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Fortsættes ...

DESCRIPTION

FIELD OF THE INVENTION

[0001] The invention relates to a method for constructing a partial or complete peptide library with cyclic peptides which can display an array of short peptides required for a partial or complete peptide library such as a tetrapeptide library, a pentapeptide library, a hexapeptide library, a heptapeptide library, or an octapeptide library, etc. The method includes constructing an expression vector for the expression of the tagged cyclic peptides. Each tagged cyclic peptide displays an array of peptides of different sizes, and the number of cyclic peptides needed for constructing a partial or complete peptide library can be dramatically reduced relative to conventional chemical peptide synthesis. Furthermore, the libraries can be easily reproduced. The cyclic peptides can also be chemically and enzymatically synthesized to construct a partial or complete peptide library. The improved peptide library preparation method can particularly be used, for example, to construct a complete pentapeptide or hexapeptide library.

BACKGROUND OF THE INVENTION

[0002] Peptide libraries, which contains a great number of peptides that have a systematic combination of amino acids, are widely applied as a powerful tool for screening large numbers of peptides in the search for critical bioactive peptides in biological research, protein related study and drug development. Peptides with low molecular weight have been known to be less allergenic and the diverse physiological roles of peptides make them suitable candidates for the development of therapeutic agents (Host A, Halken S. Hypoallergenic formulas - when, to whom and how long: after more than 15 years we know the right indication! Allergy 2004, 59 (Suppl. 78):45-52; Lax R. The future of peptide development in the pharmaceutical industry. Phar Manufacturing: Int Pept Rev 2010; Agyei D, Danquah MK. Industrial scale manufacturing of pharmaceutical grade bioactive peptides. Biotechnol Adv 2011, 29 (3):272-7). Bioactive peptides are therefore suitable candidates for a new era of pharmaceutical products, especially with the heightened concerns of side effects of small molecule drugs and the increased attention to fresher and 'greener' foods and nutraceuticals possessing health-preventing or health-promoting properties (Danquah MK, Agyei D. Pharmaceutical applications of bioactive peptides. OA Biotechnology 2012, 1(2):5).

Many kinds of bioactive peptides have been found, which include antimicrobial, anticancer, antioxidative, antihypertensive, antithrombotic, opioid, antiviral, cytomodulatory and immunomodulatory peptides, etc. (Sharma S, Singh R, Rana S. Bioactive peptide: A Review. Int. J. BioAUTOMATION 2011, 15(4):223-250; Danquah MK, Agyei D. Pharmaceutical applications of bioactive peptides. OA Biotechnology 2012, 1(2):5). Therefore, peptide drug development is one of the most promising fields in the development of the new drugs.

Since a peptide library can provide a powerful tool for drug development, protein-protein

interactions, and other biochemical as well as pharmaceutical applications, several methods have been developed to construct peptide libraries. The peptide library construction methods fall into two categories: methods involving synthetic chemistry and methods involving biotechnology approaches.

Introduced in 1985 by George P. Smith, the phage display technology allows the screening of a vast amount of different peptides (Smith, G.P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 1985, 228:1315-1317). It was found that the success of phage derived peptides essentially depends on the quality of the library screened (Lindner T, Kolmar H, Haberkorn U and Mier W. DNA Libraries for the Construction of Phage Libraries: Statistical and Structural Requirements and Synthetic Methods. Molecules 2011, 16:1625-1641), however, there is no practical method to monitor or guarantee the quality of the phage display library. Indeed, until now only few of the peptides selected by phage display have entered clinical applications (Lindner T, Kolmar H, Haberkorn U and Mier W. DNA Libraries for the Construction of Phage Libraries: Statistical and Structural Requirements and Synthetic Methods. Molecules 2011, 16:1625-1641).

Based on the solid phase synthesis developed by Merrifield, the combinatorial "split-mix synthesis" method was developed for peptide library construction (Á. Furka, F. Sebestyen, M. Asgedom, G. Dibo, General method for rapid synthesis of multicomponent peptide mixtures. Int. J. Peptide Protein Res., 1991, 37:487-493). Theoretically a huge number of peptides can be synthesized in this way to make a large library, however in practice, the number and quantity of the peptides synthesized in this way is limited due to the high cost and low production yields of synthesizing the library.

Peptides can also be produced by recombinant approaches. Short peptides are usually fused to a protein and then expressed and purified from an expression host such as E. coli. (S. Hara, M. Yamakawa, Production in Escherichia coli of moricin, a novel type antibacterial peptide from the silkworm, Bombyx mori. Biochem. Biophys. Res. Commun. 1996, 224:877-878. H.K. Kim, D.S. Chun, J.S. Kim, C.H. Yun, J.H. Lee, S.K. Hong, D.K. Kang, Expression of the cationic antimicrobial peptide lactoferricin fused with the anionic peptide in Escherichia coli. Appl. Microbiol. Biotechnol. 2006, 72:330-338). However in practice, the number and quantity of the peptides produced in this way is also limited due to the long procedure and high cost, and the capacity of peptide library constructed in this way is very low.

Compared to their linear counterparts, cyclic peptides usually show better biological activity due to the conformational rigidity and higher resistance to hydrolysis by exopeptidases due to the lack of both amino and carboxyl termini (Sang Hoon Joo, Cyclic Peptides as Therapeutic Agents and Biochemical Tools. Biomol Ther. 2012, 20 (1): 19-26), which make the cyclic peptides better candidates for drug screening. Cyclic peptides can also be produced by either synthetic chemistry or recombinant approaches based on split inteins (Sang Hoon Joo, Cyclic Peptides as Therapeutic Agents and Biochemical Tools. Biomol Ther. 2012, 20 (1):19-26. Manfredi Miraula, Charmaine Enculescu, Gerhard Schenk, Nataša Mitic, Inteins - A Focus on the Biotechnological Applications of Splicing-Promoting Proteins. American Journal of Molecular Biology 2015, 5:42-56). Just like linear peptides produced by recombination approaches, the number and quantity of the cyclic peptides produced in this way is also limited due to the long procedure and high cost, and the capacity of peptide library constructed in this way is low.

[0003] A peptide library can be constructed by synthesizing a large number of distinct peptides. Since there is no good way to predict which peptide will be a good drug candidate, it is desired to construct a complete peptide library, which contains all possible combinations of the amino acids, for high chance of finding good drug candidates during peptide screening. There are 20 natural occurring amino acids, 8,000 distinct tripeptides need to be synthesized to construct a complete tripeptide library. In the similar way, 160,000 tetrapeptides need to be synthesized to construct a complete tetrapeptide library, 3,200,000 pentapeptides need to be synthesized to construct a complete pentapeptide library, and 64,000,000 hexapeptides need to be synthesized to construct a complete hexapeptide library. Due to the high cost and low production yields to synthesize the large number of peptides, until now, only a complete tripeptide virtual library (8,000 peptides) containing all possible combinations of the 20 natural amino was synthesized using chemical method for developing a new class of COX-2 inhibitors (Ermelinda V, et al., Design, Synthesis, and Evaluation of New Tripeptides as COX-2 Inhibitors for developing a new class of COX-2 inhibitors. Journal of Amino Acids. 2013, 2013:1-7), a complete tetrapeptide library, which should contain 160,000 tetrapeptides, is even not currently available in the market, not mention a complete pentapeptide or hexapeptide library and so on.

[0004] Thus, there is still a need for a highly productive process for constructing virtual peptide libraries with large capacities. A protein display method was previously described (PEPTIDE LIBRARY CONSTRUCTING METHOD AND RELATED VECTORS, PCT/CN2015/083260) for constructing virtual peptide libraries with large capacities, with which an array of short peptides were displayed at the end of a tag protein such as GST to reduce the number of the peptides needed to build a complete tetrapeptide, pentapeptide or hexapeptide library and so on. However, with the protein display method, each tagged linear peptide, which can display many short peptides, displays much less longer peptides. For example, a tagged linear 50-mer peptide can display 47 tetrapeptides, 46 pentapeptides or 45 hexapeptides, but only two 49-mer peptides and only one 50-mer peptide, limiting the capacity of the library. Therefore, it would be desirable to have a method for constructing virtual peptide libraries with even large capacities.

US patent specification no. US 7,105,341 B2 describes compositions that relate to retroviral vectors encoding intein containing polypepetides.

PCT patent application no. WO 2017/004745 A1 describes a peptide library preparation method for constructing complete peptide libraries.

The scientific publication entitled "Split-intein mediated circular ligation used in the synthesis of cyclic peptide libraries in E. coli" (Tavassoli et al., Nature protocols, 2007, vol. 2, No. 5, page 1126) describes the production of cyclic peptide libraries of between 10^7 and 10^8 transformants.

The scientific publication entitled "Intein-mediated cyclization of randomized peptides in the periplasm of *Escherichia coli* and their extracellular secretion" (Deschuyteneer et al., ACS Chemical Biology, 2010, vol. 5, No. 7, 691 - 700) describes cysteine-mediated splicing performed in the oxidative environment of the periplasm of *Escherichia coli.*

BRIEF SUMMARY OF THE INVENTION

[0005] It is an object of this invention to provide improved methods for constructing peptide libraries with high capacities. Instead of displaying peptides at the end of a protein tag, a linear peptide is cyclized to form a cyclic peptide to display more peptides, thus to increase the capacity of the library. For example, with reasonable designing, a cyclic peptide of 50 amino acids can actually diaplay 50 distinct consecutive tripeptides, 50 distinct consecutive tetrapeptides, 50 distinct consecutive pentapeptides, 50 distinct consecutive hexapeptides, 50 distinct consecutive heptapeptides, and so on, even 50 distinct consecutive 48-mer peptides, 50 distinct consecutive 49-mer peptides and 50 50-mer peptides, which constitutes 2400 different peptides with sizes ranging from 3 to 50 amino acids.

[0006] It is another object of this invention to provide an improved method for constructing a complete tetrapeptide library. With reasonable designing, a cyclic peptide of 50 amino acids can actually display 50 distinct consecutive tetrapeptides, 3,200 cyclic 50-mer peptides instead of 160,000 synthetic tetrapeptides are only needed to construct a complete tetrapeptide library by this protein display method. In the similar way, only 2,000 cyclic 80-mer peptides are needed to construct a complete tetrapeptide library. The number of larger cyclic peptides can be further reduced to construct a complete tetrapeptide library.

[0007] It is yet another object of this invention to provide an improved method for constructing a complete pentapeptide library. With reasonable designing, a cyclic peptide of 50 amino acids can actually display 50 distinct consecutive pentapeptides, 64,000 cyclic 50-mer peptides instead of 3,200,000 synthetic pentapeptides are only needed to construct a complete pentapeptide library by this protein display method. In the similar way, only 40,000 cyclic 80-mer peptides are needed to construct a complete pentapeptide library. The number of larger cyclic peptides can be further reduced to construct a complete pentapeptide library.

[0008] It is yet another object of this invention to provide an improved method for constructing a complete hexapeptide library. With reasonable designing, a cyclic peptide of 50 amino acids can actually display 50 distinct consecutive hexapeptides, 1,280,000 cyclic 50-mer peptides instead of 64,000,000 synthetic hexapeptides are only needed to construct a complete hexapeptide library by this protein display method. In the similar way, only 800,000 cyclic 80-mer peptides are needed to construct a complete hexapeptide library. The number of larger cyclic peptides can be further reduced to construct a complete hexapeptide library.

[0009] It is yet another object of this invention to provide an improved method of constructing a peptide library with large capacity using a chemical synthesis approach, the method comprising: (i) designing a series of cyclic peptides with each cyclic peptide displaying an array of short peptides; (ii) synthesizing the linear peptides with the corresponding cyclic peptide sequences with chemical methods with or without purification; (iii) cyclizing the linear peptides to make the cyclic peptides with chemical methods or enzymatic methods; (iv) purifying the cyclic peptides and using the library for screening.
It is yet another object of this invention to provide an improved method of constructing a cyclic

peptide library with large capacity, the method comprising: (i) designing a series of cyclic peptides with each cyclic peptide containing an array of short peptides and one or more affinity tags; (ii) constructing a series of expression vectors with each vector expressing a single cyclic peptide; (iii) expressing and purifying the cyclic peptides by binding the affinity tag to a specific ligand immobilized to a solid phase; (iv) eluting the cyclic peptides from the solid phases and using the library for screening; and (v) optionally using the immobilized cyclic peptides directly for screening without being eluted from the solid phases

It is yet another object of this invention to provide a method for constructing an incomplete or partial peptide library with a significantly reduced number of cyclic peptides. For longer peptides such as heptapeptides, octapeptides, decapeptides, or even longer peptide such as 15-mer or 20-mer peptides, the complete libraries will contain huge numbers of all possible peptides, which are 1.28×10^9, 2.56×10^{10}, 1.024×10^{13}, 3.277×10^{19}, 1.049×10^{26}, respectively. Therefore, it is not practical to chemically synthesize so many peptides to make a complete peptide library. However, it is still desirable to construct a partial library since some peptides will share high sequence similarity. By rational designing, the number of peptides to construct an efficient partial peptide library can be greatly reduced. This method of the invention will further reduce the peptide number in a partial peptide library, thus to make the construction of an efficient peptide library practical.

It is a further object of this invention to provide an alternative method for constructing a complete peptide library. The DNA sequence of each cyclic peptide will be cloned into an expression vector for the expression and purification of the tagged cyclic peptide. The vector can be stored and the peptide can be expressed or reproduced from the vector readily at any time. Unlike peptide synthesis, each peptide needs to be resynthesized from scratch.

[0010] It is an object of this invention to provide a method for constructing expression vectors for the expression and purification of the tagged cyclic peptides for the peptide library construction.

[0011] Additional objects of the invention are reflected in the original claims. The details of embodiments of the disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0012] The foregoing brief summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited by the drawings presented.

[0013] In the drawings:

FIG. 1 schematically illustrates that a cyclic peptide can display many shorter peptides, and one pentapeptide, DECAF (Asp-Glu-Cys-Ala-Phe), is shown as an example;

FIG. 2 schematically illustrates the high capacity of cyclic peptides for displaying short peptides according to an embodiment of the invention;

FIG. 3 schematically illustrates a typical example of 80 different pentapeptides displayed by a single cyclic 80-mer cyclic peptide;

FIG. 4 schematically illustrates the map of a vector for split intein-based cyclic peptide expression;

FIG. 5 schematically illustrates the expression and purification of the 80-mer cyclic peptide;

DETAILED DESCRIPTION OF THE INVENTION

[0014] Unless defined otherwise, all technical and scientific terms used herein have the same

[0015] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0016] Embodiments of the present invention relate to methods useful for constructing a peptide library. In one aspect, the invention relates to a significant improvement of the construction of a peptide library by conventional chemical synthesis. For example, the present invention provides an improved method for the construction of a complete peptide library whereby the cyclic peptides are expressed and purified and the number of cyclic peptides needed to form a complete library is significantly reduced.

[0017] According to embodiments of the present invention, the amino acid sequences of the cyclic peptides are rationally designed so that each cyclic peptide can display an array of distinct peptides of different sizes, thus to increase the capacity of the library significantly. Depending on the sizes of the cyclic peptides, each cyclic peptide can display 10 to 100 or even 200 of distinct peptides of a specific size. Therefore, methods according to embodiments of the present invention greatly reduce the peptide number in a peptide library, thus the construction and screening of a peptide library will be performed at significantly reduced costs.

[0018] As used herein, the terms a "peptide", a "library", a "tag", a "protein", a "vector", "capacity", "cyclic", "complete" and an "array" are to be taken in their broadest context.

[0019] In one general aspect, the present invention relates to an improved method of constructing complete peptide libraries with reduced peptide numbers as compared with the

conventional peptide synthesis method. For example, as illustrated in FIG. 1 and 2, a cyclic peptide can display many shorter peptides. In other words, many short peptides can be displayed by a single cyclic peptide, namely "protein display". Instead of synthesizing 80 different pentapeptides, for example, one cyclic 80-mer peptide can display 80 different pentapeptides, thus to significantly reduce the number of peptides needed to construct a complete pentapeptide library and at the same time increase the screening efficiency. Compared with protein expression, chemical synthesis of an 80-mer peptide will take several days for chemical synthesis and head-to-tail peptide cyclization, while the similar cyclic peptide can be readily expressed in a bacteria host overnight. The overall yield of chemically synthesized 80-mer cyclic peptide will be low, while the similar peptide can be readily expressed in an expression host at any scale. In contrast, an embodiment of the present invention provides a method whereby a complete peptide library can be constructed with significantly reduced peptide number, these peptides can be readily expressed and purified, and these peptides can also be readily reproduced at any time using the cloned genes. The method according to the embodiment of the present invention can greatly cut down the time and the cost required for peptide library construction and screening.

[0020] Embodiments of the invention relate to protein tags useful in expressing and purifying cyclic peptides. One of the protein tags is GST (glutathione S-transferase), which is commonly used to purify a target protein fused with GST. GST can help the expression of the target protein or peptide. Another common tag is His Tag which contains a string of histidine residues for tagged peptide purification.

[0021] In one embodiment of the invention, the protein tag comprises one of the tags selected from, but not limited to, for example, GST, His Tag, Trx, SUMO, CBD, FLAG, HA, AviTag, Myc-Tag, SBP, Strep-Tag, Fc-Tag, Halo-Tag, V5, VSV, MBP, etc.

[0022] In one embodiment of the invention, the protein tag comprises a combination of the tags selected from, but not limited to, for example, GST, His Tag, Trx, SUMO, CBD, FLAG, HA, AviTag, Myc-Tag, SBP, Strep-Tag, Fc-Tag, Halo-Tag, V5, VSV, MBP, etc.

[0023] In yet a further embodiment of the invention, tags can be added at any positions of the cyclic peptide to facilitate the expression and purification of the cyclic peptide. The protein tags can be selected from, but not limited to, GST, His Tag, Trx, SUMO, CBD, FLAG, HA, AviTag, Myc-Tag, SBP, Strep-Tag, Fc-Tag, Halo-Tag, V5, VSV, MBP, etc.

[0024] A protease recognition sequence can also be added between the tag and the peptide so that the peptide can be cleaved from the tag using a protease if necessary.

[0025] It is apparent to those skilled in the art that the present invention includes modifications to the above-mentioned embodiments to further improve the library construction. These modifications include, but are not are limited to, adding one or multiple peptide sequences to the above embodiment. For example, one can add some specific amino acids in the peptide sequences to facilitate intein splicing.

[0026] A variety of methods can be used to design the peptide sequences to prepare an efficient peptide library in view of the present disclosure. For example, to facilitate the soluble expression of the tagged peptide, peptide sequences containing long stretches of hydrophobic amino acids should be avoided. It is apparent to those skilled in the art that many different trans-splicing intein systems can be used to produce cyclic peptides. For example, Npu DnaE split intein (Hideo Iwai, Sara Z
ù
ger, Jennifer Jin, Pui-Hang Tam, Highly efficient protein trans-splicing by a naturally split DnaE intein from Nostoc punctiforme. FEBS Letters. 2006, 580:1853-1858), Ssp GyrB split intein, Ssp DnaB split intein, Ssp DnaE split intein, Mxe GyrA split intein, Mtu RecA split intein, and so on, can be used to produce cyclic peptides.

It is also apparent to those skilled in the art that the cyclic peptide can be designed in such a way that the cyclic peptide can display as many as possible distinct short peptides thus to increase the capacity of the peptide library.

According to embodiments illustrated in FIG. 3, an expressed cyclic peptide of 80 amino acids can actually display 80 distinct consecutive tetrapeptide, 80 distinct consecutive pentapeptide, 80 distinct consecutive hexapeptide, 80 distinct consecutive heptapeptide, ..., 80 distinct consecutive 78-mer peptides, 80 distinct consecutive 79-mer peptides and 80 80-mer peptides, which constitutes 6160 peptides with sizes ranging from 4 to 80 amino acids.

In the above-mentioned embodiments, those skilled in the art will know that the cyclic peptide can even contain up to 200 or more amino acids, although the optimal size will be between 10 to 100. Longer peptides will have higher chances to form tertiary structures in which some peptide sequences will not be exposed for screening.

In the above-mentioned embodiments, those skilled in the art will know that instead of synthesizing 160,000 tetrapeptides chemically, 3,200 or more expressed cyclic 50-mer peptides can constitute or display a complete tetrapeptide library, or 2,000 or more expressed cyclic 80-mer peptides can constitute or display a complete tetrapeptide library.

In the above-mentioned embodiments, those skilled in the art will know that instead of synthesizing 3,200,000 pentapeptides chemically, 64,000 or more expressed cyclic 50-mer peptides can constitute or display a complete pentapeptide library, or 40,000 or more expressed cyclic 80-mer peptides can constitute or display a complete pentapeptide library.

In the above-mentioned embodiments, those skilled in the art will know that some peptides in a peptide library will share high sequence similarity and the number of peptides to construct an efficient peptide library can be greatly reduced by rational designing. Therefore, a smaller number than 3,200 of expressed 50-mer cyclic peptides can constitute an efficient tetrapeptide library. Similarly, a smaller number than 64, 000 of expressed cyclic 50-mer peptides can constitute an efficient pentapeptide library.

In the above-mentioned embodiments, those skilled in the art will know that the cyclic peptide can contain more than 80 amino acids, thus to further reduce the number of the expressed cyclic peptides. Therefore, an even smaller number of expressed peptides can constitute an efficient tetrapeptide, pentapeptide or even hexapeptide library.

In the above-mentioned embodiments, those skilled in the art will know that some peptides in a peptide library will share high sequence similarity and structure similarity, and the number of peptides to construct an efficient peptide library can be further reduced by rational designing.

Therefore, a practical number of expressed cyclic peptides can constitute an efficient polypeptide library, such as an octapeptide library, a decapeptide library, or even longer peptide libraries, such as a 15-mer peptide library, a 20-mer peptide library or even a 30-mer peptide library.

In a preferred embodiment, 2, 000 or more expressed His-tagged cyclic 80-mer peptides can be designed, cloned into an expression vector, expressed and purified to constitute a complete tetrapeptide library.

In a further preferred embodiment, 40, 000 or more expressed His-tagged cyclic 80-mer peptides can be designed, cloned into an expression vector, expressed and purified to constitute a complete pentapeptide library, which also constitute a complete tetrapeptide library.

In the above-mentioned embodiments, those skilled in the art will know that other tag or tags can also be used instead of His Tag, the tags can be selected from, but not. limited to, GST, Trx, SUMO, CBD, FLAG, HA, AviTag, , Myc-Tag, SBP, Strep-Tag, Fc-Tag, Halo-Tag, V5, VSV, MBP, etc.

In the above-mentioned embodiments, those skilled in the art will know that the tagged cyclic peptide can contain more than or less than 80 amino acids, thus to further reduce or increase the number of the expressed tagged cyclic peptides to constitute a complete peptide library.

According to embodiments illustrated in FIG. 4, an expression vector of tagged cyclic peptides can be constructed to contain optionally one or more affinity tag DNA sequences, DNA sequences for the two complementary fragments of a split intein (C-terminal intein motif Npu $DnaE_C$ and an N-terminal intein motif Npu $DnaE_N$), a peptide expression DNA sequence, and optionally a protease recognition site which can be used to linearize the cyclic peptide if necessary.

In the above-mentioned embodiments, those skilled in the art will know that the expression vector can be constructed based on, but not limited to, a bacteria expression vector, an yeast expression vector, an insect expression vector, a fungal expression vector, a mammalian expression vector, and a plant expression vector.

In another embodiment of the present invention, the tagged cyclic peptides can be expressed in and purified from an expression host. The expression host is selected from the group consisting of, but not limited to, a bacteria cell, a yeast cell, an insect cell, a fungal cell, a mammalian cell, and a plant cell.

It is readily appreciated by those skilled in the art that, similar methods can also be generally applied for constructing a cyclic peptide library. The designed cyclic peptides are expressed, purified using an affinity tag immobilized on a solid surface, and then used for screening with the peptides still attached to the solid surface. The designed cyclic peptides can also be eluted from the solid surface and then used for screening.

Various embodiments of the invention have now been described. It is to be noted, however, that this description of these specific embodiments is merely illustrative of the principles underlying the inventive concept.

[0027] The following specific examples are further illustrative of the nature of the invention, it needs to be understood that the invention is not limited thereto.

Protein Display Example

[0028] FIG. 3A shows the cyclic structure and the amino acid sequence of an 80-mer cyclic peptide, FIG. 3B shows that the 80-mer cyclic peptide can display 80 distinct pentapeptides.

[0029] The DNA sequence, as shown below, which includes the two Npu DnaE split-intein motifs for the expression of the 80-mer cyclic peptide, was cloned into the pET28a vector as shown in FIG. 4.

[0030] The DNA sequence:
ATGATCAAGATTGCTACGCGCAAATACTTGGGAAAACAGAACGTTTATGATATCGGAGTGGAA
CGTGACCACAATTTTGCTCTGAAAAACGGATTCATCGCAAGTAATTGCTGGGAAAGTGGG
AAGATGACCGGTATCGTGAAGTGGTTTAACGCTGACAAGGGGTTTGGTTTCATTA
CTCCAGATGACGGTTCTAAGGATGTTTTCGTTCACTTTTCCGCGATTCAAAACGA
CGGGTACAAATCCTTGGATGAGGGCCAAAAAGTCTCATTTACGATTGAATCCGGG
GCCAAGGGTCCCGCAGCAGGAAACGTAACTAGCTTATCCAAAACTCACCACCATC
ATCACTGTTTGTCCTACGAGACCGAGATCCTTACAGTAGAATATGGTTTGTTACCCATT
GGAAAGATCGTGGAGAAGCGTATCGAATGCACAGTGTACAGCGTTGATAACAACGGTA
ACATTTATACCCAACCCGTGGCTCAGTGGCATGACCGTGGCGAACAAGAGGTCTTCGA
GTATTGCCTTGAGGACGGGTCTCTGATCCGCGCTACAAAAGATCATAAGTTTATGACCG
TTGACGGACAGATGCTGCCTATTGACGAAATTTTTGAGCGTGAACTTGACTTAATGCGT
GTCGATAACCTGCCTAATTAA, where the Npu DnaE C-terminal motif (Npu DnaEC) is shown in *italic font,* the 80-mer cyclic peptide in **bold font,** and the Npu DnaE N-terminal motif (Npu DnaEN) in normal font.

[0031] The corresponding protein sequence:
*MIKIATRKYLGKQNVYDIGVERDHNFALKNGFIASN***CWESGKMTGIVKWFNAD**
KGFGFITPDDGSKDVFVHFSAIQNDGYKSLDEGQKVSFTIESGAKGPAAG
NVTSLSKTHHHHHCLSYETEILTVEYGLLPIGKIVEKRIECTVYSVDNNGNIY
TQPVAQWHDRGEQEVFEYCLEDGSLIRATKDHKFMTVDGQMLPIDEIFEREL DLMRVDNLPN, where the Npu DnaE C-terminal motif (Npu $DnaE_C$) is shown in *italic font,* the 80-mer cyclic peptide in **bold font,** and the Npu DnaE N-terminal motif (Npu $DnaE_N$) in normal font.

[0032] The 80-mer cyclic peptide was expressed in an *E. coli* strain using IPTG as the inducer following standard gene expression method. FIG. 5A shows that the 80-mer cyclic peptide was expressed in a soluble form, wherein Lane 1 is a protein marker with the molecular weight listed on the left, Lane 2 is the whole cell lysate without induction, and Lane 3 is the whole cell lysate with 1 mM IPTG induction, the 80-mer cyclic peptide is indicated with an arrow.

[0033] The 80-mer cyclic peptide was purified using a Ni-IDA column. The 80-mer cyclic peptide was bound onto the Ni-IDA column and eluted from the column with imidazole. FIG. 5B shows that the 80-mer cyclic peptide was purified, wherein Lane 1 is a protein marker with the

molecular weight listed on the left, Lane 2 is the whole cell lysate with IPTG induction, Lane 3 is the flow-through of the whole cell lysate sample, and Lane 4 is the purified 80-mer cyclic peptide, which is indicated with an arrow.

[0034] Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to these embodiments. Indeed, various modifications for carrying out the invention are obvious to those skilled in the art and are intended to be within the scope of the following claims.

SEQUENCE LISTING

[0035]

<110> Xiangqun, LI

<120> PEPTIDE LIBRARY CONSTRUCTING METHOD

<130> SG17P092HT

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1               5                   10                  15

gat atc gga gtg gaa cgt gac cac aat ttt gct ctg aaa aac gga ttc          96
Asp Ile Gly Val Glu Arg Asp His Asn Phe Ala Leu Lys Asn Gly Phe
            20                  25                  30

atc gca agt aat tgc tgg gaa agt ggg aag atg acc ggt atc gtg aag         144
Ile Ala Ser Asn Cys Trp Glu Ser Gly Lys Met Thr Gly Ile Val Lys
        35                  40                  45

tgg ttt aac gct gac aag ggg ttt ggt ttc att act cca gat gac ggt         192
Trp Phe Asn Ala Asp Lys Gly Phe Gly Phe Ile Thr Pro Asp Asp Gly
    50                  55                  60

tct aag gat gtt ttc gtt cac ttt tcc gcg att caa aac gac ggg tac         240
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Ser Lys Asp Val Phe Val His Phe Ser Ala Ile Gln Asn Asp Gly Tyr
65                  70                  75                  80

aaa tcc ttg gat gag ggc caa aaa gtc tca ttt acg att gaa tcc ggg  288
Lys Ser Leu Asp Glu Gly Gln Lys Val Ser Phe Thr Ile Glu Ser Gly
                85                  90                  95

gcc aag ggt ccc gca gca gga aac gta act agc tta tcc aaa act cac  336
Ala Lys Gly Pro Ala Ala Gly Asn Val Thr Ser Leu Ser Lys Thr His
            100                 105                 110

cac cat cat cac tgt ttg tcc tac gag acc gag atc ctt aca gta gaa  384
His His His His Cys Leu Ser Tyr Glu Thr Glu Ile Leu Thr Val Glu
        115                 120                 125

tat ggt ttg tta ccc att gga aag atc gtg gag aag cgt atc gaa tgc  432
Tyr Gly Leu Leu Pro Ile Gly Lys Ile Val Glu Lys Arg Ile Glu Cys
    130                 135                 140

aca gtg tac agc gtt gat aac aac ggt aac att tat acc caa ccc gtg  480

Thr Val Tyr Ser Val Asp Asn Asn Gly Asn Ile Tyr Thr Gln Pro Val
145                 150                 155                 160

gct cag tgg cat gac cgt ggc gaa caa gag gtc ttc gag tat tgc ctt  528
Ala Gln Trp His Asp Arg Gly Glu Gln Glu Val Phe Glu Tyr Cys Leu
                165                 170                 175

gag gac ggg tct ctg atc cgc gct aca aaa gat cat aag ttt atg acc  576
Glu Asp Gly Ser Leu Ile Arg Ala Thr Lys Asp His Lys Phe Met Thr
            180                 185                 190

gtt gac gga cag atg ctg cct att gac gaa att ttt gag cgt gaa ctt  624
Val Asp Gly Gln Met Leu Pro Ile Asp Glu Ile Phe Glu Arg Glu Leu
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<220>

<223> Synthetic Construct

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            20                  25                  30

Ile Ala Ser Asn Cys Trp Glu Ser Gly Lys Met Thr Gly Ile Val Lys
        35                  40                  45

Trp Phe Asn Ala Asp Lys Gly Phe Gly Phe Ile Thr Pro Asp Asp Gly
    50                  55                  60

Ser Lys Asp Val Phe Val His Phe Ser Ala Ile Gln Asn Asp Gly Tyr
65                  70                  75                  80
```

```
Lys Ser Leu Asp Glu Gly Gln Lys Val Ser Phe Thr Ile Glu Ser Gly
                85                  90                  95

Ala Lys Gly Pro Ala Ala Gly Asn Val Thr Ser Leu Ser Lys Thr His
            100                 105                 110

His His His His Cys Leu Ser Tyr Glu Thr Glu Ile Leu Thr Val Glu
        115                 120                 125

Tyr Gly Leu Leu Pro Ile Gly Lys Ile Val Glu Lys Arg Ile Glu Cys

    130                 135                 140

Thr Val Tyr Ser Val Asp Asn Asn Gly Asn Ile Tyr Thr Gln Pro Val
145                 150                 155                 160

Ala Gln Trp His Asp Arg Gly Glu Gln Glu Val Phe Glu Tyr Cys Leu
                165                 170                 175

Glu Asp Gly Ser Leu Ile Arg Ala Thr Lys Asp His Lys Phe Met Thr
            180                 185                 190

Val Asp Gly Gln Met Leu Pro Ile Asp Glu Ile Phe Glu Arg Glu Leu
        195                 200                 205

Asp Leu Met Arg Val Asp Asn Leu Pro Asn
    210                 215
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aacgacgggt acaaatcctt ggatgagggc caaaaagtct catttacgat tgaatccggg      180

gccaagggtc ccgcagcagg aaacgtaact agcttatcca aaactcacca ccatcatcac      240
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atcgtggaga agcgtatcga atgcacagtg tacagcgttg ataacaacgg taacatttat      120

acccaacccg tggctcagtg gcatgaccgt ggcgaacaag aggtcttcga gtattgcctt      180

gaggacgggt ctctgatccg cgctacaaaa gatcataagt ttatgaccgt tgacggacag      240

atgctgccta ttgacgaaat ttttgagcgt gaacttgact taatgcgtgt cgataacctg      300

cctaattaa                                                              309
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Cited references

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Patentkrav

1. Integreret fremgangsmåde til konstruktion af et delvist eller fuldstændigt peptidbibliotek med cykliske peptider.

Denne fremgangsmåde omfatter:

(i) design og fremstilling af en række cykliske peptider, idet hvert cyklisk peptid udviser et array af forskellige peptider med forskellige størrelser og et eller flere affinitetstags, idet hvert peptid indeholder et array af korte peptidsekvenser, hvor peptidet omfatter et cyklisk peptid med 50 aminosyrer, der består af 50 separate på hinanden følgende tripeptider, 50 separate på hinanden følgende tetrapeptider, 50 separate på hinanden følgende pentapeptider, 50 separate på hinanden følgende hexapeptider, 50 separate på hinanden følgende heptapeptider, 50 separate på hinanden følgende 48-mer-peptider, 50 separate på hinanden følgende 49-mer-peptider og 50 50-mer-peptider, der tilsammen udgør 2400 peptider med størrelser i intervallet fra 3 til 50 aminosyrer, hvor de cykliske peptider dannes *ved hjælp af* intein-splejsning under anvendelse af spaltede inteiner, og de tilsvarende lineære peptider fusioneres mellem et C-terminalt inteinmotiv og et N-terminalt inteinmotiv

(ii) konstruktion af en række ekspressionsvektorer, idet hver vektor udtrykker et enkelt sådant cyklisk peptid

(iii) ekspression og oprensning af de cykliske peptider ved at binde affinitetstagget til en specifik ligand, der er immobiliseret på en fast fase

(iv) eluering af de cykliske peptider fra de faste faser og anvendelse af biblioteket til screening, og

(v) eventuel anvendelse af de immobiliserede cykliske peptider direkte til screening, uden at de er blevet elueret fra de faste faser, for således at konstruere et delvist eller fuldstændigt peptidbibliotek med et væsentligt reduceret antal cykliske peptider.

2. Fremgangsmåde ifølge krav 1, hvor biblioteket med cykliske peptider er et ufuldstændigt peptidbibliotek, som indeholder nogle af de peptider, der er mulige for en specifik størrelse.

3. Fremgangsmåde ifølge krav 1, hvor affinitetstaggene er valgt fra gruppen bestående af GST, His-Tag, Trx, SUMO, CBD, FLAG, HA, AviTag, Myc-Tag, SBP, Strep-Tag, Fc-Tag, Halo-Tag, V5, VSV og MBP.

4. Fremgangsmåde ifølge krav 1, hvor ekspressionsværten er valgt fra gruppen bestående af en bakteriecelle, en gærcelle, en insektcelle, en svampecelle, en pattedyrcelle og en plantecelle.

5. Fremgangsmåde ifølge krav 1, hvor ekspressionsvektoren er valgt fra gruppen bestående af en bakterieekspressionsvektor, en gærekspressionsvektor, en insektekspressionsvektor, en svampeekspressionsvektor, en pattedyrekspressionsvektor og en planteekspressionsvektor.

6. Fremgangsmåde ifølge krav 1, hvor de cykliske peptider udtrykkes enten i opløselig form eller uopløselige inklusionslegemer eller både i opløselig form og uopløselige inklusionslegemer i en bakteriecelle såsom *E. coli*.

7. Fremgangsmåde ifølge krav 1, hvor der konstrueres en række ekspressionsvektorer, idet hver vektor udtrykker et enkelt cyklisk peptid, hvor vektoren omfatter: en vektorrygrad, en C-terminal inteinmotivsekvens, eventuelt en eller flere affinitetstag-DNA-sekvenser, en peptid-DNA-sekvens, en N-terminal inteinmotivsekvens og eventuelt et eller flere proteasegenkendelsessteder.

8. Vektorkonstruktion ifølge krav 7, hvor oprensningstagget eller -taggene er placeret hvor som helst mellem det C-terminale inteinmotiv og det N-terminale inteinmotiv.

9. Vektorkonstruktion ifølge krav 8, hvor de to eller flere oprensningstags er forbundet til hinanden eller adskilt.

10. Fremgangsmåde til konstruktion af et ufuldstændigt eller kompletpeptidbibliotek med cykliske peptider, hvor fremgangsmåden omfatter:

(i) design af en række cykliske peptider, idet hvert cyklisk peptid udviser et array af forskellige peptider med forskellige størrelser, idet hvert peptid indeholder et array af korte peptidsekvenser, hvor peptidet omfatter et cyklisk peptid med 50 aminosyrer, der indeholder 50 separate tripeptider, 50 separate på hinanden følgende tetrapeptider, 50 separate på hinanden følgende pentapeptider, 50 separate på hinanden følgende hexapeptider, 50 separate på hinanden følgende heptapeptider, 50 separate på hinanden

følgende 48-mer-peptider, 50 separate på hinanden følgende 49-mer-peptider og 50 50-mer-peptider, der tilsammen udgør 2400 peptider med størrelser i intervallet fra 3 til 50 aminosyrer

(ii) syntese af de lineære peptider med de tilsvarende cykliske peptidsekvenser ved brug af kemiske fremgangsmåder med eller uden oprensning

(iii) ringslutning af de lineære peptider for at fremstille de cykliske peptider ved brug af kemiskeeller enzymatiske fremgangsmåder

(iv) oprensning af de cykliske peptider og anvendelse af biblioteket til screening.

DRAWINGS

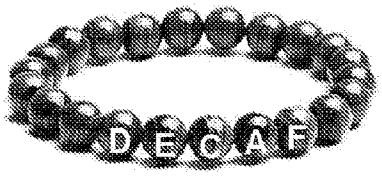
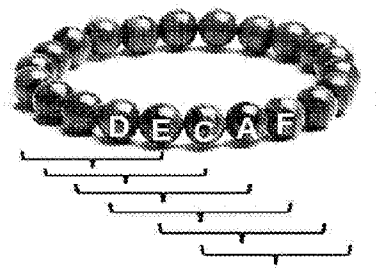


FIG. 1



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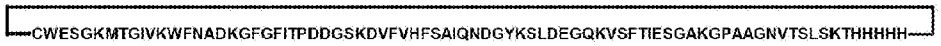

(A)

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B	VKWFN	KWFNA	WFNAD	FNADK	NADKG	ADKGF	DKGFG	KGFGF	GFGFI	FGFIT
C	GFITP	FITPD	ITPDD	TPDDG	PDDGS	DDGSK	DGSKD	GSKDV	SKDVF	KDVFV
D	DVFVH	VFVHF	FVHFS	VHFSA	HFSAI	FSAIQ	SAIQN	AIQND	IQNDG	QNDGY
F	NDGYK	DGYKS	GYKSL	YKSLD	KSLDE	SLDEG	LDEGQ	DEGQK	EGQKV	GQKVS
G	QKVSF	KVSFT	VSFTI	SFTIE	FTIES	TIESG	IESGA	ESGAK	SGAKG	GAKGP
H	AKGPA	KGPAA	GPAAG	PAAGN	AAGNV	AGNVT	GNVTS	NVTSL	VTSLS	TSLSK
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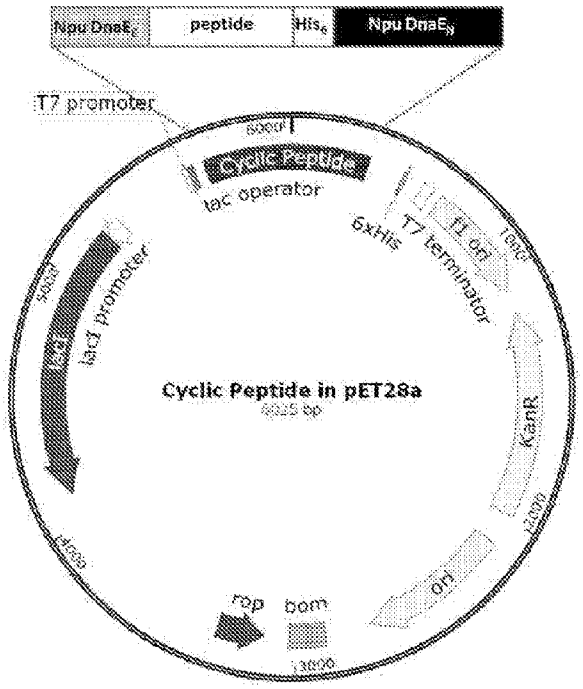
(B)

FIG.3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peptide
His$_6$
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T7 promoter
Cyclic Peptide
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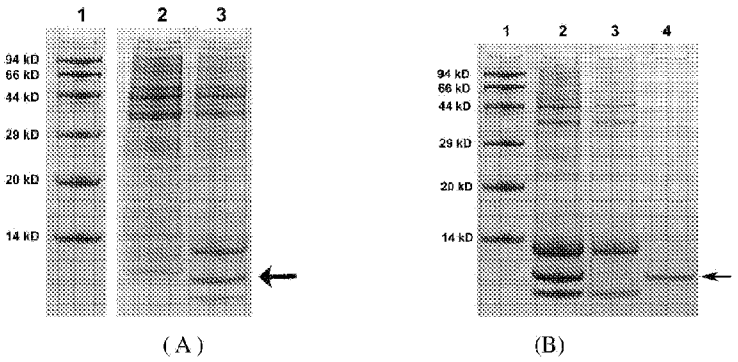
lacI promoter
Cyclic Peptide in pET28a
5315 bp
KanR
rop
pBR322 ori

FIG. 4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3
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1
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44 kD
29 kD
20 kD
14 kD
(B)

FIG.5