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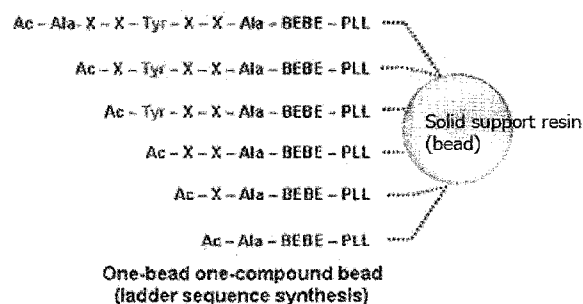
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[Continued on next page]

(54) Title: PROCESS FOR IDENTIFICATION OF KINASE SUBSTRATE SPECIFICITY BY USING PEPTIDE LIBRARY

[Fig. 1]



(57) Abstract: The present invention relates to a process for the identification of a kinase substrate specificity by using a peptide library. More particularly, the present invention is directed to a process for identifying a kinase substrate specificity, comprising: i) linking a plurality of peptides, having a same amino acid sequence, to the surface of a solid support resin to obtain a peptide library which has peptides, with the same amino acid sequence, on the surface of the solid support resin; ii) repeating the step i) with changing the amino acid sequence to give a random mixture of peptide libraries, wherein each solid support resin has peptides with a same amino acid sequence, however the amino acid sequence linked to each solid support is different from one another; iii) phosphorylating selectively a specific amino acid of a peptide which has a certain amino acid sequence by reacting the random mixture of the peptide libraries with a kinase, to give a phosphorylated peptide library; iv) reacting the phosphorylated peptide library with an antibody which is conjugated with one of a phosphatase and a peroxidase and binds selectively to a phosphorylated amino acid, followed by the addition of a substrate selectively acting on the thus prepared reaction mixture; v) selecting a solid support resin which has a peptide library of which property has been changed, by detecting the change of property due to the reaction of the substrate with one of the phosphatase and the peroxidase; and vi) separating a peptide from the selected solid support resin followed by analysing a sequence of the separated peptide.

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【DESCRIPTION】**【Invention Title】**

PROCESS FOR IDENTIFICATION OF KINASE SUBSTRATE SPECIFICITY BY USING
PEPTIDE LIBRARY

【Technical Field】

<1>

The present invention relates to a process for the identification of a kinase substrate specificity by using a peptide library. More particularly, the present invention is directed to a process for indentifying a kinase substrate specificity, comprising: i) linking a plurality of peptides, having a same amino acid sequence, to the surface of a solid support resin to obtain a peptide library which has peptides, with the same amino acid sequence, on the surface of the solid support resin; ii) repeating the step i) with changing the amino acid sequence to give a random mixture of peptide libraries, wherein each solid support resin has peptides with a same amino acid sequence, however the amino acid sequence linked to each solid support is different from one another; iii) phosphorylating selectively a specific amino acid of a peptide which has a certain amino acid sequence by reacting the random mixture of the peptide libraries with a kinase, to give a phosphorylated peptide library; iv) reacting the phosphorylated peptide library with an antibody which is conjugated with one of a phosphatase and a peroxidase and binds selectively to a phosphorylated amino acid, followed by the addition of a substrate selectively acting on the thus prepared reaction mixture; v) selecting a solid support resin which has a peptide library of which property has been changed, by detecting the change of property due to the reaction of the substrate with one of the phosphatase and the peroxidase; and vi) separating a peptide from the selected solid support resin followed by analysing a sequence of the separated peptide.

【Background Art】

<2>

In the context of the present invention, the term "kinase" refers to an enzyme that transfers phosphate groups from ATP to serine, threonine or tyrosine residues of a protein, and the term "tyrosine kinase" refers to an

enzyme that phosphorylates specifically a tyrosine residue of a protein.

<3>

The term "antibody" refers to an immunoglobulin that is found in blood or other bodily fluids and is a protein which binds to an antigen. Antigenic specificity of the antibody is diverse. The term "substrate specificity" refers to a characteristic feature of enzyme activity in relation to the kind of substrate on which the enzyme or catalytic molecule reacts. The substrate specificity is defined by a protein portion of an enzyme.

<4>

The term "phosphorylation" refers to a reaction that introduces a phosphate group to a protein molecule or a small molecule such as tyrosine, serine or threonine. The term "KIPP-MS (kinase phosphorylation profiling by mass spectrometry)" refers to a method for analysing a phosphorylation reaction of a kinase using a mass spectrometry. Also, the term "MALDI-TOF (matrix assisted laser desorption/ionization time-of flight)" refers to a soft ionization technique used in mass spectrometry, allowing the analysis of biomolecules (biopolymers such as proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules), which tend to be fragile and fragment when ionized by more conventional ionization methods. Further, the term "phosphatase" refers to an enzyme that removes a phosphate group from its substrate by hydrolysing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group.

<5>

The term "peptide library" refers to a collection of cloned free peptides, frequently consisting of all possible combinations of amino acids making up an n-amino acid peptide. In addition, the term "ladder peptide sequence" refers to a peptide that is synthesized in a shape of a ladder in which a series of polypeptides are anchored to one bead. Further, the term "split and pool synthesis" refers to a method for synthesizing a peptide library that involves splitting a sample of solid supports into a given number of fractions, charging each subset to its own reaction vessel for reaction, collecting and thoroughly mixing the solid supports back together,

with successive splitting, reacting, and remixing.

<6> The term "OBOC (one-bead one-component)" refers to a amino acid library that one bead contains one amino acid library of a specific amino acid sequence. Further, the term "photolabile linker" refers to a compound that is designed to facilitate the functional connection of a peptide to a bead and is liable to be decomposed by an ultra violet ray of a certain wavelength. A solid support resin made of polystyrene is used as a bead of the present invention.

<7> The term "docking" refers to a method which, using a computer program, predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex, the reaction between proteins and ligands, and the interaction between reactive residues and ligands. Also, the term "docking energy" refers to an energy that is calculated from the interaction between proteins and ligands, such as a hydrogen bond, electron affinity, electron repulsion, etc., by using a quantum chemical program package. In addition, the term "flexible docking" refers to a docking method that allows the protein and/or ligand to change their molecular conformation throughout the course of the simulation.

<8> The term "FlexX" refers to a commercial docking software that predicts protein-ligand interactions and the geometry of the complex as well as an estimate for the strength of binding for a given protein and a ligand so as to give a optimal docking mode.

<9> The term "P-O distance" refers to a distance between a phosphorous atom of the phosphate group of adenosine triphosphate (ATP) and an oxygen atom of the hydroxyl group of the substrate such as tyrosine.

<10> The term "RMS gradient" refers to an energy change rate accompanied by the energy change according to the structure change when calculating the structure of protein. The most stable structure can be found in which the energy change is minimized.

<11> The present invention is directed to a method for fast identification of a kinase substrate specificity by using a peptide library and a mass

spectrometer. Therefore, the method can be applied to find out the phosphorylation of a kinase of which a substrate specificity is not known thereby investigating the signal transduction activated by the phosphorylation. Also, the method can be applied to the research into pretease or phosphatase which participates in the signal transduction in a cell as well as the study of a protein kinase.

<12> An enzyme has a substrate specificity that it is extremely selective for its substrate since it binds specifically the substrate by recognizing the specific structure of the substrate. Therefore, it is very important to verify the substrate specificity of an enzyme in order to understand the function of the enzyme within a living organism.

<13> A kinase is a kind of a phosphotransferase that transfers phosphate groups from nucleotide triphosphate, such as ATP, to specific target molecules (substrates), and activates a substrate. There are a variety of kinases, such as a adenylate kinase, a pyruvate kinase, a phosphokinase, etc. Among these kinases, a protein kinase plays an important role in the metabolic ragulation in a human body by the activation or inactivation of an enzyme.

<14> A tyrosine kinase is likely to be associated with the regulation of cell proliferation. Most cell growth factor receptors such as an epithelial growth factor receptor has a tyrosine kinase. A protein produced by the representative oncogene, src, is alos a kind of a tyrosine kinase.

<15> Lam et al. disclose first a method for synthesizing an OBOC peptide library in which the library is synthesized on a TentaGel bead, and this method has been recently used for identifying bioactive materials (Lam; K. S., Salmon; S. E., Hersh; E. M., Hruby; V., Kazmierski; W. M., Knapp; R. J.. Nature 354, 82 (1991)).

<16> However, when analyzing sequences, the OBOC peptide library developed by Lam et al. cannot be used with a photolabile linker due to the intrinsically uniform distribution of functional groups of the TentaGel, and has a disadvantage that peptides are extricated by using chemicals and

therefore an additional peptide purification procedure is required (Franz; A. H., Liu; R., Song; A., Lam; K. S., Lebrilla; C. B., J. Comb. Chem. 5, 125 (2003)).

<17> St. Hilaire et al. used a photochemical method for extricating peptides from a peptide library in order to identify the substrate specificity of protease, in 2002. The method developed by St. Hilaire et al. utilizes large PEGA resins which have sizes up to from 300 nm to 80 nm, and therefore it is difficult to synthesize millions of peptide libraries (St. Hilaire; Alves; L. C., Herrera; F., Renil; M., Sanderson; S. J., Mottram; J. C.; Coombs; G. H., Juliano; M. A., Juliano; L., Arevalo; J., Meldal; M. J. Med. Chem. 45, 1971 (2002)).

<18> A kinase is associated with the mechanism of disease transfer and it is very important to understand exactly the function of the kinase based on the clear understanding of the signal transduction by the phosphorylation. It is also very important to sequencing the substrate to which the kinase acts in order to analyze a novel kinase and map the gene of the novel kinase according to the Human Kinome Map thereby using as a marker for drug discovery and development.

<19> Up to the present, various methods for identifying the substrate specificity of a kinase has been developed. However, the conventional methods are carried out with limited peptide libraries to analyze the sequence of the substrate of the kinase, and are usually labor-intensive and time-consuming.

<20> A variety of methods for detecting phosphorylated sequences have been recently developed by means of combinatorially synthesized peptide library. For example, site-specific microarrays for amino acid-scanning mutagenesis, amino acid deletion and positional scanning, were produced on the surface of glass with the synthesis of respective peptide libraries (Uttamchandani; M., Chan; E. W., Chen; G. Y., Yao; S. Q., Bioorg. Med. Chem. Lett. 13, 2997 (2003)).

<21> With the use of the site-specific microarray, fluorescein-labeled

antiphosphotyrosine antibody was used to analyze the phosphorylation pattern of a substrate, and the study for the analysis of the kinase substrate specificity was performed with analyzing the active profile of the fluorescein-labeled antiphosphotyrosine antibody (Mahesh, U. (2003), US Patent No. 6,806,056).

<22> In addition, a method for detecting the degree of phosphorylation using a radioactive isotope instead of an antibody was developed (Jessica E. H. (2004)), and Edman degradation was used for sequencing amino acids in a peptide. Moreover, methods based on bioinformatics were used.

<23> However, such methods are time-consuming and have shortcomings such as limitations on the use of radioactive rays. The abovementioned positional scanning method has a difficulty in determining the exact sequence of the kinase substrate since the positional scanning method optimizes only the single site sequence close to the phosphorylated amino acid residue.

<24> Franz et al. discloses a method for preparing a small-sized compound library on a solid support resin and extricating the library using chemicals J. Comb. Chem., 2003, Vol. 5, 125). Franz et al. prepared the bioactive compound library with TentaGel and a methionine linker, and finally extricated the analyte using cyanogen bromide (CNBr).

<25> However, the prior art by Franz et al. does not utilize a photolabile linker due to the intrinsically uniform distribution of functional groups of the TentaGel, and therefore employed a chemoselective material, CNBr, in order to extricate the analyte. Consequently, the prior art by Franz et al. has a defect that additional purification procedure is required after extricating the analyte.

<26> The present inventors have recognized that, after synthesizing a peptide on a core-shell bead as a ladder sequence, phosphorylating the synthesized peptide with a kinase, reacting the phosphorylated peptide library with an antibody which is conjugated with one of a phosphatase and a peroxidase and binds selectively to a phosphorylated amino acid, the phosphorylated peptide sequence can be more easily and exactly identified

than the prior arts by paying attention to the selection of a solid support resin by detecting the change of property, e.g. color, due to the reaction of the substrate with one of the phosphatase and the peroxidase. Also, the present inventors have recognized that the mass analysis of the peptide can be made easy by the introduction of a photolabile linker to the core-shell bead. The present invention has been completed.

【Disclosure】

【Technical Problem】

<27>

Therefore, the primary object of the present invention is to provide a process for indentifying a kinase substrate specificity, comprising: i) linking a plurality of peptides, having a same amino acid sequence, to the surface of a solid support resin to obtain a peptide library which has peptides, with the same amino acid sequence, on the surface of the solid support resin; ii) repeating the step i) with changing the amino acid sequence to give a random mixture of peptide libraries, wherein each solid support resin has peptides with a same amino acid sequence, however the amino acid sequence linked to each solid support is different from one another; iii) phosphorylating selectively a specific amino acid of a peptide which has a certain amino acid sequence by reacting the random mixture of the peptide libraries with a kinase, to give a phosphorylated peptide library; iv) reacting the phosphorylated peptide library with an antibody which is conjugated with one of a phosphatase and a peroxidase and binds selectively to a phosphorylated amino acid, followed by the addition of a substrate selectively acting on the thus prepared reaction mixture; v) selecting a solid support resin which has a peptide library of which property has been changed, by detecting the change of property due to the reaction of the substrate with one of the phosphatase and the peroxidase; and vi) separating a peptide from the selected solid support resin followed by analysing a sequence of the separated peptide.

<28>

Another object of the present invention is to provide a peptide library represented by the following formula:

<29> Z-X-X-Y-X-X-Z-Spacer-Linker-solid support resin

<30> where Z represents one of the essential amino acids excluding cysteine; X represents an amino acid which is one of the essential amino acids excluding cysteine and not identical to Y; Y represents tyrosine, threonine or serine; Spacer represents a linear or branched peptide which consists of 1 to 6 amino acids and contains alkyl and/or ether; and Linker represents a 2-nitrobenzyl derivative, a 2-(2-nitrophenyl)propyl derivative, a benzoin derivative or a phenacyl derivative.

【Technical Solution】

<31> The abovementioned primary object of the present invention can be achieved by providing a process for indentifying a kinase substrate specificity, comprising: i) linking a plurality of peptides, having a same amino acid sequence, to the surface of a solid support resin to obtain a peptide library which has peptides, with the same amino acid sequence, on the surface of the solid support resin; ii) repeating the step i) with changing the amino acid sequence to give a random mixture of peptide libraries, wherein each solid support resin has peptides with a same amino acid sequence, however the amino acid sequence linked to each solid support is different from one another; iii) phosphorylating selectively a specific amino acid of a peptide which has a certain amino acid sequence by reacting the random mixture of the peptide libraries with a kinase, to give a phosphorylated peptide library; iv) reacting the phosphorylated peptide library with an antibody which is conjugated with one of a phosphatase and a peroxidase and binds selectively to a phosphorylated amino acid, followed by the addition of a substrate selectively acting on the thus prepared reaction mixture; v) selecting a solid support resin which has a peptide library of which property has been changed, by detecting the change of property due to the reaction of the substrate with one of the phosphatase and the peroxidase; and vi) separating a peptide from the selected solid support resin followed by analysing a sequence of the separated peptide.

<32> The present invention employs a mass spectrometer to identify a

kinase substrate specificity. In addition, the present invention employs a OBOC synthetic technique including a ladder peptide to investigate fast and effectively the phosphorylation of the kinase. The process according to the present invention can be applicable to not only a tyrosine kinase but also a serine and threonine kinase. The substrate specificity of p60^{c-src} and ZAP-70 was identified by the process of the present invention.

<33> KIPP-MS according to the present invention is described in below.

<34> In the first step, an OBOC ladder peptide is synthesized. The peptide library is synthesized by the split and pool synthesis using a resin in the form of a core-shell. The photolysis efficacy of the core-shell resin is at least twice higher than that of the TentaGel since amino groups are present on the surface of the core-shell resin (Kim, H., Cho, J. K., Chung, W. J., Lee, Y. S., Org. Lett., 6, 3273 (2004); Korean patent application No. 10-2004-0078860).

<35> The peptide library is randomly synthesized with 18 amino acids excluding cysteine and tyrosine at two sites respectively on either side of tyrosine, total four sites. Alanine is introduced at N-terminal and C-terminal of the peptide such that the kinase approaches easily the peptide synthesized on the bead. The amino acid at N- and C-terminal can be any of all the amino acids excluding alanine.

<36> When the peptide is analyzed by MALDI-TOF mass spectrometer, a photolabile linker is introduced onto the surface of the bead in order to dissociate the peptide from the bead. The spacer composed of (β -Ala)-(ϵ -ACA)-(β -Ala)-(ϵ -ACA) (BEBE) is also introduced next to the linker in order to avoid coinciding the mass of the peptide with that of matrix, an auxiliary agent, used for mass analysis. The photolabile linker may be selected from a 2-nitrobenzyl derivative, a 2-(2-nitrophenyl)propyl derivative, a benzoin derivative or a phenacyl derivative and the spacer may be a linear or branched peptide which consists of 1 to 6 amino acids and contains alkyl and/or ether. Consequently, the peptide library sequence is

Ala-X-X-Tyr-X-X-Ala-BEBE-NH₂ (Fig. 1). About 30 mg of the synthesized beads has more than about 100,000 random sequences, and therefore the beads can fully contain four amino acid combinations.

<37> In the second step, peptide libraries are prepared. The beads are washed with a phosphate-buffered solution (PBS), and are treated with a blocking solution in order to prevent a non-specific protein adsorption.

<38> In the third step, phosphorylation takes place. The phosphorylation is proceeded by mixing a Tris-HCL buffer, ATP and a kinase. After the phosphorylation is completed, the reacting solution is removed and the beads are washed with Type III water.

<39> In the fourth step, an antibody (Ab) is conjugated with the bead and the antibody used is an anti-phosphotyrosine Ab to which an alkaline phosphatase is bound. When the Ab is reacted with the bead, the Ab is conjugated with only the phosphorylated bead. After reaction, the resultants are washed.

<40> In the fifth step, the selection of the peptide library is performed. That is, when the substrates are reacted with the alkaline phosphatase conjugated with the Ab used in the step iv), phosphorylated beads can be detected by their color change. The alkaline phosphatase is reacted with the beads in the PBS solution in which BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) and NBT (nitro-blue tetrazolium chloride) are dissolved. The phosphorylated beads conjugated with the Ab becomes clear red as the reaction of the phosphatase progresses. The reaction stops by washing the beads. When peroxidase is used, a color change can be observed by reacting the peroxidase with 3,3'-diaminobenzidine.

<41> In the sixth step, UV rays with a wavelength of 365 nm is irradiated to the color-changed beads which has been selected one by one with forceps and a microscope and then the peptides linked to the beads are dissociated. The dissociated peptides are analyzed by MALDI-TOF. The organic auxiliary agent used during the analysis is DHB (2,5-dihydroxybenzoic acid in 70% MeOH).

<42> A molecular modeling study was performed to analyze the difference of reactivity between the tyrosine kinase and the optimal substrates. The molecular modeling was carried out by using the molecular modeling software package, SYBYL 7.1. The structure of p60^{c-src} and ZAP-70 kinases were obtained from the Protein Data Bank. Flexible docking was performed by using FlexX in order to predict X-ray crystal structure of the kinase-substrate complex. The optimal structure of the kinase-substrate complex can be obtained by minimizing the RMS gradient of amino acid residues within the range of 15 Å from the active site so as to be below 0.05 kcal/mol/Å.

<43> Another object of the present invention can be achieved by providing a peptide library represented by the following formula:

<44> Z-X-X-Y-X-X-Z-Spacer-Linker-solid support resin

<45> where Z represents one of the essential amino acids excluding cysteine; X represents an amino acid which is one of the essential amino acids excluding cysteine and not identical to Y; Y represents tyrosine, threonine or serine; Spacer represents a linear or branched peptide which consists of 1 to 6 amino acids and contains alkyl and/or ether; and Linker represents a 2-nitrobenzyl derivative, a 2-(2-nitrophenyl)propyl derivative, a benzoin derivative or a phenacyl derivative.

<46> Preferably, the solid support resin has a core-shell structure.

【Advantageous Effects】

<48> First, the kinase substrate specificity can be fast determined by the combination of MALDI-TOF mass spectrometry and the synthetic technique of the OBOC peptide library comprising the ladder peptide according to the present invention.

<49> Secondly, the thus obtained substrate sequence is well in accord with the motif on which a kinase actually acts in vivo, which is very important to understand exactly the function of the protein kinase and is able to give significant information to develop a protein kinase inhibitor.

<50> Thirdly, the processes for the rapid analysis and screening of proteins by using the peptide library are related to drug discovery and development, and therefore can play an important role in searching a novel protein marker and inhibitor. It is a key area of study to develop a kinase inhibitor in a medical and medicinal field.

<51> Fourthly, the process of the present invention can be applicable to the serine and threonine kinases as well as to the tyrosine kinase.

【Description of Drawings】

<52> Fig. 1 is a schematic diagram of the library bead according to the present invention.

<53> Fig. 2 is a flow diagram of the identification of the kinase substrate specificity by using KIPP-MS according to the present invention.

<54> Fig. 3 shows the results of the mass analysis of Example 5 of the present invention.

<55> Fig. 4 shows the sequence of the $p60^{c-src}$ phosphorylated by the tyrosine kinase.

<56> Fig. 5 is a graph showing the number of appearance of amino acid according to the position of the tyrosine kinase, $p60^{c-src}$, which is obtained by the statistical calculation.

<57> Fig. 6 shows the results of the analysis of the sequences phosphorylated by the tyrosine kinase, ZAP-70, by using MALDI-TOF.

<58> Fig. 7 is a graph showing the number of appearance of amino acid according to the position of the tyrosine kinase, ZAP-70, which is obtained by the statistical calculation.

<59> Fig. 8 shows proteins of which sequence is in accord with the substrate specificity sequence of the tyrosine kinase, ZAP-70, which is identified by NCBI human protein database and KIPP-MS.

<60> Fig. 9 (a) shows the results of the three dimensional docking of the optimal substrate peptide obtained by KIPP-MS on the active site of the tyrosine kinase, p60^{c-src}, and Fig. 9 (b) shows the results of the three dimensional docking of the optimal substrate peptide obtained by KIPP-MS on the active site of the tyrosine kinase, ZAP-70.

【Best Mode】

<61> Hereinafter, the present invention will be described in greater detail with reference to the following examples and drawings. However, the examples and drawings are given only for illustration of the present invention and not to be limiting the present invention.

<62> Example 1. Preparation of a support resin to which a photolabile linker and a spacer is introduced

<64> In order to introduce the photolabile linker and the spacer on the core-shell resin, HiCore support resin (0.3 mmol/g) was swelled within 30 mL of NMP (N-methyl-2-pyrrolidone), and then 6 mmol of Fmoc-photolabile linker (Fmoc-PLL, Fmoc-4-[4-(1-aminoethyl)-2-methoxy-5-nitrophenoxy]butyric acid), 0.6 mmol of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, BOP), 0.6 mmol of 1-hydroxybenzotriazole (HOBt), and 1.2 mmol of diisopropylethylene (DIEA) were dissolved in 10 mL of NMP.

<65> The thus prepared solution was maintained to react for 1 - 2 hr at room temperature, and then the solution was filtered and washed with NMP, DCM and MeOH to obtain support resins bound by Fmoc-PLL. The completion of the reaction was determined using the Kaiser's ninhydrin test. The Fmoc protecting groups were removed by the reaction with piperidine/NMP solution

for 3 min and 17 min respectively, and then the solution was washed as described above. The reaction of the PLL linked support resin with Fmoc-e-ACA (Fmoc-e-aminocaproic acid; 2 equivalents) and Fmoc-b-Ala (2 equivalents), in which BOP (2 equivalents), HOBt (2 equivalents) and DIEA (4 equivalents) were used in turn to introduce a spacer, was repeated twice to give the support resin introduced with the photolabile linker and the spacer.

<66>

<67> Example 2. Preparation of a peptide library

<68>

The peptide library was prepared using split and pool synthesis. Alanine was introduced at N-terminal and C-terminal of the peptide and tyrosine was introduced in the middle of the peptide to synthesize the peptide library having a structure of Ala-X-X-Tyr-X-X-Ala-BEBE-PLL-HiCore, in order that the kinase approaches easily the peptide synthesized on the bead. As shown in Fig. 1, the peptide sequence of one-bead one-sequence, was synthesized as a form of a ladder.

<69>

PLL is a photolabile linker. The PLL is decomposed by the exposure to 365 nm UV rays. The BEBE which is a spacer is introduced to distinguish from matrix used for mass analysis. The X represents 18 amino acids excluding cysteine and tyrosine.

<70>

The HiCore support resin to which the photolabile linker and the spacer prepared in Example 1, was divided into 18 equal parts of 50 mg each, and then each part was added into a reaction tube (5 mL). In turn, Fmoc-amino acid (excluding cysteine and tyrosine, 2 equivalents), acetic acid (0.2 equivalents), BOP (2.2 equivalents), HOBt (2.2 equivalents) and DIEA (4.4 equivalents) dissolved in 3 mL of NMP were added into the reaction tube.

<71>

The thus prepared solution was maintained to react for 1 hr to 4 hr at room temperature, and then filtered to remove the filtrate. The filtrand was washed with NMP, DCM and MeOH. Each support resin was put into one vessel and 20% piperidine/NMP solution was added to the vessel to react for

3 min and 17 min respectively thereby removing the Fmoc protecting group. Then the resin was washed and dried in a vacuum oven for 6 to 12 hours.

<72> The above procedure was repeated and finally reagent K (85% trifluoroacetic acid (TFA), 5% pheno, 2.5% ethanedithiol, and 2.5% water) was added and reacted with the dried resin for 1 to 2 hours to give a peptide library support resin.

<73>

<74> Example 3. Preparation of a phosphorylated peptide library

<75> The peptide library support resin was washed with 50 mM PBS. In order to prevent non-specific absorptions of proteins, blocking solution (3% BSA, Tween20 in PBS) was treated for 2 to 4 hr at room temperature and then beads were washed with the blocking solution. Kinase was reacted with 25 mM Tris-HCl buffer solution (pH 7.4, 7 mM MgCl₂, 0.5 mM EGTA), 100 M ATP and 2 units of p60^{c-src} or ZAP-70 for 1 to 2 hr at 20 to 30 °C. After the reaction, the reaction solution was removed and the beads were washed with Type III water.

<76>

<77> Example 4. Conjugation of an antibody with the phosphorylated peptide library

<78> An anti-phosphotyrosine Ab to which an alkaline phosphatase was bound, was reacted with the beads to conjugate the antibody with the phosphorylated beads. The beads were reacted with 100 ng/ml of the antibody for 1 to 2 hr at room temperature and then washed with 50 mM PBS and Type III water.

<79>

<80> Example 5. Selecting the phosphorylated peptide library which reacted with the phosphatase and Sequencing the amino acids of the selected peptide

<81> In order to select the phosphorylated beads through the color change due to the reaction of the alkaline phosphatase bound to the antibody, the alkaline phosphatase was dissolved in the 50 mM PBS solution in which 0.1 mg/mL of BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) and

0.1 mg/mL of NBT (nitro-blue tetrazolium chloride) had been dissolved, and then the solution was reacted with the beads for 30 min to 1 hr at 37°C followed by the washing with Type III water.

<82> The color of the phosphorylated beads conjugated with the antibody was changed to clear red by the reaction of the phosphatase and the color-changed beads were picked out by using a pincette and a microscope. Then, the selected beads were irradiated with 365 nm UV rays (50 mW/cm^2) for 30 min to 1 hr to cleave the synthesized peptide from the bead.

<83> As shown Fig. 2 (A), the OBOC library beads were phosphorylated by the reaction with the kinase. Then, the phosphorylated beads were reacted with the anti-phosphoamino acid Ab bound to a phosphatase. The phosphatase bound to the antibody was used for detecting the phosphorylated bead. That is, when the phosphatase was bound to the antibody and then the phosphatase substrate was added to the library bead, the color of the bead phosphorylated with the phosphatase was changed to clear red. The color-changed beads were picked out. In the step of Fig. 2 (B), the peptides were cleaved from the selected beads by the irradiation of UV rays, and analyzed by using MALDI-TOF.

<84> The cleaved peptides (5 μL) which was mixed with the auxiliary agent, DHB (2,5-dihydroxybenzoic acid 1 μL , 30 mg/mL, MeOH:water=70:30), was sequenced by the MALDI-TOF. The results of the above analysis are shown in Fig. 3, and the peptides of the form of a ladder has six peaks. When the mass differences of each peak are calculated, each amino acid of the peptide can be determined.

<85>

<86> Example 6. Efficacy test of KIPP-MS using p60^{c-src} tyrosine kinase

<87> The tyrosin kinase, p60^{c-src}, of which substrate specificity is well-known, was tested in order to confirm that the KIPP-MS of the present invention can be used for the identification of a kinase substrate

specificity. When using $p60^{c-src}$, one hundred of beads of which color had changed to red were selected and photolyzed, and then analyzed by the MALDI-TOF. Among those, eighty one of spectra which had been analyzed exactly were obtained. The sequences of the peptides which reacted with the kinase were shown in Fig. 4.

<88> Through the statistical analysis based on the above sequences, the principal sequence of each position, which exerts an influence on the phosphorylation reaction of $p60^{c-src}$, was determined (Fig. 5).

<89> It can be noted that the kinase substrate specificity of $p60^{c-src}$ is characterized in that the phosphorylation reaction of $p60^{c-src}$ most effectively takes place when acidic amino acids (Glu and Asp) are located at +1 and +2 positions and Ile is located at -1 position. This result was the same as the prior study and proved that the KIPP-MS of the present invention can be generally applied to the identification of tyrosine kinase specificity.

<90>

<91> Example 7. Identification of the kinase specificity of ZAP-70 by KIPP-MS

<92> In order to generalize the KIPP-MS of the present invention, the kinase specificity of ZAP-70 was identified by the same method of Example 6. As yet, the kinase specificity of ZAP-70 has not been found, however ZAP-70 is classified into the same kind as $p60^{c-src}$. The analytical efficiency of sequencing the peptide of ZAP-70 was about 92%.

<93> It can be ascertained that in the sequenced peptides, Glu is appeared at +1 position (21%) and +2 position (49%) (Fig. 6). It can be also ascertained that No. of appearance of Asp at +2 position is about 29% and this is next to Glu in the order of No. of appearance. At -1 position, Glu and Asp appeared most frequently, and at -2 position selectivity of a certain amino acid was not detected (Fig. 7). Through such statistical data analysis, it could be understood that ZAP-70 well recognized and

phosphorylated the acidic amino acids on either side of the tyrosine. Therefore, through the KIPP-MS of the present invention, it was identified that the optimum substrates for ZAP-70 at -1, +1 and +2 positions are all Glu.

<94>

<95> Example 8. Molecular modeling for analyzing the differences of reactivity between the tyrosine kinase and the optimum substrates

<96> Molecular modeling for analyzing the differences of reactivity between the tyrosine kinase and the optimum substrates was performed. The molecular modeling was performed by using SYBYL 7.1 molecular modeling software package. The structures of p60^{c-src} and ZAP-70 was obtained from the Protein Data Bank. The flexible docking for predicting the X-ray crystallography of the kinase-substrate complex was carried on by FlexX.

<97> The optimal structure of the kinase-substrate complex was obtained by minimizing the RMS gradient of amino acid residues within the range of 15 Å from the active site so as to be below 0.05 kcal/mol/Å.

<98>

<99> Example 9. Study on the three dimensional docking regarding the experimental results of the kinase substrate specificity of p60^{c-src}

<100> Study on the three dimensional docking regarding the experimental results of the kinase substrate specificity of p60^{c-src} was performed. Ile-Tyr-Glu-Glu was selected as the optimum substrate through such experiments. Ile at -1 position interacted electrostatically with Gly406 of p60c-src, and the P-O distance between the hydroxyl group of the substrate, Tyr, and the phosphate group of ATP was as close as about 4.16 Å, and consequently the hydrogen bond between Arg388 and Asp286 which were located within the active site of the kinase was formed.

<101> The docking energy (ΔG) of the optimum substrate was -15.76 kcal/mol. When Ile at -1 position was changed with Glu, the second optimum sequence, the docking energy (ΔG) became -11.22 kcal/mol and the P-O

distance 4.34 Å (Fig. 9(a)). Also, when Glu at +1 and +2 positions were changed with other amino acid, substrates were not able to be inserted into the active site of p60^{c-src}. These results proved that the KIPP-MS of the present invention can be generally used for the identification of the kinase substrate specificity.

<102>

<103> Example 10. Study on the three dimensional docking regarding the experimental results of the kinase substrate specificity of ZAP-70

<104> Study on the three dimensional docking regarding the experimental results of the kinase substrate specificity of ZAP-70 was carried out. Glu at -1 position interacted with Ser497 located at the boundary of the binding pocket of the kinase, in the vicinity of the Ser497. Glu at +1 position made and hydrogen bonding with Lys500. The hydroxyl group of Tyr made hydrogen bondings simultaneously with the polar residues of both Asp476 and Asp461 located within the active site.

<105> The docking energy (ΔG) of the optimum substrate was -28.17 kcal/mol and the P-O distance was 3.53 Å (Fig. 9(b)). When Glu at -1 position was exchanged with Asp, the hydroxyl group of Tyr made an hydrogen bonding only with Asp476 for the active site. Therefore, the P-O distance increased as to be 4.44 Å.

<106>

<107> Example 11. Identification of ZAP-70 kinase by KIPP-MS

<108> As a result of BLAST (basic local alignment search tool) search by using the active peptide sequences of ZAP-70 of Example 7, it was shown that the sequences belonged to portions of T-cell receptor zeta chain, proto-oncogene Cbl and Vav proteins (Fig. 9). With this result, the kinase substrate specificity of ZAP-70 has first been revealed by the KIPP-MS of the present invention.

【CLAIMS】**【Claim 1】**

<110> A process for indentifying a kinase substrate specificity, comprising:

<111> i) linking a plurality of peptides, having a same amino acid sequence, to the surface of a solid support resin to obtain a peptide library which has peptides, with the same amino acid sequence, on the surface of the solid support resin;

<112> ii) repeating the step i) with changing the amino acid sequence to give a random mixture of peptide libraries, wherein each solid support resin has peptides with a same amino acid sequence, however the amino acid sequence linked to each solid support is different from one another;

<113> iii) phosphorylating selectively a specific amino acid of a peptide which has a certain amino acid sequence by reacting the random mixture of the peptide libraries with a kinase, to give a phosphorylated peptide library;

<114> iv) reacting the phosphorylated peptide library with an antibody which is conjugated with one of a phosphatase and a peroxidase and binds selectively to a phosphorylated amino acid, followed by the addition of a substrate selectively acting on the thus prepared reaction mixture;

<115> v) selecting a solid support resin which has a peptide library of which property has been changed, by detecting the change of property due to the reaction of the substrate with one of the phosphatase and the peroxidase; and

<116> vi) separating a peptide from the selected solid support resin followed by analysing a sequence of the separated peptide.

【Claim 2】

<117> The method of Claim 1, wherein the solid support resin has a core-shell structure.

【Claim 3】

<118> The method of Claim 1, wherein a photolabile linker is bound to the

surface of the solid support resin.

【Claim 4】

<119> The method of Claim 2, wherein a spacer is further connected to the photolabile linker bound to the surface of the solid support.

【Claim 5】

<120> The method of Claim 1, wherein the kinase is selected from the group consisting of a tyrosine kinase, a serine kinase, and a threonine kinase.

【Claim 6】

<121> The method of Claim 1, wherein the kinase is a tyrosine kinase.

【Claim 7】

<122> The method of Claim 6, wherein the tyrosine kinase is p60^{c-src} or ZAP-70.

【Claim 8】

<123> The method of Claim 1, wherein the change of property in the step v) is the change of color of the solid support resin.

【Claim 9】

<124> The method of Claim 3, wherein the photolabile linker is selected from the group consisting of a 2-nitrobenzyl derivative containing Fmoc-4-[4-(1-aminomethyl)-2-methoxy-5-nitrophenoxy]butanoic acid, a 2-(2-nitrophenyl)propyl derivative, a benzoin derivative and a phenacyl derivative.

【Claim 10】

<125> The method of Claim 4, wherein the spacer is a linear or branched peptide which consists of 1 to 6 amino acids and contains alkyl or ether compositely.

【Claim 11】

<126> A peptide library represented by the following formula:

<127> Z-X-X-Y-X-X-Z-Spacer-Linker-solid support resin

<128> where Z represents one of the essential amino acids excluding cysteine; X represents an amino acid which is one of the essential amino acids excluding cysteine and not identical to Y; Y represents tyrosine, threonine or serine;

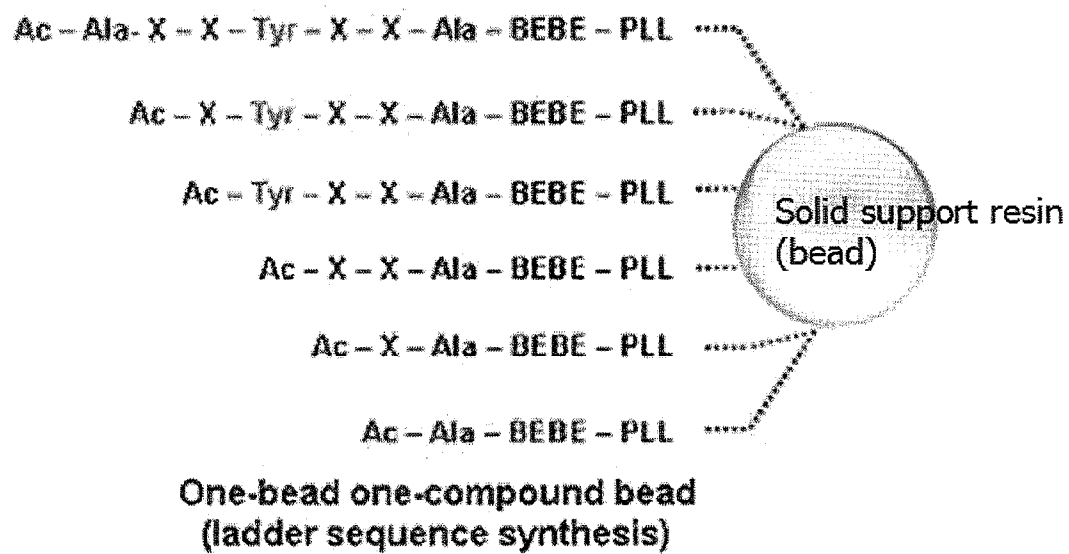
Spacer represents a linear or branched peptide which consists of 1 to 6 amino acids and contains alkyl and/or ether; and Linker represents a 2-nitrobenzyl derivative, a 2-(2-nitrophenyl)propyl derivative, a benzoin derivative or a phenacyl derivative.

【Claim 12】

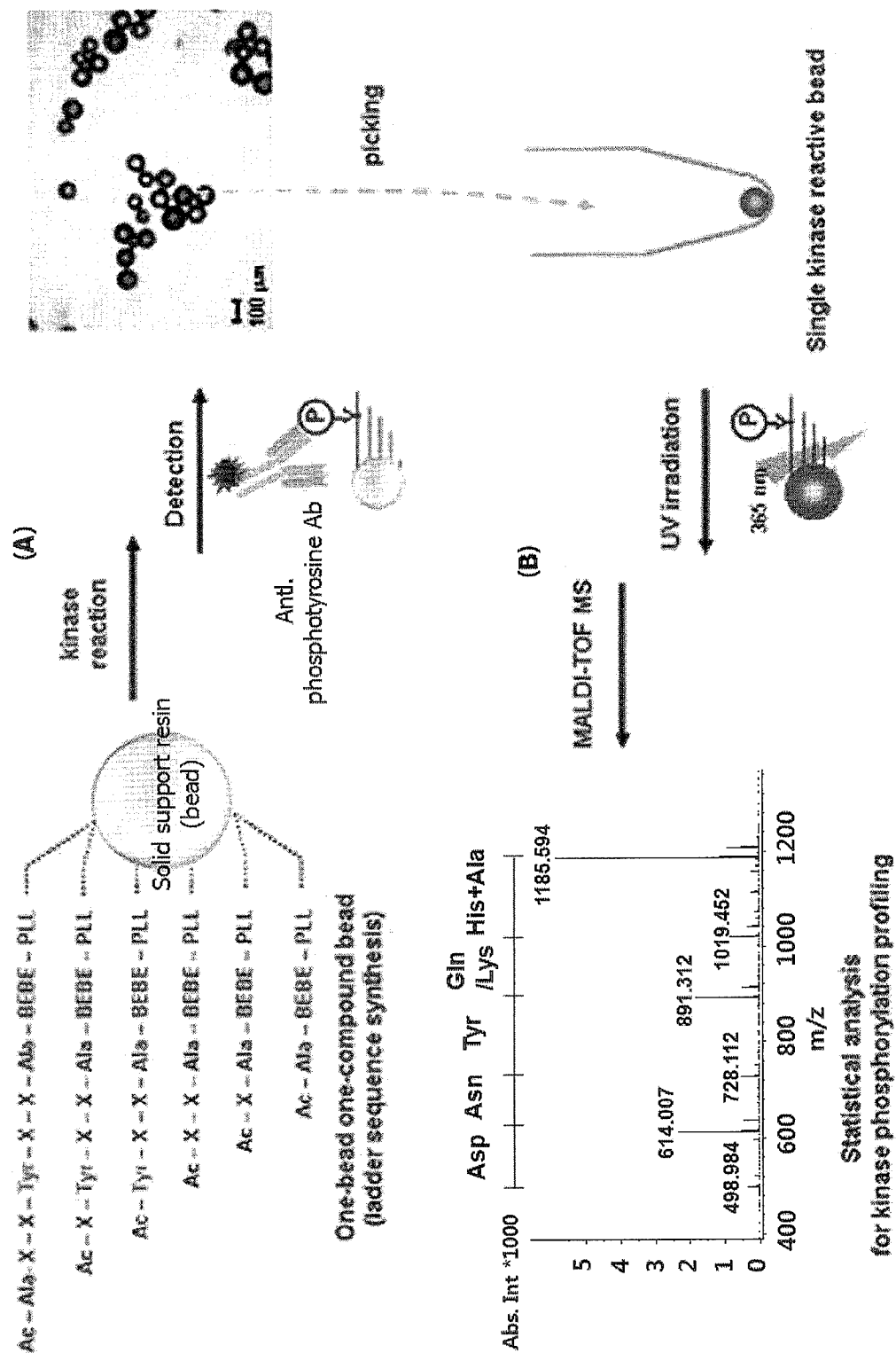
<129>

The peptide library of Claim 11, wherein the solid support resin has a core-shell structure.

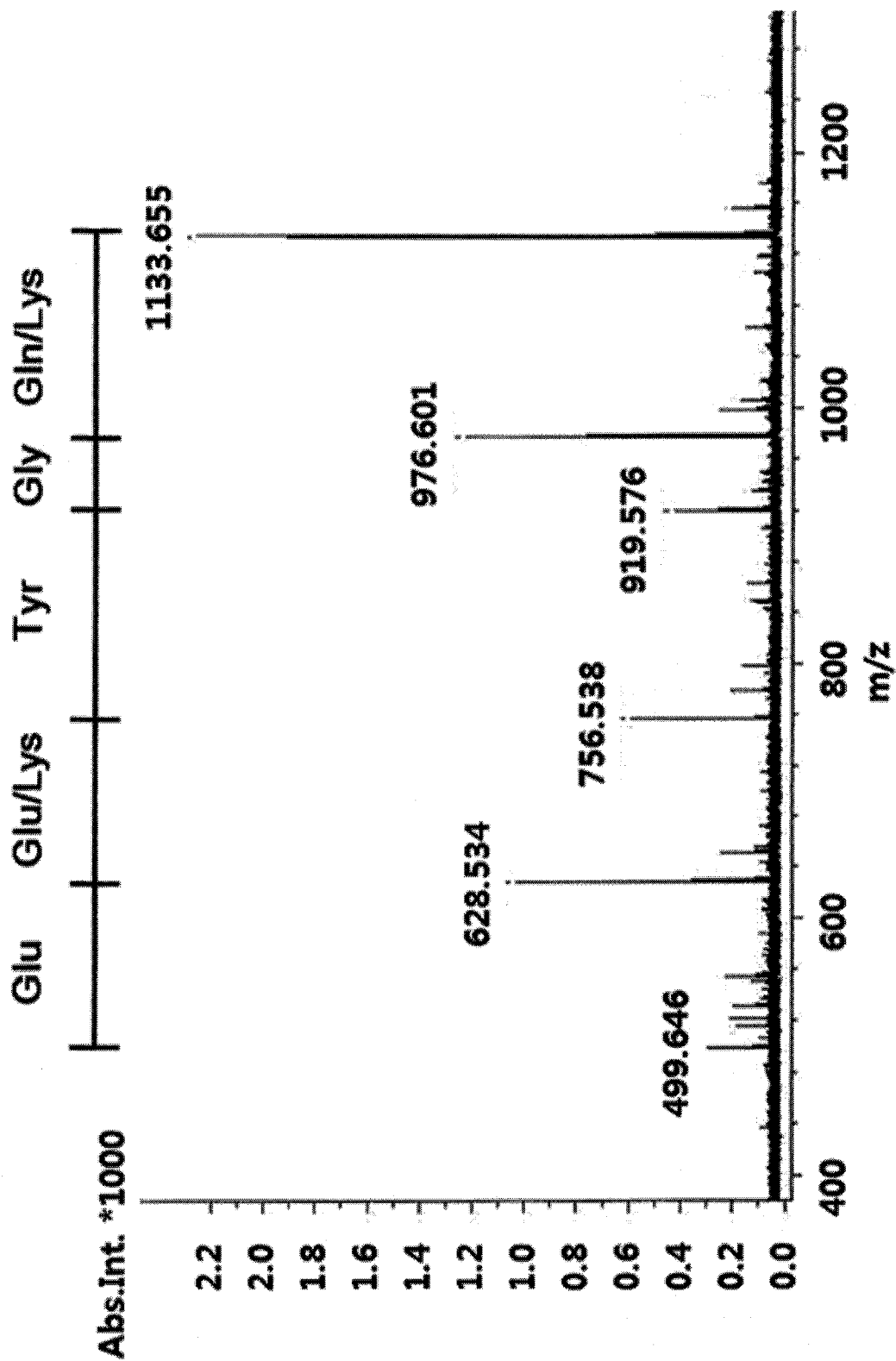
[Fig. 1]



[Fig. 2]



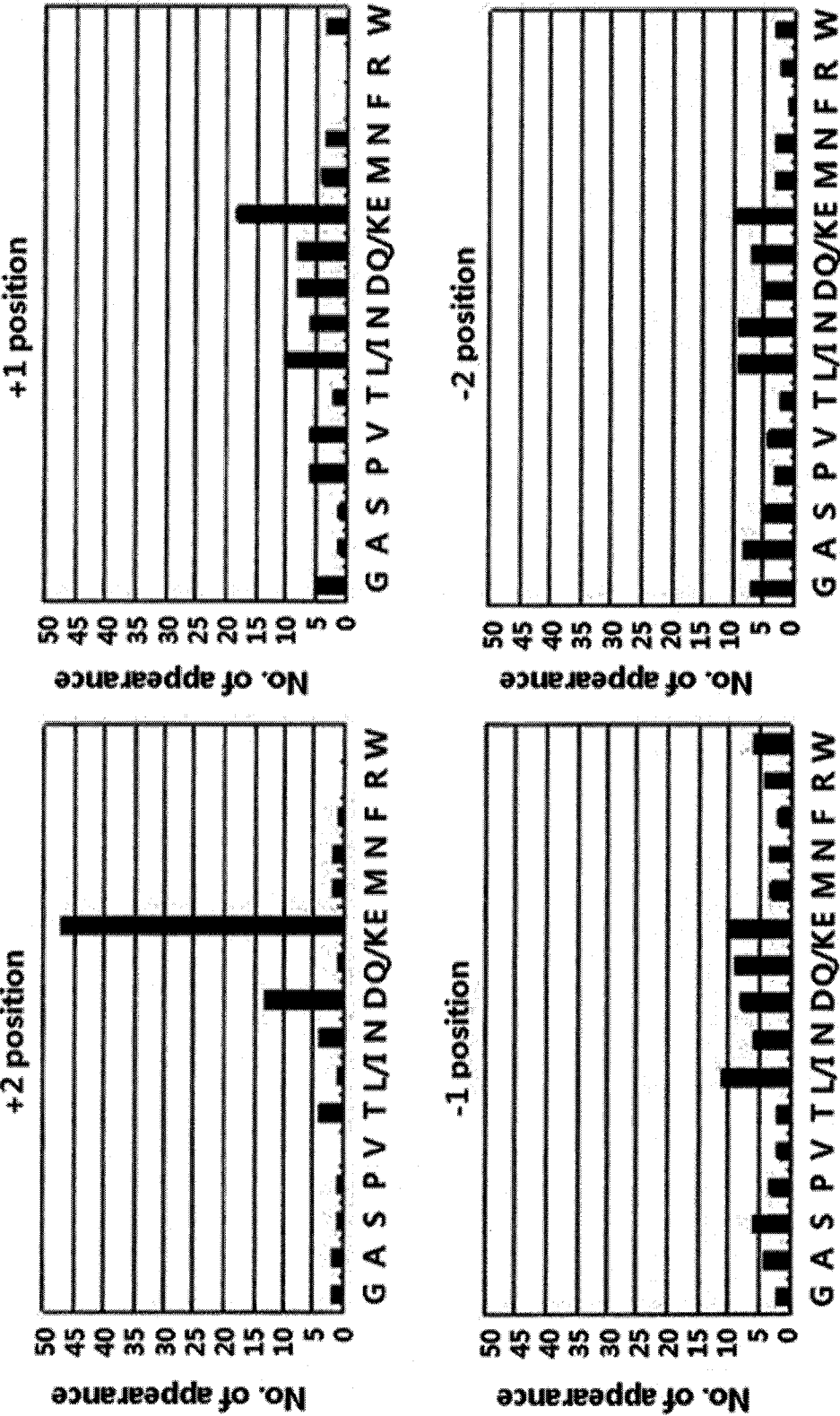
[Fig. 3]



[Fig. 4]

NH ₂ - Ala - X ₍₋₂₎ - X ₍₋₁₎ - Tyr - X ₍₊₁₎ - X ₍₊₂₎ - Ala - linker - bead				
(Q/K)GY(I/L)E	NWY(I/L)H	AEYVE	EDY(Q/K)D	M(I/L)YNE
WNYEP	(I/L)NYEE	NEYE(I/L)	(I/L)AYGE	(Q/K)PYPE
G(Q/K)YDE	NPY(I/L)E	ENYAE	(I/L)PYMD	TWYHE
VDY(Q/K)E	(I/L)NYGE	M(I/L)YNF	PSYED	A(I/L)YEE
HRYHE	(Q/K)(Q/K)Y(I/L)D	SMYDE	ERYEG	E(I/L)YPN
(I/L)EYPE	GEY(Q/K)E	N(Q/K)YVE	NNYEM	REYVE
ARYPE	(Q/K)AY(Q/K)E	(Q/K)WYDE	NFYEE	W(Q/K)YES
N(I/L)YVE	(Q/K)TY(I/L)E	SSYGG	EMY(I/L)E	DEYPN
MTYHE	SEYME	HEYTD	DMYDT	E(Q/K)Y(I/L)N
H(Q/K)YND	GDYED	TDYED	G(Q/K)YDE	V(I/L)YEE
SWYGE	AHYEE	D(I/L)YTE	(I/L)AYEA	F(Q/K)Y(Q/K)E
PDYEE	(I/L)DYNE	RHYSE	E(I/L)YWD	E(I/L)YME
AVYDE	AWYDE	D(I/L)YET	VSYWE	ASYWE
NWYET	SSYED	(Q/K)GYPD	DDY(I/L)E	GEYVE
A(Q/K)YDE	GEY(I/L)D	PNY(Q/K)M	WWYVN	EFY(Q/K)E
WSYMA	GAY(I/L)E	(I/L)HYND	(I/L)RY(Q/K)T	N(I/L)YG(Q/K)
EDYNH				

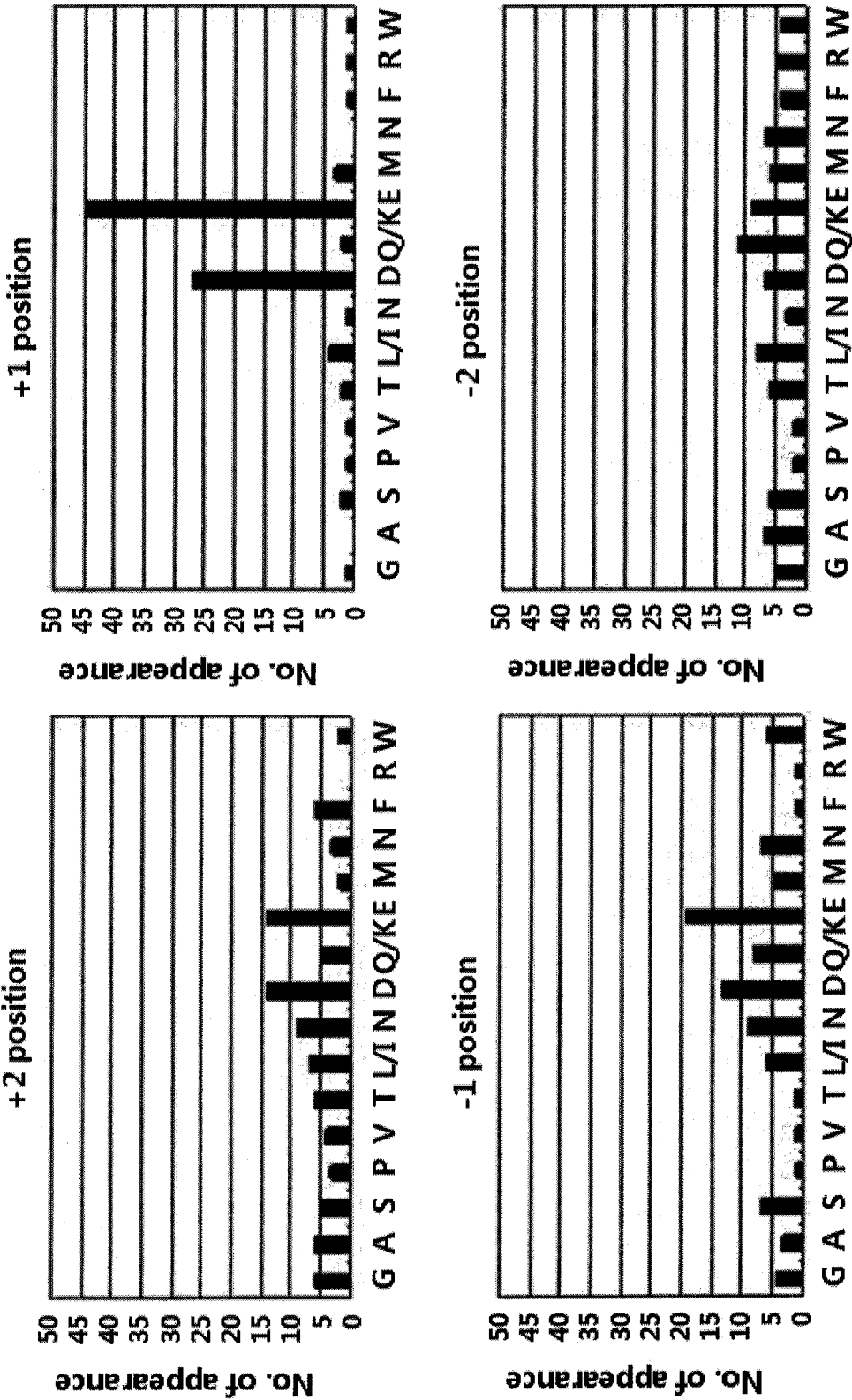
[Fig. 5]



[Fig. 6]

NH ₂ - Ala - X ₍₋₂₎ - X ₍₋₁₎ - Tyr - X ₍₊₁₎ - X ₍₊₂₎ - Ala - linker - bead				
MNYDE	(Q/K)EYND	(Q/K)NYDE	WTYED	(Q/K)EYNE
GNYEM	RVYTE	(I/L)(Q/K)YWE	HDYEW	M(I/L)YET
PGYEV	SEYWE	WWYEE	HTYED	AEY(I/L)(I/L)
GWYSD	DTYDE	GFYDE	HNYED	(Q/K)VYDE
(I/L)TY(Q/K)D	(I/L)GYD(I/L)	(Q/K)NYNE	(Q/K)(I/L)YDE	EDY(Q/K)D
TSYNE	NDYDE	TDY(Q/K)D	HGYEF	FSYNE
D(I/L)Y(I/L)E	(I/L)(I/L)YDE	DDYHD	EEYMD	(I/L)NYNE
AAY(Q/K)T	SDY(I/L)D	GFYED	(I/L)MYHE	MMYME
MEYGP	EGYGE	WAYSE	NEYSD	FVY(I/L)E
ENYGD	ADY(I/L)(Q/K)	E(I/L)YED	EDYVD	(I/L)FY(I/L)E
AEYWE	SAYWD	MFY(Q/K)E	RTYED	MEYE(I/L)
AEYD(I/L)	TNYRE	GFYNE	TNYGD	R(Q/K)YHE
DDYNE	(Q/K)AYDD	(Q/K)SYHE	SPYDE	TFYED
(Q/K)EY(Q/K)R	E(Q/K)YDM	(I/L)GYES	HTYEM	NEYFD
PEYES	(Q/K)AYME	VDY(Q/K)E	AAYWE	WSY(Q/K)D
VWYNE	DDYSE	HPYAE	A(I/L)YAE	EDYSE
EGYHD	FHYSG	F(Q/K)YAE	R(I/L)YME	TSYME
DDYHD	SHYPE	DPYEN	HEYHD	RHYSD
SDYWE	(Q/K)(Q/K)YE(Q/K)			

[Fig. 7]

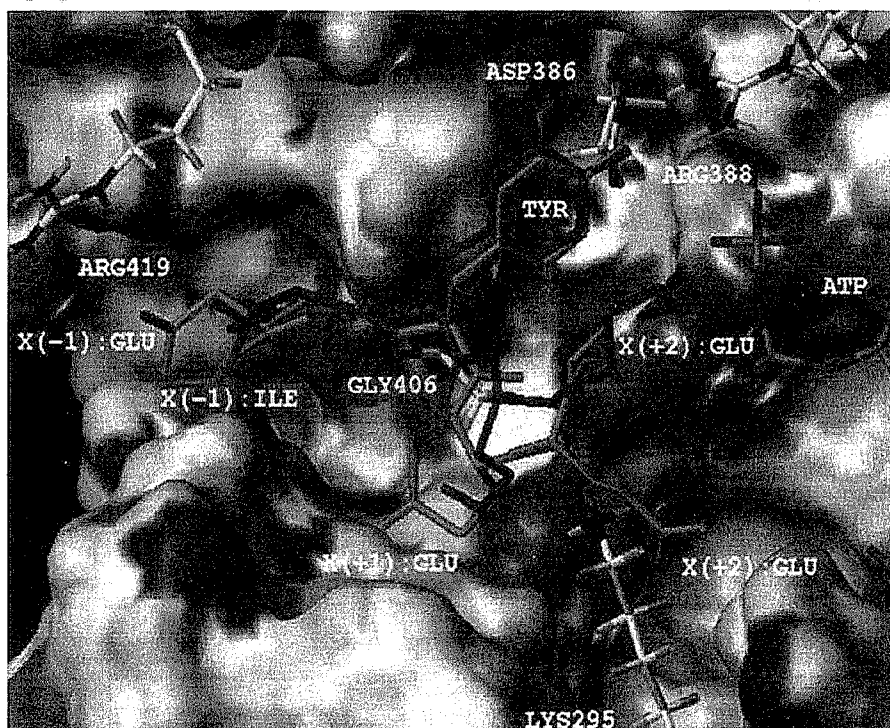


[Fig. 8]

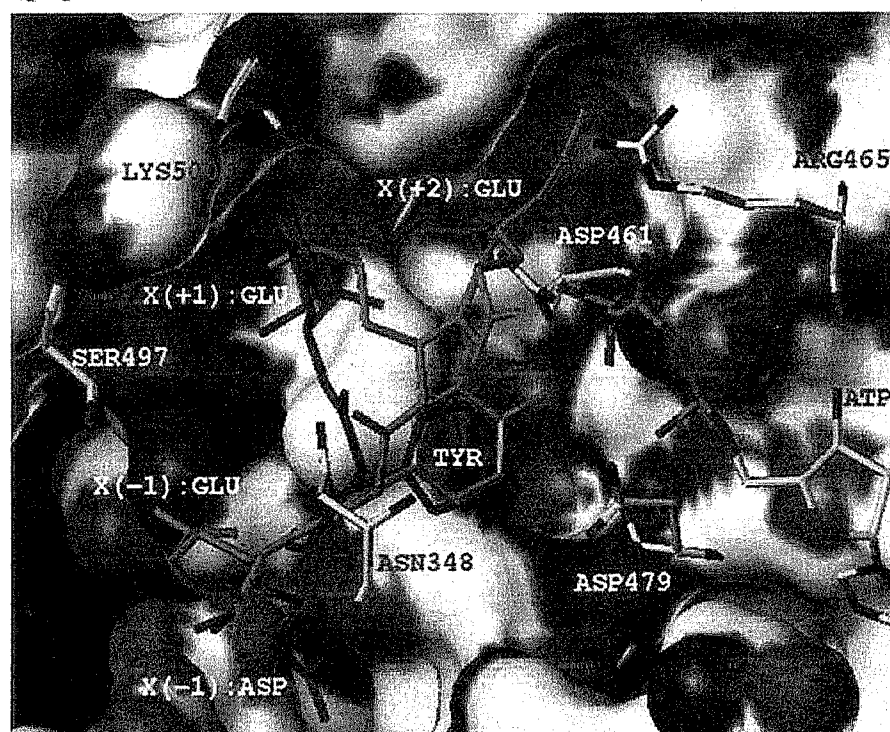
Protein tyrosine kinase	Identified sequence	Protein name(s)
ZAP-70	DTYDE	T-cell receptor zeta chain
	EIYED	Proto-oncogene CBL-B / c-CBL
		Proto-oncogene Vav
	LIYDE	Proto-oncogene tyrosine protein kinase receptor ret (C-ret)
	HTYED	Ephrin type-A receptor 5 (Tyrosine-protein kinase receptor EHK-1)

[Fig. 9]

(a)



(b)



A. CLASSIFICATION OF SUBJECT MATTER*C12Q 1/00(2006.01)i*

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8: C12Q 1/00, C40B 40/02

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS, WPI, USPTO, PAJ "kinase, peptide library, ladder peptide, OBOC, etc."

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2006-0134697 A1 (KIT S. LAM and XIAOBING WANG, US) 22 Jun. 2006 See the whole document.	1-12
A	ADAMS L. GARSKE and JOHN M. DENU, 'SIRT1 top 40 hits: use of one-bead, one-compound acetyl-peptide libraries and quantum dots to probe deacetylase specificity', In: Biochemistry, January 2006, Vol.45(1), pp.94-101. See the whole document.	1-12
A	SCOTT E. MARTIN and BLAKE R. PETERSON, 'A colorimetric enzyme-linked on-bead assay for identification of synthetic substrates of protein tyrosine kinases', In: Journal of Peptide Science, May 2002, Vol.8(5), pp.227-233. See the whole document.	1-12
A	RUIWU LIU et al., 'Development and applications of topologically segregated bilayer beads in one-bead one-compound combinatorial libraries', In: QSAR & Combinatorial Science, December 2005, Vol.24(10), pp.1127-1140. See the whole document.	1-12
A	US 5,510,240 A (KIT S. LAM and E. SALMON, US) 23 Apr. 1996 See the whole document.	1-12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

23 OCTOBER 2008 (23.10.2008)

Date of mailing of the international search report

23 OCTOBER 2008 (23.10.2008)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-
gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

Heo, Joo-Hyung

Telephone No. 82-42-481-8150



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2008/002632

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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