation by Fibrinogen (GenBank Accession No. gi:11761629)
30 is associated with hereditary renal amyloidosis (Sacchettini and Kelly (2002) Nat Rev Drug Discov 1:267-75). Based on the proposed role of aromatic residues in amyloid

self-assembly, the amyloidogenic features of a Fibrinogen-derived peptide, SGIFTN (SEQ ID NO: 41) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Fibrinogen -derived peptide to
5 form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 34, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that SGIFTN of Fibrinogen is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide
10 sequences.

EXAMPLE 32

Identification of an amyloidogenic peptide from Insulin

Amyloid fibril formation by Insulin (GenBank Accession No. gi:229122) is
15 associated with injection-localized amyloidosis [Sacchettini and Kelly (2002) Nat Rev Drug Discov 1:267-75]. Based on the proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic features of an insulin-derived peptide, ERGFF (SEQ ID NO: 42) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the Insulin-derived peptide to form
20 fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 35, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that ERGFF of insulin is important for the polypeptide self-assembly. These results further substantiate the
25 ability of the present invention to predict amyloidogenic peptide sequences.

EXAMPLE 33

Identification of an amyloidogenic peptide from prolactin

Amyloid fibrils formation by prolactin (GenBank Accession No. gi:4506105)
30 is associated with pituitary-gland amyloidosis (Sacchettini and Kelly (2002) Nat Rev Drug Discov 1:267-75). Based on the proposed role of aromatic residues in amyloid

self-assembly, the amyloidogenic features of a prolactin-derived peptide, RDFLDR (SEQ ID NO: 43) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the prolactin-derived peptide to form
5 fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 36, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that RDFLDR of prolactin is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide sequences.

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EXAMPLE 34

Identification of an amyloidogenic peptide from Beta-2-microglobulin

Amyloid fibrils formation by beta-2-microtubulin (GenBank Accession No. gi:70065) is associated haemodialysis-related amyloidosis (Sacchettini and Kelly
15 (2002) Nat Rev Drug Discov 1:267-75). Based on the proposed role of aromatic residues in amyloid self-assembly, the amyloidogenic features of a beta-2-microtubulin-derived peptide, SNFLN (SEQ ID NO: 44) were studied.

Materials and Experimental Procedures – Described in Examples 7 and 10.

Results – To characterize the ability of the beta-2-microtubulin-derived peptide
20 to form fibrillar supramolecular ultrastructures, negative staining electron microscopy analysis was effected. As shown in Figure 37, under mild conditions, filamentous structures were observed for the selected peptide, suggesting that SNFLN of beta-2-microtubulin is important for the polypeptide self-assembly. These results further substantiate the ability of the present invention to predict amyloidogenic peptide
25 sequences.

EXAMPLE 35

Inhibition of amyloid formation an amyloidogenic peptide identified according to the teachings of the present invention

30 The ability of amyloidogenic peptides of IAPP, identified according to the teachings of the present invention to inhibit amyloid formation by the full-length polypeptide was tested by the addition of beta-breaker proline residues to the

recognition sequence as set forth in the peptide sequence NFLVHPP (SEQ ID NO: 45).

The degree of amyloid fibrils formation with and without the inhibitor was assessed using thioflavin T (ThT) as molecular indicator. The degree of fluorescence of the ThT dye is directly correlated with the amount of amyloid fibrils in the solution [LeVine H 3rd. (1993) Protein Sci. 2:404-410. IAPP solutions (4 μ M hIAPP in 10 mM Tris buffer pH 7.2), were incubated in the presence or absence of 40 μ M of the modified peptide (i.e., NFLVHPP) at room temperature. Fibril formation was determined by a ten fold dilution of the solutions into a solution that contained 3 μ M thioflavin T (ThT) in 50 mM sodium phosphate pH 6.0 and determination of fluorescence at 480 nm with excitation at 450 nm using a LS50B spectrofluorimeter (Perkin Elmer, Wellesley, MA). As a control 10 mM Tris buffer pH 7.2 were diluted into the ThT solution and fluorescence was determined as described.

Result – As shown in Figure 38, while the IAPP alone showed high levels of ThT fluorescence as expected for amyloidogenic protein, there was a significant increase in fluorescence in the presence of the inhibitory peptide. Thus, these results validate the NFLVH sequence as the amyloidogenic determinant in the IAPP polypeptide.

20

EXAMPLE 36

Significance of hydrophobic residues in amyloid assembly

The significance of an aromatic residue in the basic amyloidogenic unit of IAPP has been demonstrated in Examples 1-5. As described, substitution of a phenylalanine to an alanine abolished the ability of an amyloidogenic fragment (NAGAIL, SEQ ID NO: 9) to form amyloid fibrils *in vitro*. Based on this observation, the remarkable occurrence of aromatic residues in other short amyloid related sequences (Examples 12-35), and the well-known role of π -stacking in processes of self-assembly in chemistry and biochemistry, it was suggested that stacking of aromatic residues may play a role in the process of amyloid fibrils formation [Gazit (2002) FASEB J. 16:77-83].

30

The study was further extended to indicate whether the phenylalanine residue is critical due to aromaticity thereof, or rather due to its hydrophobic nature. The

effect of phenylalanine substitution with hydrophobic residues on the self assembly of the basic amyloidogenic unit of IAPP (i.e., NFGAIL peptide) was addressed.

The list of peptides used in the study and designation thereof is presented in Table 6, below.

5

Table 6

| <i>Amino acid substitution and coordinates on hIAPP</i> | <i>Peptide sequence</i> | <i>SEQ ID NO:</i> |
|--|--------------------------------|--------------------------|
| WT 22-29 | NH ₂ -NFGAILSS-COOH | 46 |
| F>I 22-29 | NH ₂ -NIGAILSS-COOH | 47 |
| F>L 22-29 | NH ₂ -NLGAILSS-COOH | 48 |
| F>V 22-29 | NH ₂ -NVGAILSS-COOH | 49 |
| F>A 22-29 | NH ₂ -NAGAILSS-COOH | 89 |

It will be appreciated that while these hydrophobic amino acids are similar or even slightly more hydrophobic than phenylalanine [Wolfenden (1981) Biochemistry 20:849-855; Kyte (1982) J. Mol. Biol. 157:105-132; Radzicka (1988)], they are not aromatic. Furthermore, valine and isoleucine, are considered to be very strong β -sheet formers [Chou (1974) Biochemistry 13:211-222; Chou (1978) Annu. Rev. Biochem. 47:251-276], which is assumed to be important to the formation of β -sheet rich amyloid fibrils.

15

EXAMPLE 37

Characterization of the aggregation kinetics of hydrophobically modified hIAPP peptide fragments as monitored by turbidity measurements

Experimental Procedures – Effected as described in Example 8.

Results

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To get insight into the aggregation potential of the hydrophobically-modified IAPP-derived peptide analogues, turbidity assay was performed. Freshly made stock solutions of the wild-type peptide and the various peptide mutants were made in DMSO. The peptides were then diluted to a buffer solution and the turbidity was monitored by following the absorbance at 405 nm as a function of time. As shown in Figure 39, significant increase in turbidity was observed for the wild-type NFGAILSS octapeptide within minutes following dilution thereof into the aqueous solution. The shape of the aggregation curve resembled that of a saturation curve, with a rapid increase in turbidity in the first hour, followed by a much slower increase

25

in turbidity over the entire incubation time monitored. This probably reflects a rapid aggregation process, with the number of free building blocks as the rate limiting factor. In contrast, none of the analogue peptides revealed any significant aggregative behavior and the turbidity of all the hydrophobic analogues as well as the alanine-substituted analogues remained very low for at least 24 hours (Figure 39).

To determine whether the non-aggregative behavior of the hydrophobic analogues is a result of extremely slow kinetics, peptide analogue solutions were incubated for 1 week in the same experimental conditions and endpoint turbidity values were determined. As shown in Figure 30, some low degree of turbidity was observed with the NIGAILSS, and lower extent for the NLGAILSS, NAGAILSS, and NVGAILSS peptides in decreasing order of turbidity. However, even for the NIGAILSS, the degree of turbidity was significantly lower as compared to the wild-type NFGAILSS protein (Figure 40). Moreover, there was no correlation between aggregation potential and hydrophobicity or β -sheet forming tendency, since the lower degree of aggregation was observed with the substitution to the highly hydrophobic and β -sheet former, valine. The slight decrease in the endpoint turbidity value of the NFGAILSS wild-type peptide, as compared to the values obtained after 24 hours incubation, could reflect the formation of very large aggregates that adhere to the cuvette surface.

EXAMPLE 38

Ultrastructural analysis of hydrophobically modified hLAPP peptide fragments

Electron microscopy analysis was effected as described in Example 10.

An ultrastructural visualization of any possible structures formed by the various analogous peptides was effected following five days of incubation. This structural analysis represents the most sensitive method since various aggregates were visualized individually. For that aim, the occurrence and characteristics of the formed structures were studied by electron microscopy using negative staining, with the same of peptide solution which were incubated in the aggregation assay (Example 32). As expected, well-ordered fibrils were observed with the wild-type peptide NFGAILSS peptide fragment (Figures 41a-b). Some amorphous aggregates could be also seen with the modified fragments (Figures 41c-f). However, those structures

were significantly less abundant on the microscope grid. Larger aggregative structures were observed with the more hydrophobic substitutions as compared to the alanine analogues. Yet, unlike the ordered fibrillar structures that were seen with the NFGAILSS peptide, as mentioned above, these aggregates were quite rare and did not have ordered structures (Figures 41c-f). Those irregular and sporadic structures are consistent with some degree of non-specific aggregation as expected after long incubation of rather hydrophobic molecules.

EXAMPLE 39

10 *Determination of the specific function of phenylalanine in the IAPP self assembly*

To determine the specific role of the phenylalanine residue in IAPP-self assembly, a membrane-based binding assay was preformed in order to systematically explore the molecular determinants that facilitate the ability of the full-length hIAPP to recognize the “basic amyloidogenic unit”. To this end, the ability of MBP-IAPP (see Example 6) to interact with an array of peptides in which the phenylalanine position was systematically altered (SEQ ID NOs. 91-110), was addressed.

Materials and Experimental Procedures – see Examples 6-7.

Results

A peptide array corresponding to the SNNXGAILSS motif (SEQ ID NO: 90), where X is any natural amino-acid but cysteine was constructed. As shown in Figure 42a, binding of MBP-IAPP was clearly observed to peptides which contained the aromatic tryptophan and phenylalanine residues at the X position (Figure 42a). Interestingly, binding was also observed upon substitution of phenylalanine with basic amino acids such as arginine and lysine. In contrast, no binding was observed with any of the hydrophobic substitutions of the position, even after long exposure of the membrane (Figure 42b).

The short exposure binding was assessed using densitometry (Figure 42c). It will be appreciated though, that the measured binding should be interpreted as semiquantitative since the coupling efficiency during synthesis and therefore the amount of peptide per spot may vary. In this case, however, the marked difference in binding between the various peptide variants was very clear.

Taken together, all these observations substantiate the role of aromatic residues in the acceleration of amyloid formation processes.

Although the invention has been described in conjunction with specific
5 embodiments thereof, it is evident that many alternatives, modifications and
variations will be apparent to those skilled in the art. Accordingly, it is intended to
embrace all such alternatives, modifications and variations that fall within the spirit
and broad scope of the appended claims. All publications, patents, and patent
applications mentioned in this specification are herein incorporated in their entirety
10 by reference into the specification, to the same extent as if each individual
publication, patent, or patent application was specifically and individually indicated
to be incorporated herein by reference. In addition, citation or identification of any
reference in this application shall not be construed as an admission that such
reference is available as prior art to the present invention.

WHAT IS CLAIMED IS:

1. A peptide comprising at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

2. The peptide of claim 1, wherein said amino acid sequence further includes a polar uncharged amino acid selected from the group consisting of serine, threonine, asparagine, glutamine and natural derivatives thereof.

3. The peptide of claim 1, wherein said amino acid sequence further includes at least one positively charged amino acid and at least one negatively charged amino acid.

4. The peptide of claim 3, wherein said at least one positively charged amino acid is selected from the group consisting of lysine, arginine and natural and synthetic derivatives thereof.

5. The peptide of claim 3, wherein said at least one negatively charged amino acid is selected from the group consisting of aspartic acid, glutamic acid and natural and synthetic derivatives thereof.

6. The peptide of claim 1, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-45.

7. The peptide of claim 1, wherein the peptide is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.

8. The peptide of claim 1, further comprising at least two serine residues at a C-terminus thereof.

9. The peptide of claim 1, wherein the peptide is a linear or cyclic peptide.

10. The peptide of claim 1, wherein said peptide further includes at least one beta-breaker amino acid.

11. The peptide of claim 10, wherein said beta-breaker amino acid is proline.

12. A peptide comprising at least 3 amino acid residues and less than 15 amino acid residues, the peptide including an amino acid sequence selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-44 wherein the peptide is capable of self-aggregating under physiological conditions.

13. The peptide of claim 12, further comprising at least two serine residues at a C-terminus thereof.

14. The peptide of claim 12, wherein the peptide is a linear or cyclic peptide.

15. The peptide of claim 12, wherein said peptide further includes at least one beta-breaker amino acid.

16. The peptide of claim 15, wherein said beta-breaker amino acid is proline.

17. A peptide selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25.

18. The peptide of claim 17, wherein the peptide further includes at least two serine residues attached to a C-terminus thereof.

19. The peptide of claim 17, wherein the peptide is a linear or cyclic peptide.

20. The peptide of claim 17, wherein said peptide further includes at least one beta-breaker amino acid.

21. The peptide of claim 20, wherein said beta-breaker amino acid is proline.

22. A peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-44.

23. The peptide of claim 22, further comprising at least two serine residues at a C-terminus thereof.

24. The peptide of claim 22, wherein the peptide is a linear or cyclic peptide.

25. The peptide of claim 22, wherein said peptide further includes at least one beta-breaker amino acid.

26. The peptide of claim 25, wherein said beta-breaker amino acid is proline.

27. A peptide having an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 10-11 and 21-22.

28. The peptide of claim 27, further comprising at least two serine residues at a C-terminus thereof.

29. The peptide of claim 27, wherein the peptide is a linear or cyclic peptide.

30. The peptide of claim 27, wherein said peptide further includes at least one beta-breaker amino acid.

31. The peptide of claim 30, wherein said beta-breaker amino acid is proline.

32. A method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual a therapeutically effective amount of a peptide having at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

33. The method of claim 32, wherein said peptide is an active ingredient of a pharmaceutical composition which also includes a physiologically acceptable carrier.

34. The method of claim 32, wherein said peptide is expressed from a nucleic acid construct.

35. The method of claim 32, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-45.

36. The method of claim 32, wherein said peptide is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.

37. The method of claim 32, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

38. The method of claim 32, wherein said peptide is a linear or cyclic peptide.

39. The method of claim 32, wherein said amino acid sequence further includes a polar uncharged amino acid selected from the group consisting of serine, threonine, asparagine, glutamine and natural and synthetic derivatives thereof.

40. The method of claim 32, wherein said amino acid sequence further includes at least one positively charged amino acid and at least one negatively charged amino acid.

41. The method of claim 40, wherein said at least one positively charged amino acid is selected from the group consisting of lysine, arginine and natural and synthetic derivatives thereof.

42. The method of claim 40, wherein said at least one negatively charged amino acid is selected from the group consisting of aspartic acid, glutamic acid and natural and synthetic derivatives thereof.

43. The method of claim 32, wherein said peptide further includes at least one beta-breaker amino acid.

44. The method of claim 43, wherein said beta-breaker amino acid is proline.

45. A method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual therapeutically effective amount of a peptide having at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45.

46. The method of claim 45, wherein said peptide is an active ingredient of a pharmaceutical composition, which also includes a physiologically acceptable carrier.

47. The method of claim 45, wherein said peptide is expressed from a nucleic acid construct.

48. The method of claim 45, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

49. The method of claim 45, wherein said peptide is a linear or cyclic peptide.

50. The method of claim 45, wherein said peptide further includes at least one beta-breaker amino acid.

51. The method of claim 50, wherein said beta-breaker amino acid is proline.

52. A method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual a therapeutically effective amount of a peptide selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25, wherein said peptide is an active ingredient of a pharmaceutical compositions which also includes a physiologically acceptable carrier.

53. The method of claim 52, wherein said peptide is expressed from a nucleic acid construct.

54. The method of claim 52, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

55. The method of claim 52, wherein said peptide is a linear or cyclic peptide.

56. The method of claim 52, wherein said peptide further includes at least one beta-breaker amino acid.

57. The method of claim 56, wherein said beta-beaker amino acid is proline.

58. A method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual a therapeutically effective amount of a peptide selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45.

59. The method of claim 58, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

60. The method of claim 58, wherein said peptide is a linear or cyclic peptide.

61. The method of claim 58, wherein said peptide is an active ingredient of a pharmaceutical composition, which also includes a physiologically acceptable carrier.

62. The method of claim 58, wherein said peptide further includes at least one beta-breaker amino acid.

63. The method of claim 62, wherein said beta-beaker amino acid is proline.

64. A method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual therapeutically effective amount of a peptide having at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25, wherein said peptide is an active ingredient of a pharmaceutical composition which also includes a physiologically acceptable carrier.

65. The method of claim 64, wherein said peptide is expressed from a nucleic acid construct.

66. The method of claim 64, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

67. The method of claim 64, wherein said peptide is a linear or cyclic peptide.

68. The method of claim 64, wherein said peptide further includes at least one beta-breaker amino acid.

69. The method of claim 68, wherein said beta-breaker amino acid is proline.

70. A pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide having at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence as set forth in SEQ ID NO: 7 and a pharmaceutically acceptable carrier or diluent.

71. The pharmaceutical composition of claim 70, wherein said amino acid sequence is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.

72. The pharmaceutical composition of claim 70, wherein said peptide is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.

73. The pharmaceutical composition of claim 70, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

74. The pharmaceutical composition of claim 70, wherein said peptide is a linear or cyclic peptide.

75. The pharmaceutical composition of claim 70, wherein said amino acid sequence further includes a polar uncharged amino acid selected from the group consisting of serine, threonine, asparagine, glutamine and natural and synthetic derivatives thereof.

76. The pharmaceutical composition of claim 70, wherein said amino acid sequence further includes at least one positively charged amino acid and at least one negatively charged amino acid.

77. The pharmaceutical composition of claim 76, wherein said at least one positively charged amino acid is selected from the group consisting of lysine, arginine and natural and synthetic derivatives thereof.

78. The pharmaceutical composition of claim 76, wherein said at least one negatively charged amino acid is selected from the group consisting of aspartic acid, glutamic acid and natural and synthetic derivatives thereof.

79. The pharmaceutical composition of claim 76, wherein said peptide further includes at least one beta-breaker amino acid.

80. The pharmaceutical composition of claim 79, wherein said beta-breaker amino acid is proline.

81. A pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25 and a pharmaceutically acceptable carrier or diluent.

82. The pharmaceutical composition of claim 81, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

83. The pharmaceutical composition of claim 81, wherein said peptide is a linear or cyclic peptide.

84. The pharmaceutical composition of claim 81, wherein said peptide further includes at least one beta-breaker amino acid.

85. The pharmaceutical composition of claim 84, wherein said beta-breaker amino acid is proline.

86. A pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide having at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 10-11, 21-22 and 25 and a pharmaceutically acceptable carrier or diluent.

87. The pharmaceutical composition of claim 86, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

88. The pharmaceutical composition of claim 86, wherein said peptide is a linear or cyclic peptide.

89. The pharmaceutical composition of claim 86, wherein said peptide further includes at least one beta-breaker amino acid.

90. The pharmaceutical composition of claim 89, wherein said beta-breaker amino acid is proline.

91. A pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide having at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45 and a pharmaceutically acceptable carrier or diluent.

92. The pharmaceutical composition of claim 91, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

93. The pharmaceutical composition of claim 91, wherein said peptide is a linear or cyclic peptide.

94. The pharmaceutical composition of claim 91, wherein said peptide further includes at least one beta-breaker amino acid.

95. The pharmaceutical composition of claim 94, wherein said beta-breaker amino acid is proline.

96. A pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient a peptide selected from the group consisting of SEQ ID NOs: 4, 12-19 and 27-45 and a pharmaceutically acceptable carrier or diluent.

97. The pharmaceutical composition of claim 96, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

98. The pharmaceutical composition of claim 96, wherein said peptide is a linear or cyclic peptide.

99. The pharmaceutical composition of claim 96, wherein said peptide further includes at least one beta-breaker amino acid.

100. The pharmaceutical composition of claim 99, wherein said beta-breaker amino acid is proline.

101. A nucleic acid construct comprising a polynucleotide segment encoding a peptide having at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

102. The nucleic acid construct of claim 101 further comprising a promoter.
103. The nucleic acid construct of claim 101, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.
104. The nucleic acid construct of claim 101, wherein said amino acid sequence further includes a polar uncharged amino acid selected from the group consisting of serine, threonine, asparagine, glutamine and natural and synthetic derivatives thereof.
105. The nucleic acid construct of claim 101, wherein said amino acid sequence further includes at least one positively charged amino acid and at least one negatively charged amino acid.
106. The nucleic acid construct of claim 105, wherein said at least one positively charged amino acid is selected from the group consisting of lysine, arginine and natural and synthetic derivatives thereof.
107. The nucleic acid construct of claim 105, wherein said at least one negatively charged amino acid is selected from the group consisting of aspartic acid, glutamic acid and natural and synthetic derivatives thereof.
108. The nucleic acid construct of claim 101, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-45.
109. The nucleic acid construct of claim 101, wherein the peptide is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.
110. The nucleic acid construct of claim 101, wherein said peptide further includes at least one beta-breaker amino acid.

111. The nucleic acid construct of claim 110, wherein said beta-beaker amino acid is proline.

112. A nucleic acid construct comprising a polynucleotide segment encoding a peptide selected from the group consisting of SEQ ID NOs: 8, 10–11, 21–22 and 25.

113. The nucleic acid construct of claim 112 further comprising a promoter.

114. The nucleic acid construct of claim 112, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

115. The nucleic acid construct of claim 112, wherein said peptide further includes at least one beta-breaker amino acid.

116. The nucleic acid construct of claim 115, wherein said beta-beaker amino acid is proline.

117. A nucleic acid construct comprising a polynucleotide segment encoding a peptide selected from the group consisting of SEQ ID NOs: 4, 12–19 and 27–45.

118. The nucleic acid construct of claim 117 further comprising a promoter.

119. The nucleic acid construct of claim 117, wherein said peptide further includes at least two serine residues attached to a C-terminus thereof.

120. The nucleic acid construct of claim 117, wherein said peptide further includes at least one beta-breaker amino acid.

121. The nucleic acid construct of claim 120, wherein said beta-beaker amino acid is proline.

122. An antibody or an antibody fragment comprising an antigen recognition region capable of binding a peptide including at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

123. The antibody or the antibody fragment of claim 122, wherein said amino acid sequence further includes a polar uncharged amino acid selected from the group consisting of serine, threonine, asparagine, glutamine and natural derivatives thereof.

124. The antibody or the antibody fragment of claim 122, wherein said amino acid sequence further includes at least one positively charged amino acid and at least one negatively charged amino acid.

125. The antibody or the antibody fragment of claim 124, wherein said at least one positively charged amino acid is selected from the group consisting of lysine, arginine and natural and synthetic derivatives thereof.

126. The antibody or the antibody fragment of claim 124, wherein said at least one negatively charged amino acid is selected from the group consisting of aspartic acid, glutamic acid and natural and synthetic derivatives thereof.

127. The antibody or the antibody fragment of claim 122, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-45.

128. The antibody or the antibody fragment of claim 122, wherein said peptide is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.

129. The antibody or the antibody fragment of claim 122, wherein said peptide further includes at least two serine residues at a C-terminus thereof.

130. The antibody or the antibody fragment of claim 122, wherein said peptide is a linear or cyclic peptide.

131. The antibody or the antibody fragment of claim 122, wherein said peptide further includes at least one beta-breaker amino acid.

132. The antibody or the antibody fragment of claim 131, wherein said beta-breaker amino acid is proline.

133. A pharmaceutical composition for treating or preventing an amyloid-associated disease comprising as an active ingredient an antibody or an antibody fragment having an antigen recognition region capable of binding a peptide including at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

134. The pharmaceutical composition of claim 133, wherein said amino acid sequence further includes a polar uncharged amino acid selected from the group consisting of serine, threonine, asparagine, glutamine and natural derivatives thereof.

135. The pharmaceutical composition of claim 133, wherein said amino acid sequence further includes at least one positively charged amino acid and at least one negatively charged amino acid.

136. The pharmaceutical composition of claim 135, wherein said at least one positively charged amino acid is selected from the group consisting of lysine, arginine and natural and synthetic derivatives thereof.

137. The pharmaceutical composition of claim 135, wherein said at least one negatively charged amino acid is selected from the group consisting of aspartic acid, glutamic acid and natural and synthetic derivatives thereof.

138. The pharmaceutical composition of claim 133, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-45.

139. The pharmaceutical composition of claim 133, wherein said peptide is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.

140. The pharmaceutical composition of claim 133, wherein said peptide further includes at least two serine residues at a C-terminus thereof.

141. The pharmaceutical composition of claim 133, wherein said peptide is a linear or cyclic peptide.

142. The pharmaceutical composition of claim 133, wherein said peptide further includes at least one beta-breaker amino acid.

143. The pharmaceutical composition of claim 142, wherein said beta-breaker amino acid is proline.

144. A method of treating or preventing an amyloid-associated disease in an individual, the method comprising providing to the individual therapeutically effective amount of an antibody or an antibody fragment having an antigen recognition region capable of binding a peptide including at least 3 amino acid residues and less than 15 amino acid residues, said peptide including an amino acid sequence as set forth in SEQ ID NO: 7.

145. The method of claim 144, wherein said amino acid sequence further includes a polar uncharged amino acid selected from the group consisting of serine, threonine, asparagine, glutamine and natural derivatives thereof.

146. The method of claim 144, wherein said amino acid sequence further includes at least one positively charged amino acid and at least one negatively charged amino acid.

147. The method of claim 146, wherein said at least one positively charged amino acid is selected from the group consisting of lysine, arginine and natural and synthetic derivatives thereof.

148. The method of claim 146, wherein said at least one negatively charged amino acid is selected from the group consisting of aspartic acid, glutamic acid and natural and synthetic derivatives thereof.

149. The method of claim 144, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 4, 12-19 and 27-45.

150. The method of claim 144, wherein said peptide is selected from the group consisting of SEQ ID NOs. 4, 12-19 and 27-45.

151. The method of claim 144, wherein said peptide further includes at least two serine residues at a C-terminus thereof.

152. The method of claim 144, wherein said peptide is a linear or cyclic peptide.

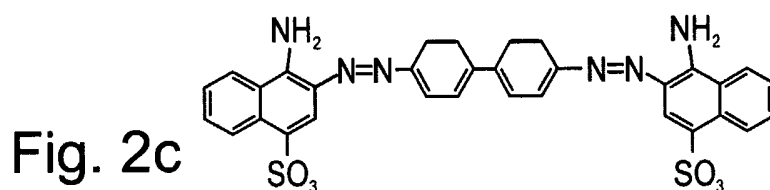
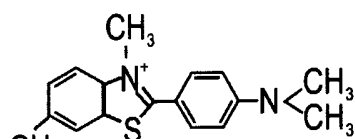
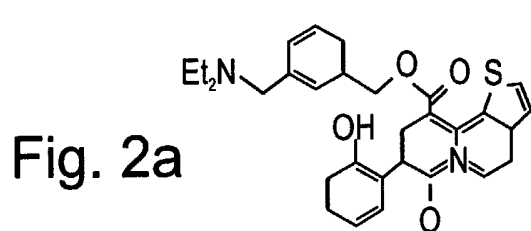
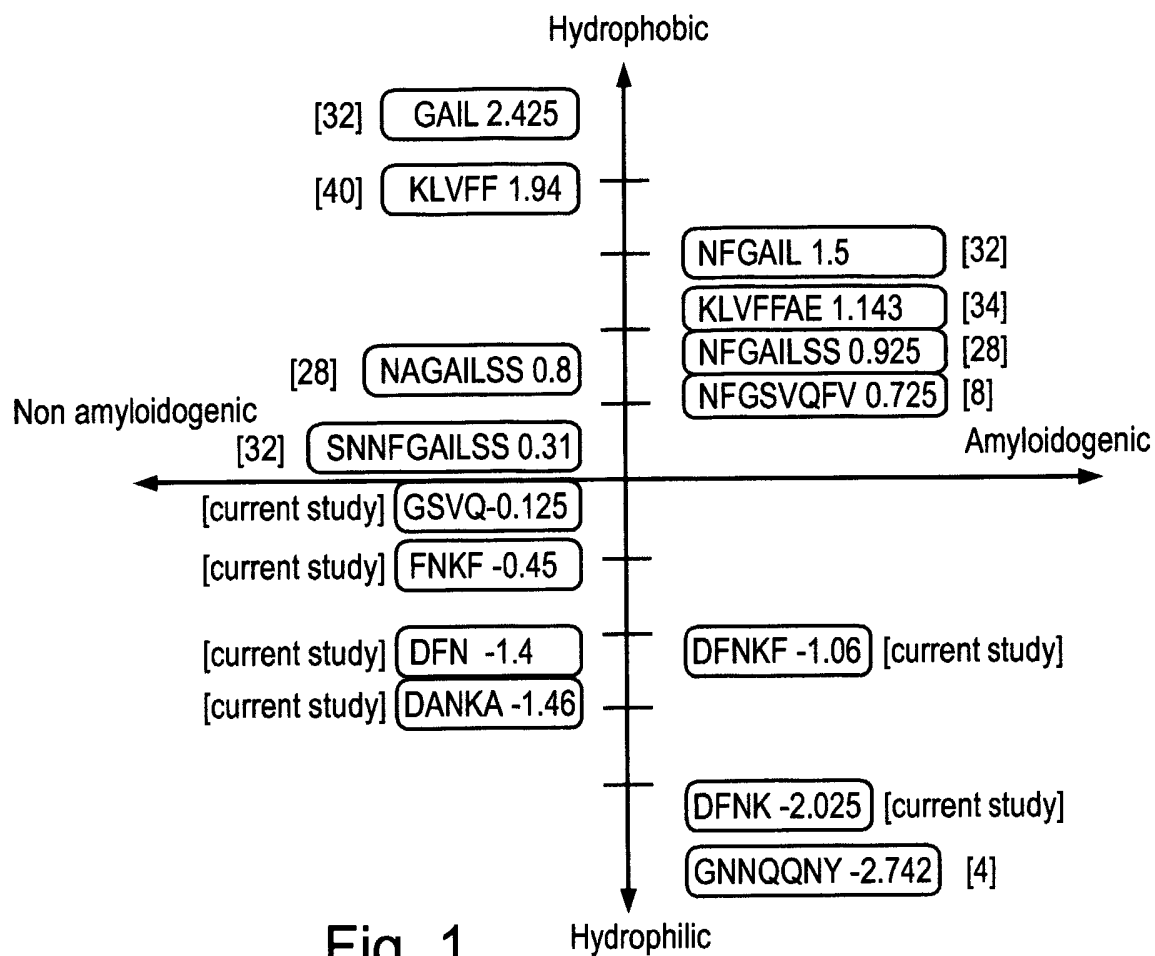
153. The method of claim 144, wherein said peptide further includes at least one beta-breaker amino acid.

154. The method of claim 153, wherein said beta-breaker amino acid is proline.

155. The method of claim 144, wherein said antibody or antibody fragment is an active ingredient of a pharmaceutical composition, which also includes a

physiologically acceptable carrier.

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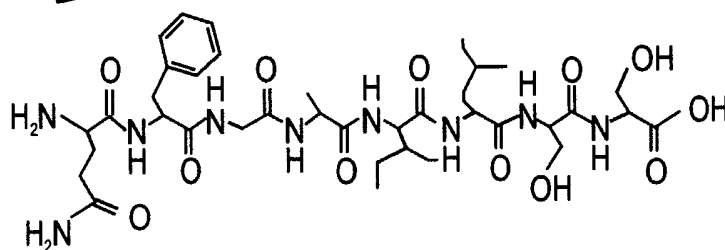


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Fig. 3a

¹
 Rodent KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY³⁷
 Human KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY

Fig. 3b



| | |
|-----|--|
| wt | NH ₂ -NFGAILSS-COOH |
| N1A | NH ₂ -N <u>A</u> FGAILSS-COOH |
| F2A | NH ₂ -NAGAILSS-COOH |
| G3A | NH ₂ -NFAAILSS-COOH |
| I5A | NH ₂ -NFGA <u>A</u> LSS-COOH |
| L6A | NH ₂ -NFGAI <u>A</u> SS-COOH |

Fig. 3c

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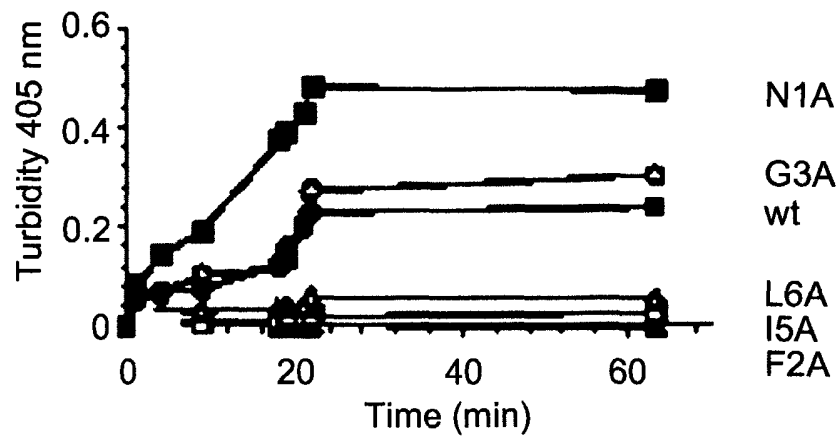


Fig. 4a

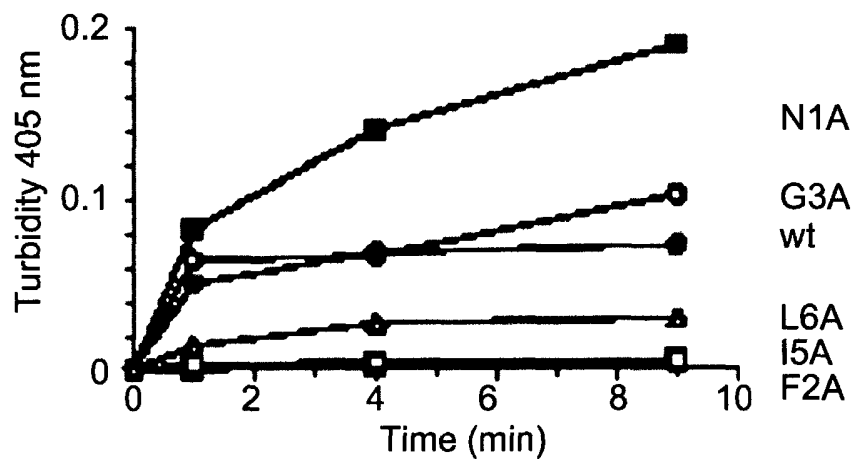


Fig. 4b

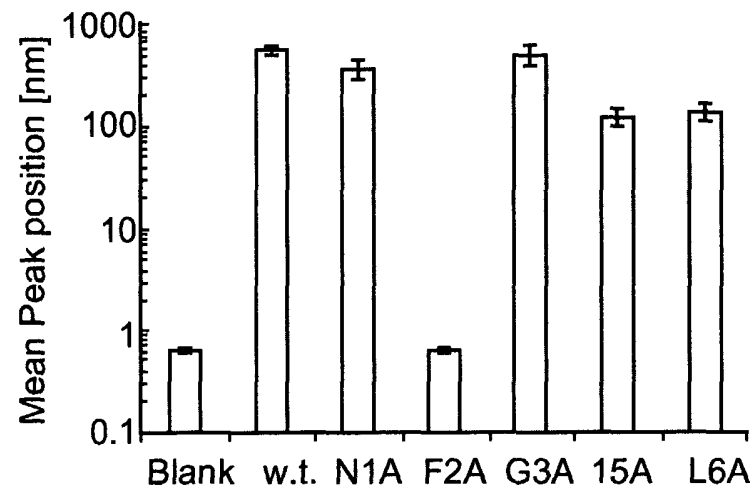


Fig. 5

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Fig. 6a

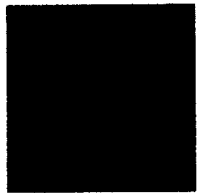


Fig. 6b

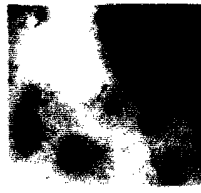


Fig. 6c

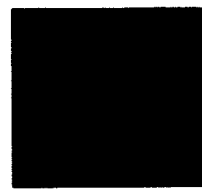


Fig. 6d

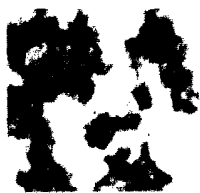


Fig. 6e



Fig. 6f

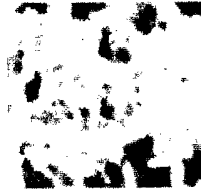


Fig. 6g



Fig. 6h

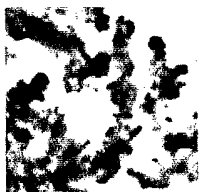


Fig. 6i



Fig. 6j

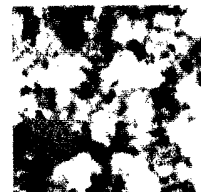


Fig. 6k

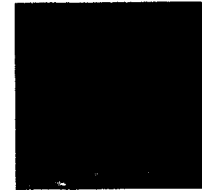


Fig. 6l



Fig. 6m

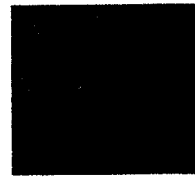


Fig. 6n

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Fig. 7a

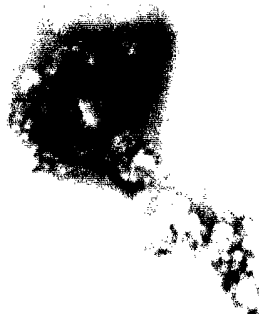


Fig. 7b



Fig. 7c



Fig. 7d



Fig. 7e



Fig. 7f

| | | | | | | | | | | | | | | |
|------------------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Synthetic gene Human cDNA | M | K | C | N | T | A | T | C | A | T | Q | R | L | A |
| | ATGAAATGCAACACCGCGACCTGCGCGACCCAGCGCCTGGCG | | | | | | | | | | | | | |
| | ATGAAATGCAACACTGCCACATGTGCAACCCAGCGCCTGGCA | | | | | | | | | | | | | |
| | M | F | L | V | H | S | S | N | N | F | G | A | I | L |
| | AAC <u>TTTCT</u> GGTGCATAGCAGCAACAACTTTGGCGGATTC | | | | | | | | | | | | | |
| | AATTTT <u>TT</u> AGTTCAATCCAGCAACAACTTTGGTGCCATTCTC | | | | | | | | | | | | | |
| | S | S | T | N | V | G | S | N | T | Y | | | | |
| | AGCAGCACCAACGTGGGCGAGCAACACCTAT | | | | | | | | | | | | | |
| | TCATCTACCAACGTGGGATCCAATACATAT | | | | | | | | | | | | | |

Fig. 8a

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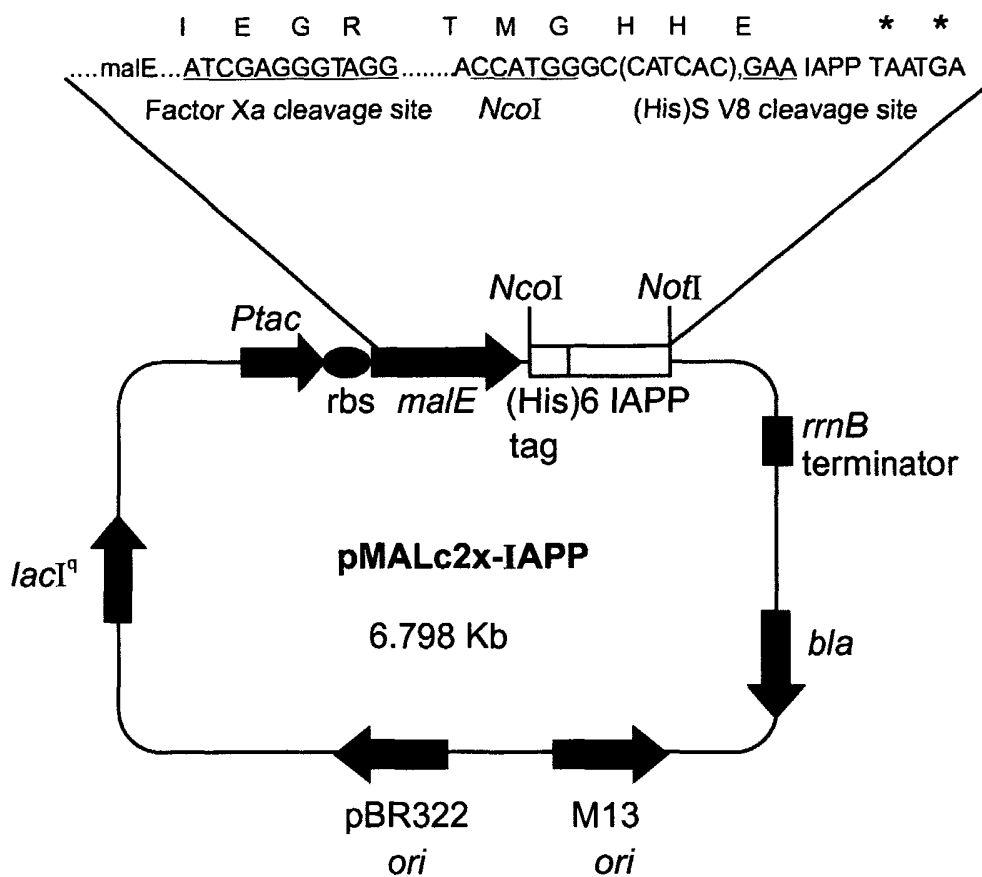


Fig. 8b

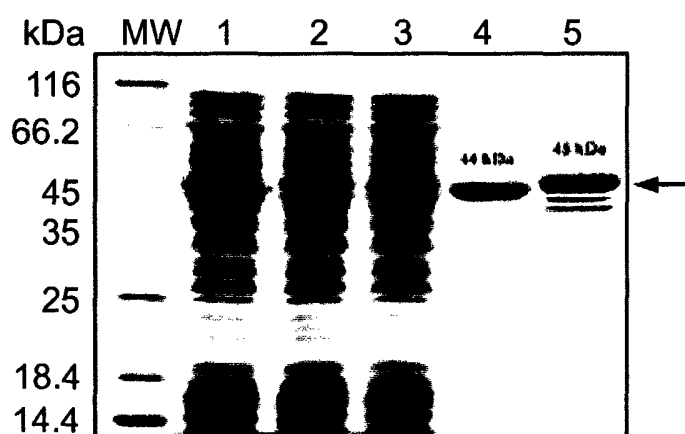


Fig. 9

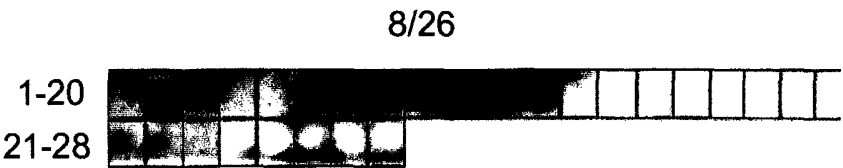


Fig. 10a

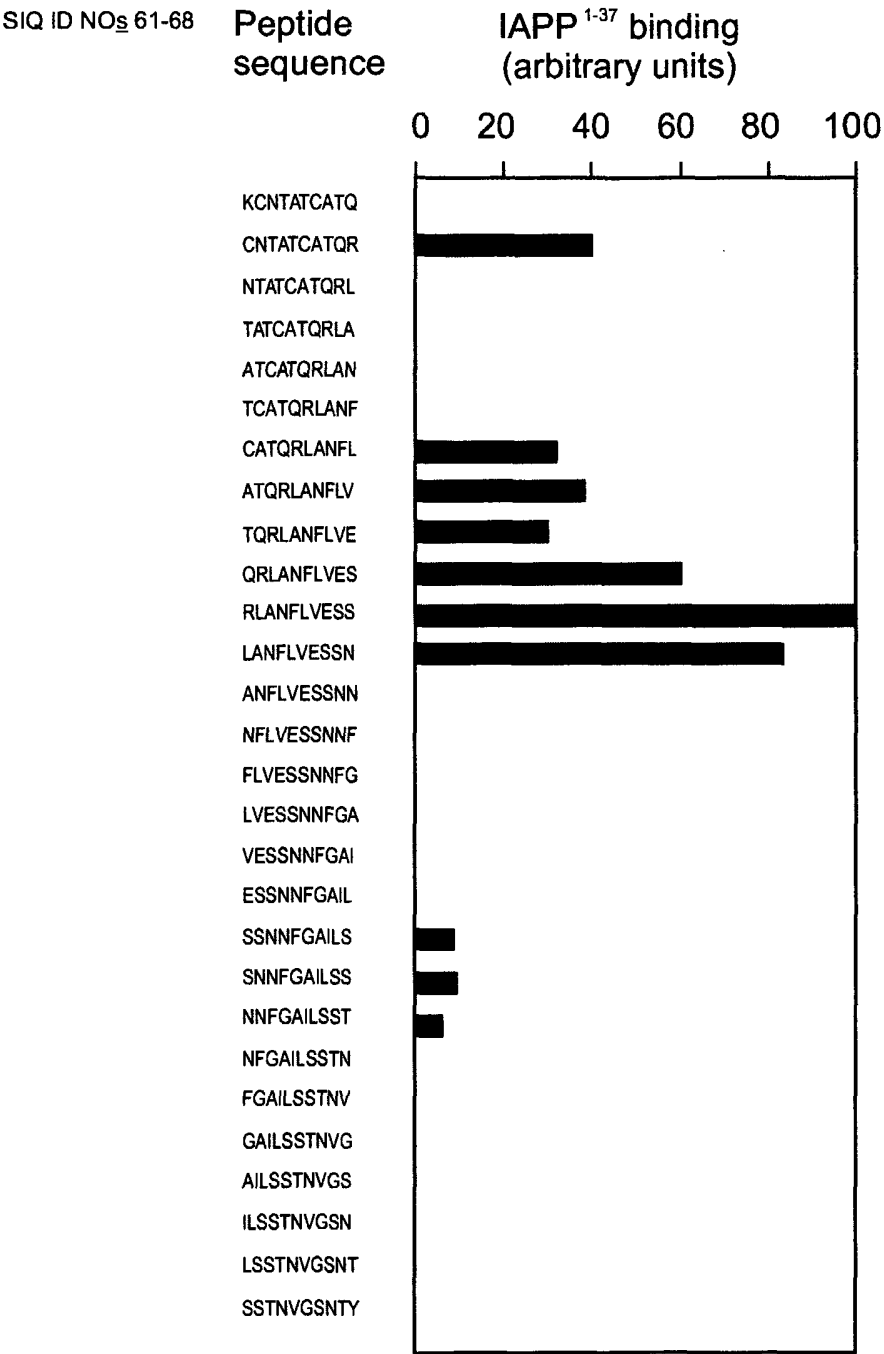


Fig. 10b

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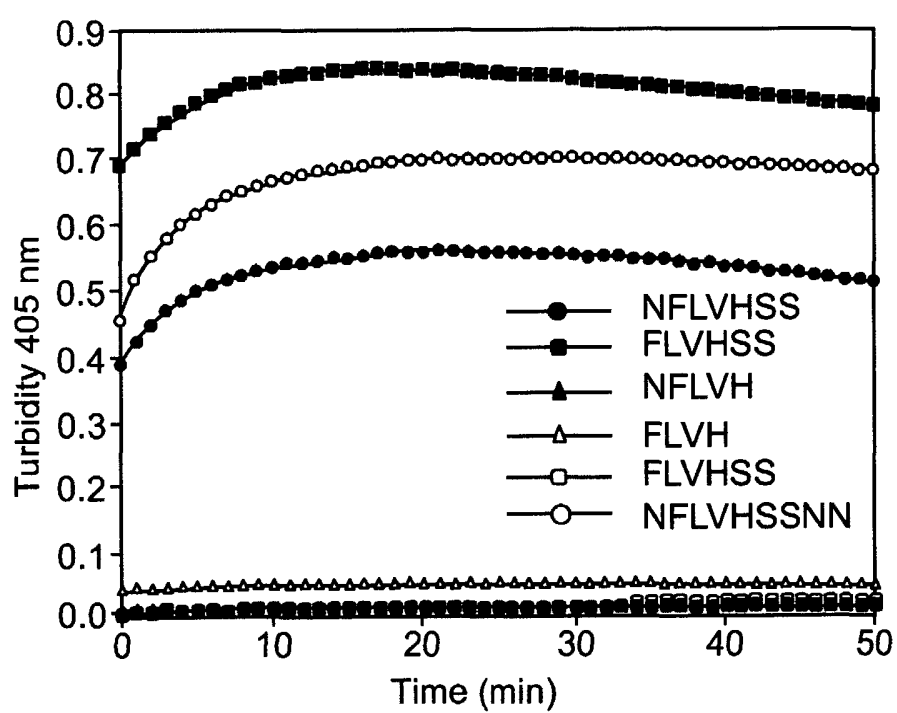


Fig. 11

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Fig. 12a



Fig. 12b

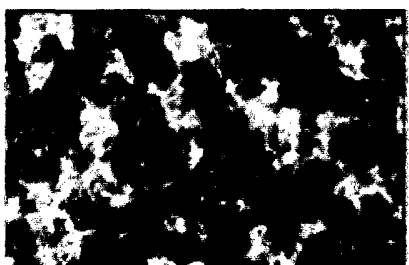


Fig. 12c



Fig. 12d

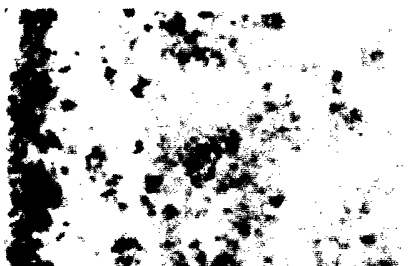


Fig. 12e

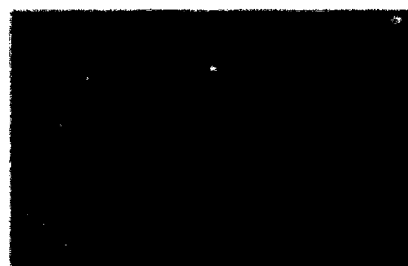


Fig. 12f

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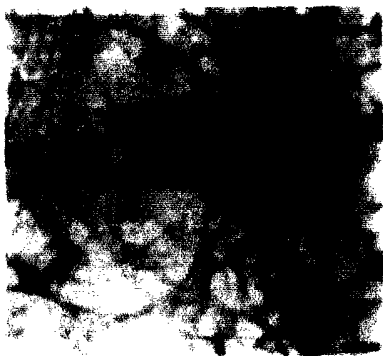


Fig. 13a



Fig. 13b



Fig. 13c

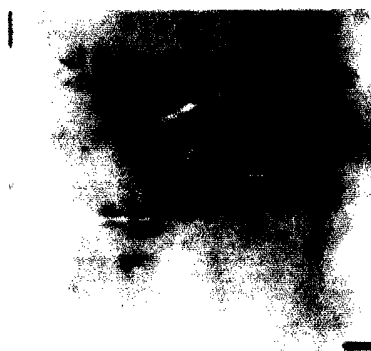


Fig. 13d



Fig. 13e

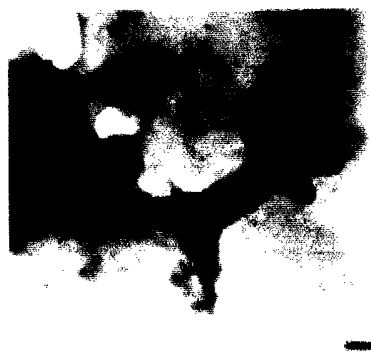


Fig. 13f

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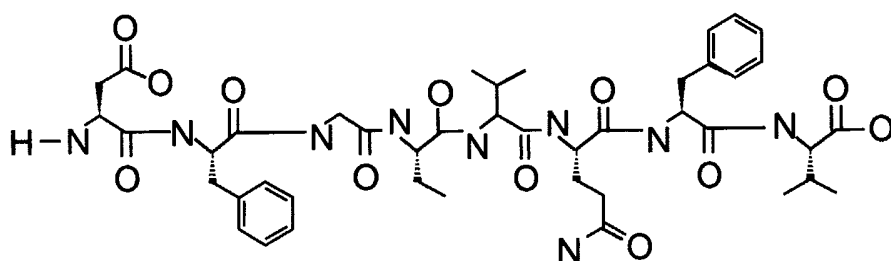
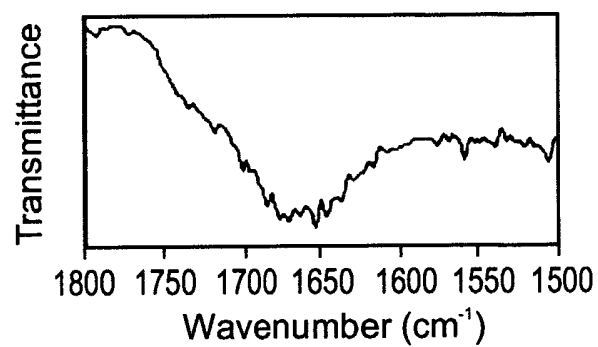
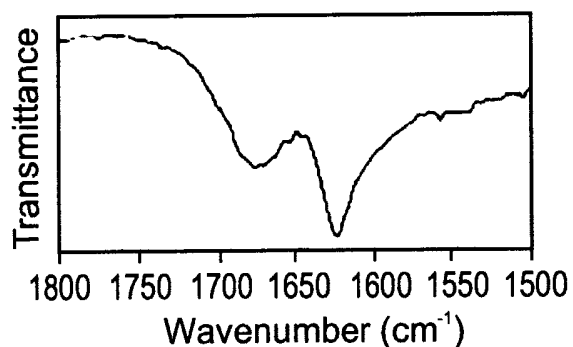
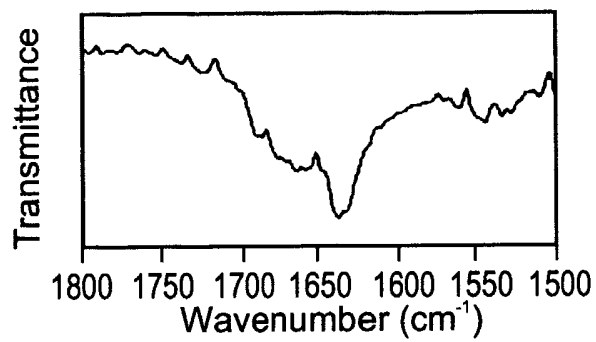
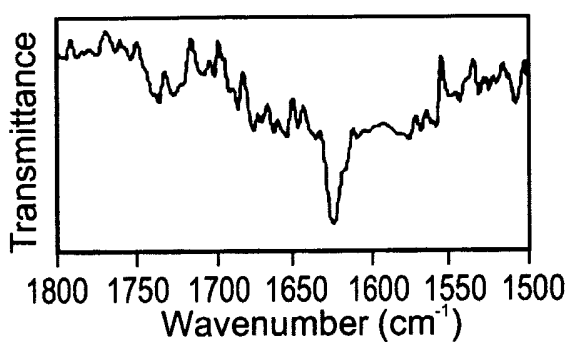
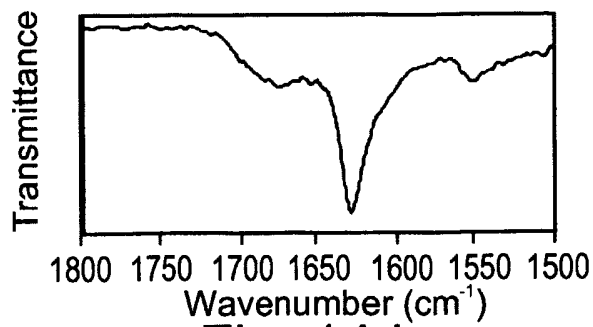
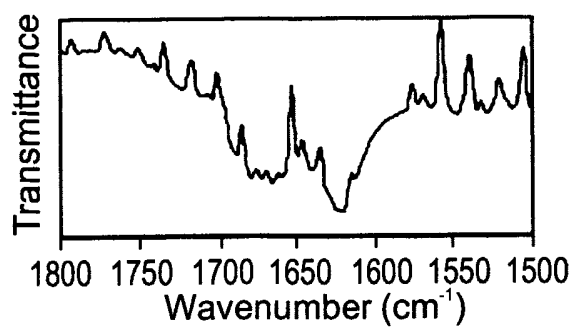


Fig. 15

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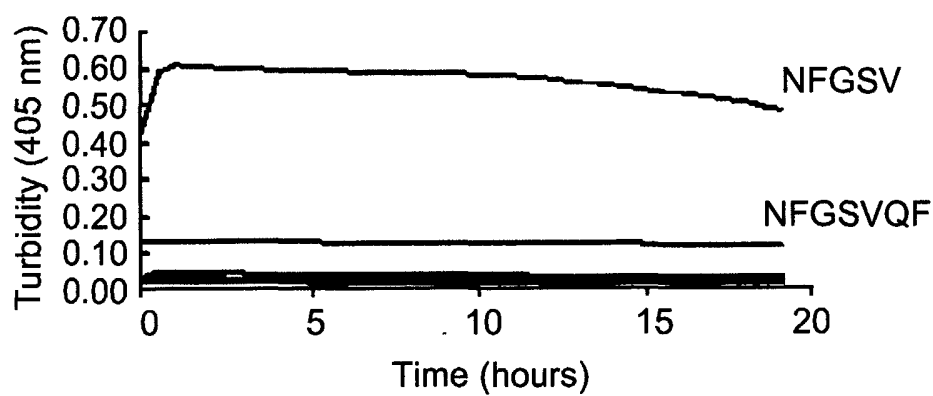


Fig. 16a

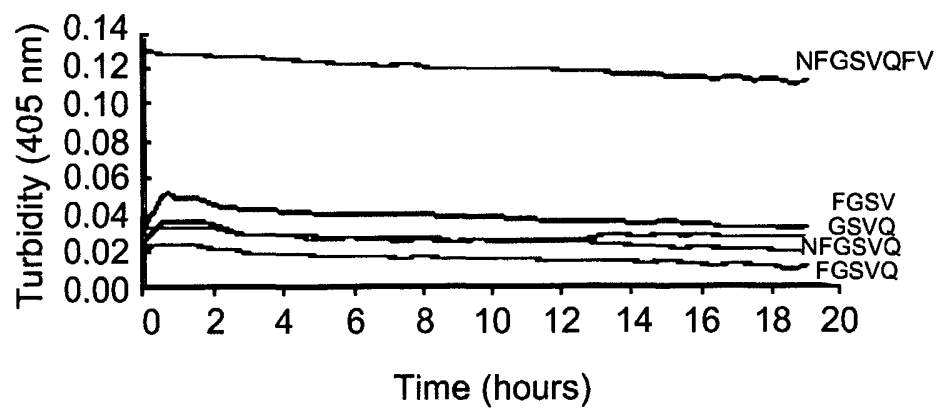


Fig. 16b

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Fig. 17a



Fig. 17b

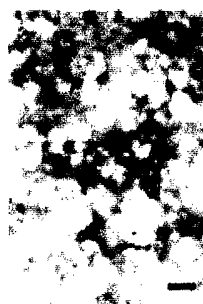


Fig. 17c



Fig. 17d



Fig. 17e



Fig. 17f

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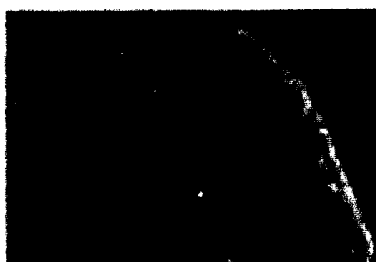


Fig. 18a



Fig. 18b



Fig. 18c



Fig. 18d



Fig. 18e

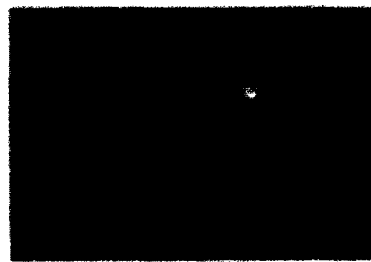


Fig. 18f

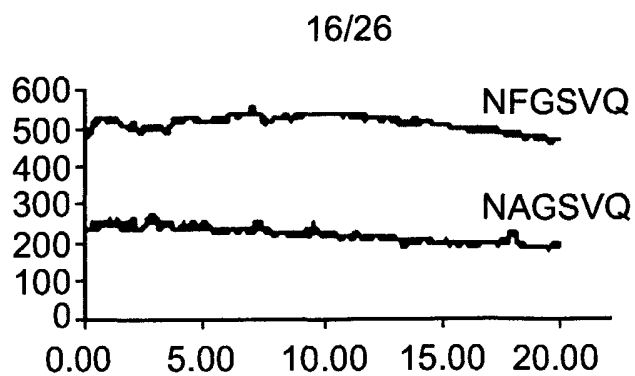


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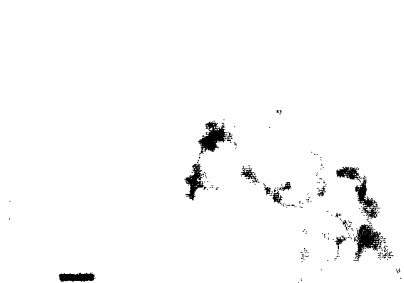


Fig. 19b

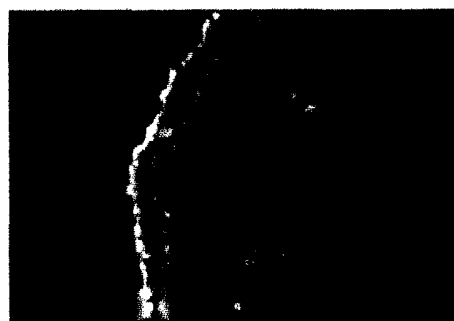
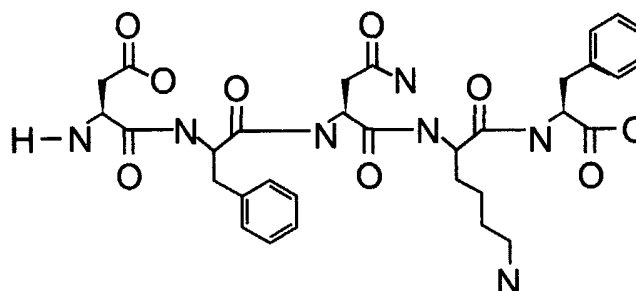


Fig. 19c



Fig. 20b



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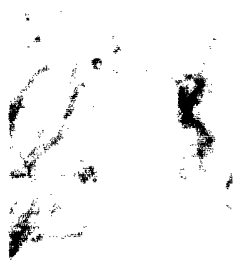


Fig. 21a



Fig. 21b



Fig. 21c

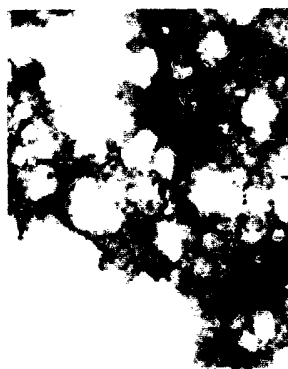


Fig. 21d

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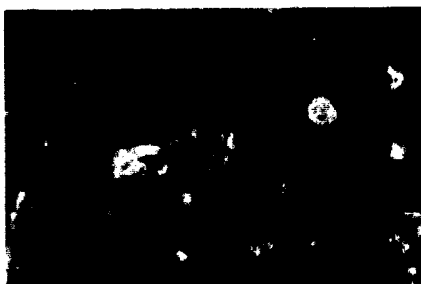


Fig. 22a



Fig. 22b



Fig. 22c



Fig. 22d

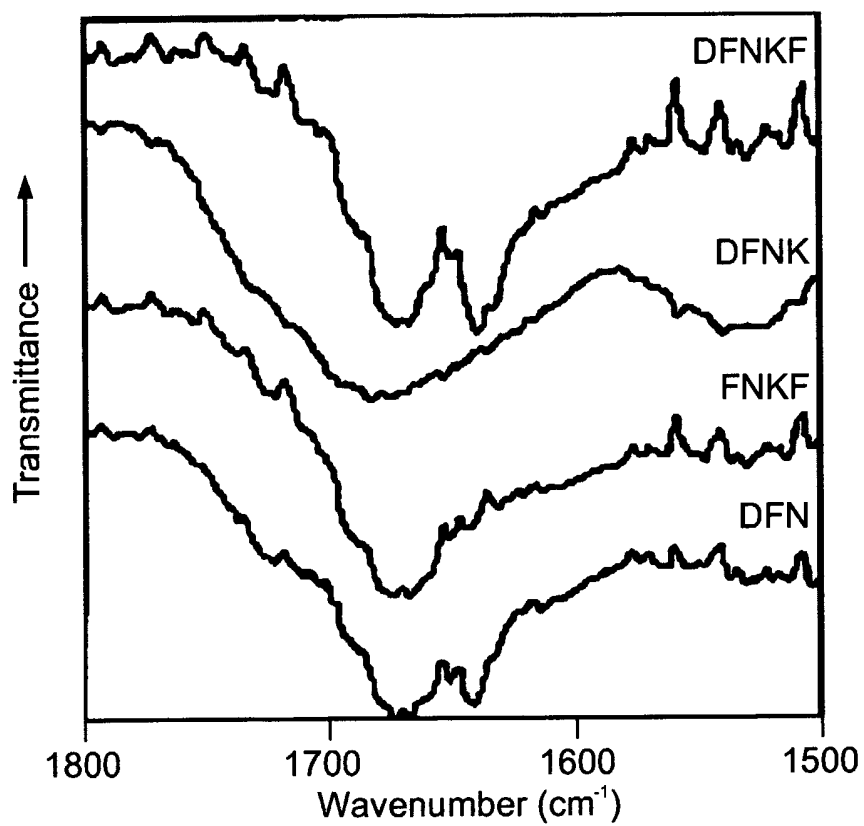


Fig. 23

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Fig. 24a

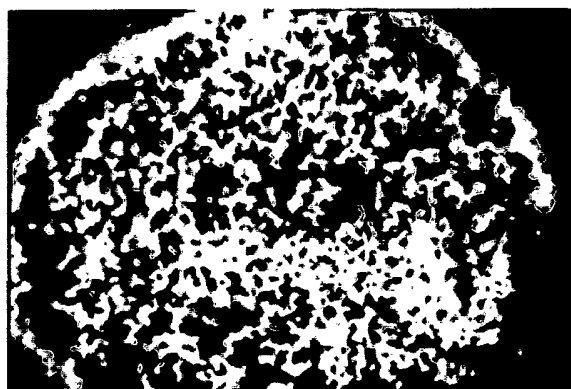


Fig. 24b

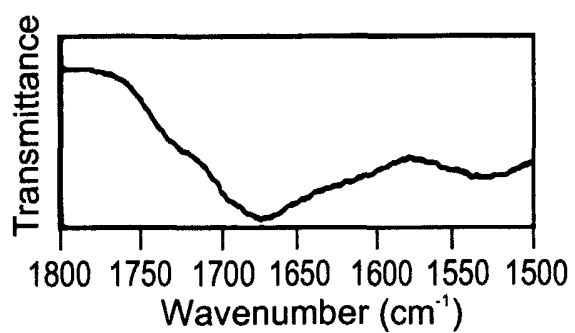


Fig. 24c

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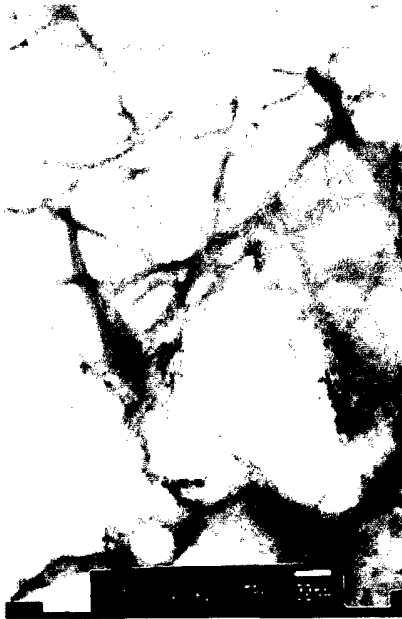


Fig. 25

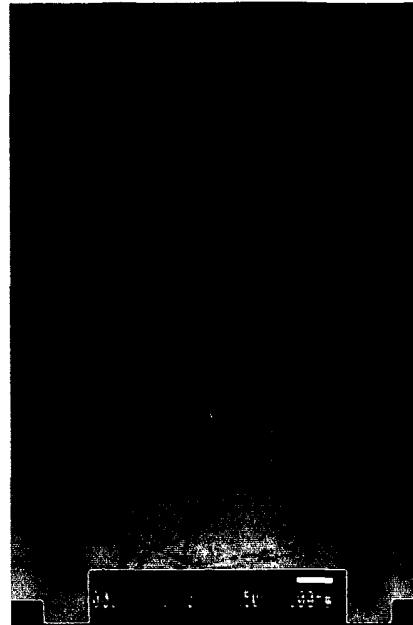


Fig. 26



Fig. 27



Fig. 28

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Fig. 29



Fig. 30

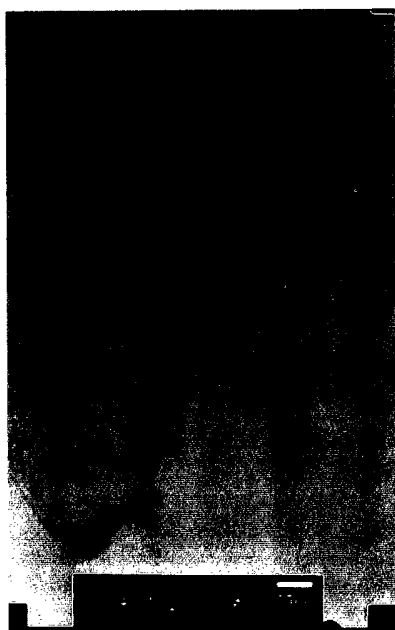


Fig. 31



Fig. 32

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Fig. 33



Fig. 34



Fig. 35



Fig. 36

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Fig. 37

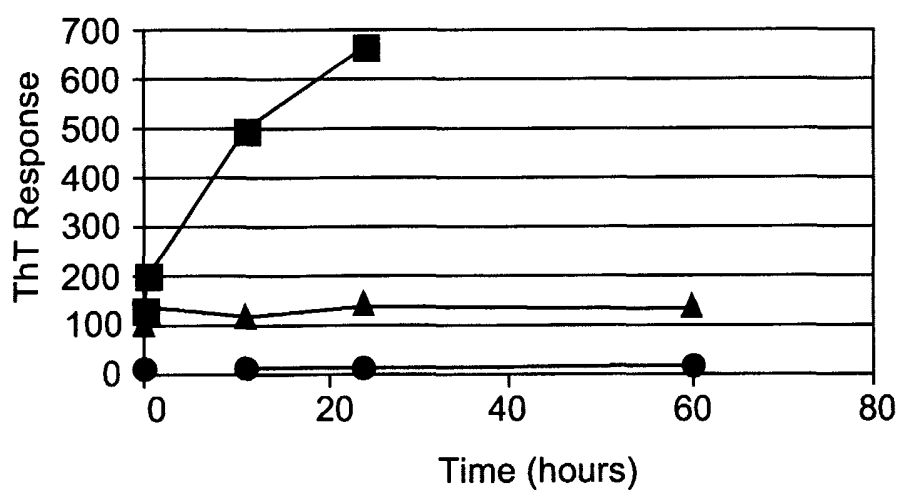


Fig. 38

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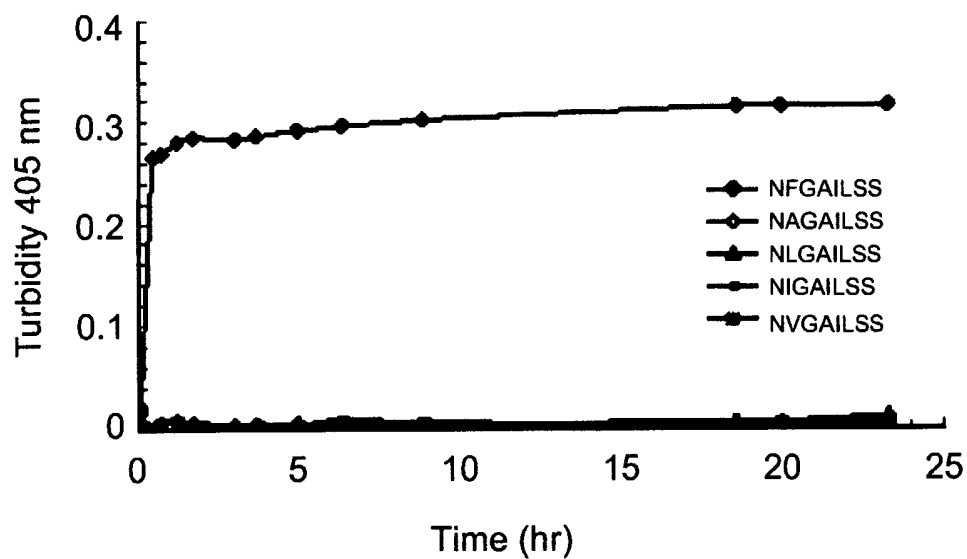


Fig. 39

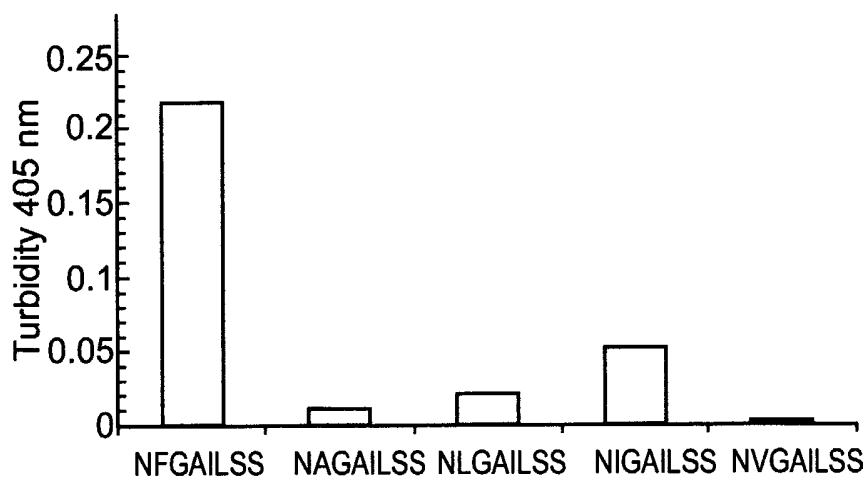


Fig. 40

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Fig. 41a



Fig. 41b

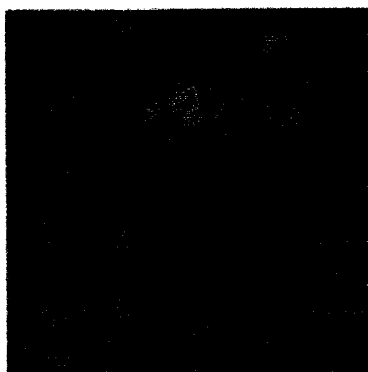


Fig. 41c



Fig. 41d



Fig. 41e



Fig. 41f

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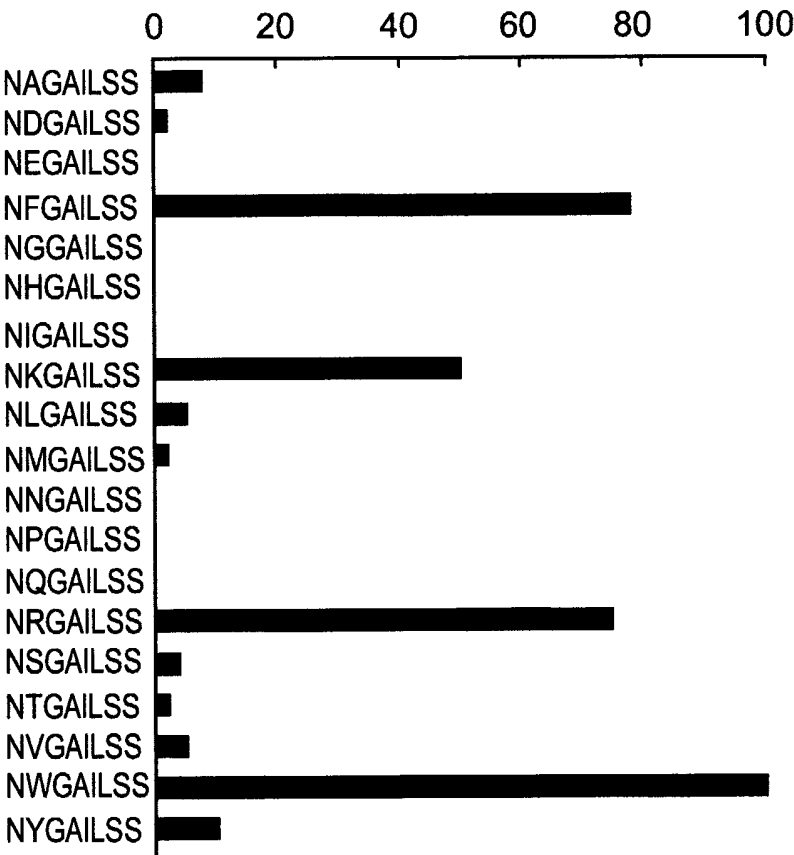
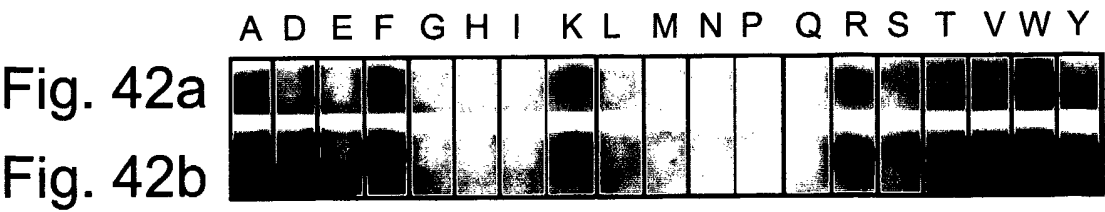


Fig. 42c

1
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Asn Ile Gly Ala Ile Leu Ser Ser
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Asn Thr Gly Ala Ile Leu Ser Ser
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Asn Phe Gly Ala Ile Leu
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