

METHOD FOR DETECTING PROTEIN HAVING CHANGES IN ENERGY STATE, OR AFFINITY OF LIGAND TO PROTEIN

FIELD OF THE INVENTION

[0001] The present invention belongs to the research field of functional proteomics (proteomics category). Specifically, the present invention involves detecting proteins with changes in energy state, identifying a ligand-binding protein, or determining the local affinity between a ligand and proteins in complex protein mixtures such as cell or tissue lysates.

BACKGROUND OF THE INVENTION

[0002] Proteins are the primary players in biological processes. Traditional quantitative proteomics is commonly used to assess differences in protein abundance among different samples. However, many diseases, such as Parkinson's disease and Alzheimer's disease, or the interaction of ligands (including drugs, endogenous metabolites, etc.) with proteins, result in changes in the protein folding status, post-translational modifications, or alterations in protein function (De Souza N, et al., *Curr Opin Struct Biol*, 2020, 60:57-65; Cappelletti V, et al., *Cell*, 2021, 184(2):545-559), rather than in the abundance of specific proteins. These changes in protein properties beyond abundance can affect the thermodynamic stability, that is, the energy states of proteins. Therefore, the development of methods capable of detecting changes in the energy states of proteins is of significant importance in fields such as disease diagnosis, analysis of metabolic molecular pathways, and drug target discovery.

[0003] Top-down mass spectrometry analysis allows for direct analysis of intact proteins, to monitor changes in the energy states of proteins (Sheng Y et al., *International Journal of Mass Spectrometry*, 2010, 300 (2011) 118-122). However, this method has low throughput and is mainly applicable to purified proteins, making it challenging to apply in complex protein mixtures. There are also non-mass spectrometry methods available to detect changes in the energy states of proteins, such as fluorescence resonance energy transfer (Heyduk T, et al., *Curr. Opin. Biotechnol*, 2002, 13, 292–296), nuclear magnetic resonance (Sakakibara D, et al., *Nature*, 2009, 458, 102–105), etc. These techniques are suitable for purified proteins and therefore cannot be applicable at the proteomic level.

[0004] Bottom-up mass spectrometry is a technique that involves enzymatically digesting

large protein fragments into smaller peptides for subsequent mass spectrometry analysis. This technique allows for the identification or quantification of thousands, or even tens of thousands of proteins simultaneously. In recent years, a series of high-throughput methods combining bottom-up mass spectrometry techniques to analyze changes in protein energy states have received widespread attention both domestically and internationally. These methods are based on the altered tolerance to heat or denaturants following changes in protein energy states. Consequently, proteins with lower energy states are more likely to maintain their original conformation, making them less prone to precipitation or structural unfolding when subjected to the same intensity of thermal or denaturant stimuli. By analyzing the differences in the amount of precipitated or unfolded proteins between two or more groups of samples, proteins with changes in energy state can be identified. Such techniques include Cellular Thermal Shift Assay (CETSA, Martinez Molina et al., *Science*, 2013, 341(6141):84-87), Thermal Proteome Profiling (TPP, Savitski M et al., *Science*, 2014, 346(6205): 1255784), and Pulse Proteolysis (PP, Chiwook P et al., *Nature Methods*, 2005, 2(3):207-212), etc. These methods for detecting changes in protein energy states have successfully been applied to identify drug target proteins (Savitski M et al., *Science*, 2014, 346(6205): 1255784; Perrin J et al., *Nature Biotechnology*, 2020, 38(3):303-308), analyze protein-protein interactions (Tan C S et al., *Science*, 2018, 359(6380):1170-1177), and study functional post-translational modifications (Huang J X et al., *Nature Methods*, 2019, 16, 894-901). However, these methods analyze changes in protein energy states at the protein level and cannot be used to determine the specific regions where protein energy states undergo changes. In addition, for a protein with multiple domains, changes in local stability may have minimal impact on the overall stability of the protein, making it difficult to capture stability changes at the protein level (Mateus A, et al., *Proteome Sci*, 2016, 15:13).

[0005] Limited Proteolysis (LiP) has long been used to study protein conformation. This method is based on the differential susceptibility of a protein to proteolysis in different conformations. By using a small amount of nonspecific enzyme, the protein is slightly cleaved, primarily targeting the disordered regions in high energy states and generating large protein fragments. When the protein undergoes conformational changes (e.g., disordered regions becoming more ordered), the susceptibility of the conformationally changed regions to proteolysis changes accordingly. The conformationally changed regions of the protein are identified by detecting the abundance differences of the resulting large protein fragments through immunoblotting. Since changes in protein energy states are often accompanied by conformational changes, this method can also be used to study changes in protein energy states

and to identify the regions where protein energy states change (Antonio Aceto, et al., *The International Journal of Biochemistry & Cell Biology*, 1995, 27(10):1033-1041; Polverino de Laureto, et al., *J Mol Biol*, 2003, 334(1):129-141). However, due to the difficulty of directly analyzing large protein fragments in a bottom-up mass spectrometry analysis mode for high-throughput quantification, this technique is limited to the characterization of purified proteins.

[0006] Paola et al. combined Limited Proteolysis (LiP) with bottom-up mass spectrometry analysis (Feng YH, et al., *Nature Biotechnology*, 2014, 32(10):1036-1044). The large fragments generated from limited proteolysis (i.e., the aforementioned large protein fragments) were subjected to denaturation using denaturing agents and then to a second complete trypsin digestion, to generate semi-tryptic peptides containing a nonspecific cleaved site and fully tryptic peptides without a nonspecific cleaved site. By quantifying the abundance differences of semi-tryptic or fully tryptic peptides in two or more groups of samples using bottom-up quantitative proteomics, the proteins with conformational changes were determined. Additionally, by analyzing the positions of these peptides on the protein, the conformationally changed regions were determined. This method has to some extent achieved large-scale analysis of changes in protein energy state and captured interactions between metabolites and proteins in relatively simple biological systems (Piazza I, et al., *Cell*, 2018, 172(1-2):358-372) as well as protein structural changes due to post-translational modifications, heat stimulation, and osmotic pressure (Cappelletti V, et al., *Cell*, 2021, 184(2):545-559). However, the two-step digestion in this method significantly increased the complexity of the samples and was prone to generating interfering peptides. Therefore, this method is mostly applied in relatively simple biological systems. Paola et al. (2020) further developed LiP-Quant (Piazza I, et al., *Nature Communications*, 2020, 11(1):4200), a method that improves the reliability of target protein identification based on LiP-MS with multiple ligand concentrations. LiP-Quant has to some extent achieved the identification of ligand-binding proteins and their binding regions in mammalian cell lysates. However, due to the two-step digestion, it is challenging to identify semi-tryptic peptides that contain a nonspecific cleaved site. Additionally, peptides with a nonspecific cleaved site exhibit a relatively weak response to ligand binding, resulting in very limited sensitivity for the identification of ligand-binding target proteins. For instance, in the identification of target proteins for the broad-spectrum kinase inhibitor staurosporine, only 9 kinase target proteins in HeLa cell lysates were identified by LiP-Quant (Piazza I, et al., *Nature Communications*, 2020, 11(1):4200), compared to 111 kinase target proteins in HeLa cell lysates by the present invention (see Example 3), when requiring that >80% of all identified candidate target proteins are kinases.

[0007] The aforementioned proteolysis-based methods for studying protein conformation involve using a relatively small amount of enzyme, which confines the cleavage site to a high-energy disordered region. Different conformations of the protein result in distinct large protein fragments. The proteins with conformational changes are determined by detecting the differences in these generated protein fragments through immunoblotting or denaturant-assisted secondary digestion. There is no hint of using a larger amount of enzyme to induce disruptive digestion of the proteins (i.e., first digesting structures in the high-energy states of proteins, to induce the exposure of cleavage sites in low-energy states, and then digesting structures in low-energy states in the presence of a substantial amount of enzyme) to directly generate small peptides with a molecular weight less than 5 kDa having two cleaved sites that reflect the stability of protein regions. In the bottom-up mass spectrometry analysis mode, quantitative proteomics is used to analyze the abundance differences between these peptides to determine proteins and protein regions with altered energy states, or the local binding affinity of a ligand to a protein. To address these issue, we have invented a method that can sensitively determine the proteins and protein regions with change in energy state, and measure the local affinity of a ligand to a protein in complex protein mixtures.

[0008] This method utilizes disruptive digestion to directly generate small peptides that are suitable for bottom-up mass spectrometry analysis and contain two cleaved sites both reflecting the local stability of protein, significantly reducing sample complexity compared to the products obtained by two-step digestion with two kinds of enzymes used in LiP-MS. Furthermore, during the generation of these peptides, the protein undergoes a relatively thorough digestion (i.e., protein structures in high energy states are initially disrupted, inducing further digestion of protein structures in low energy states). Within this cascading process, the cumulative differences in digestion rates at each stage result in small peptides exhibiting large magnitudes of responses to the changes in protein energy states or binding with a ligand, thereby substantially increasing the range of changes in the detectable peptide fragments. Therefore, this method exhibits high sensitivity in detecting changes in protein energy states. For example, compared to LiP-Quant, when requiring the kinase proportion to be greater than 80% among all identified target proteins for staurosporine, 111 kinases in HeLa cell lysates can be identified by this method (see Example 3), which is a 12.3-fold improvement over LiP-Quant (9 kinases can be identified). This method outputs quantitative results at a peptide level and can be used to calculate the local binding affinity of protein to a ligand at multiple doses.

SUMMARY

[0009] A purpose of the present invention is to develop a method capable of sensitively determining proteins or protein regions with changes in energy state in a complex system, or local binding affinity of ligands to proteins. This method is referred to as PEptide-centric Local Stability Assay (PELSA).

[0010] The principle for this invention is that when the energy state of a protein changes, its local or global stability also changes, altering its ability to resist external disruption. The present invention exploits the fact that the enzymatic digestion is a disruptive process where changes in the protein energy state affect resistance to disruption caused by enzymatic digestion. At high concentrations of protease, the protein is first cleaved into large subunits. Due to the disruptive nature of enzymatic digestion, the subunit structure in a high-energy state or without binding to ligand are disrupted and quickly unfold, inducing further enzymatic digestion of the structure itself. This leads to the destruction of the structure in the low energy states and ultimately producing small peptides (< 5 kDa) that are suitable for bottom-up mass spectrometry analysis and contains two cleaved sites both capable of reflecting the stability of protein region. On the other hand, the protein region in a low-energy state or binding to ligand maintains a stable structure and is less likely to enter the active pocket of the protease, making it difficult to generate small peptides. A protein with altered energy states can be determined by comparing the abundance differences of such peptides. A protein region with altered energy state can be determined by analyzing the locations of different peptides on the protein. In this method, the protein directly generates small peptides containing two cleaved sites that both capable of reflecting protein's local stability under a non-denaturing condition. Due to the relatively thorough cascading digestion process of generating small peptides, the difference in enzymatic digestion rates at every stage in this cascading process accumulates, which result in the small peptides exhibiting large magnitudes of responses to the changes in protein energy state or binding with ligands. Furthermore, since this method involves extensive disruptive enzymatic digestion, the cleavage sites are not limited to a high-energy disordered region, resulting in a greater number of peptides that reflect changes in protein stability. Additionally, using only one protease in this process significantly reduces the complexity of the generated peptides, allowing for interrogating a large number of proteins. These characteristics make this method highly sensitive.

[0011] When a ligand is exposed to a complex protein sample, the binding of the ligand with its target protein will result in the change of the energy state of a ligand-binding region in the

target protein. Therefore, the protein binding to the ligand and the ligand-binding region thereof could be determined by analyzing the change of the energy state of the intact protein with this method.

[0012] The principle of this technique for determining the local affinity of the protein to the ligand is based on the dose-dependent changes in the local stability of the target protein with respect to the dose of the added ligand, as long as saturation concentration is not reached. By detecting the abundance of cleaved small peptides in samples treated with different ligand concentrations, the local affinity of the ligand to the protein region can be calculated.

[0013] The small peptides detected by this technique possess two cleaved sites that both reflect the protein local stability. Different stability levels of various regions in the protein under its tested conformation result in varying degrees of susceptibility in enzymatic digestion. The cleaved sites generated under such conditions are referred to as cleaved sites reflecting the protein local stability.

[0014] The present invention comprises the following technical scheme: (a) preparing a solution of proteins in different energy states or a solution of proteins incubated with ligands of varying concentrations, and then subjecting the solution to disruptive enzymatic digestion at a specific ratio of enzyme to protein, allowing most proteins to generate small peptides with a molecular weight less than 5 kDa. These peptides contain two cleaved sites both capable of reflecting the protein's local stability; (b) isolating the cleaved peptides and quantitatively analyzing the cleaved peptides using a bottom-up mass spectrometry analysis mode; and (c) analyzing the differences in abundance of the cleaved peptides to determine the proteins and protein regions with changes in energy state, as well as the local binding affinity of ligands to the protein.

[0015] Specifically, the technical scheme includes the following steps.

[0016] 1) Retaining the protein conformation to be tested in the solution mentioned in step (a). If tissue or cell lysate is obtained through lysis, one or more gentle lysis methods, such as repeated freezing and thawing with liquid nitrogen, liquid nitrogen grinding, or homogenization, may be employed. The lysis solution used also preserves the protein conformation to be tested.

[0017] 2) Dividing the biological samples containing proteins into two groups during the investigation of ligand-binding proteins. The experimental group is treated with a specific concentration of the ligand, while the control group is treated with a blank solvent. However, it is not limited to only two groups; the experimental group may also include multiple groups with

varying concentrations of ligands.

[0018] 3) Adding enzyme to the solution at a weight ratio of enzyme to total proteins ranging from 1:1 to 1:50 and digesting the proteins at a certain temperature for 0.5 min to 60 min, using an oscillating shaker at a speed of 1000 rpm to 1500 rpm.

[0019] 4) Heating the digested samples from step 3) at 100 °C in a metal bath for 5 min to terminate the digestion.

[0020] 5) Dissolving the heating-induced precipitates in guanidine hydrochloride at a final concentration of 6 M followed by adding a final concentration of 10 mM tris(2-carboxyethyl)phosphine (TCEP) and a final concentration of 40 mM chloroacetamide (CAA) at 95 °C for 5 min to alkylate the digested samples.

[0021] 6) Transferring the alkylated samples from step 5) to a 10 kDa ultrafiltration unit and centrifuging at 14000 g for 20 min to 1 h to isolate the small peptides. The technical scheme further includes subsequently washing the ultrafiltration membrane with 200 µL pH 8.2 HEPES buffer followed by another centrifugation at 14000 g to isolate the residual peptides, and combining the residual peptides with the peptides collected in the initial centrifugation.

[0022] 7) Quantifying the collected peptides from step 6) using mass spectrometry, by utilizing either label-based or label-free quantification methods.

[0023] 8) Analyzing the quantification results at the peptide level, with the significance of or fold change of the first/second/third/fourth -rank peptide (ranking by significance or fold change) are used as indicators for the significance or fold change of the protein. This approach is employed to screen reliable target proteins.

[0024] 9) Assigning the peptides to their respective protein sequences or structures to identify the protein regions with changes in energy state, fitting fold changes of the peptides at different ligand concentrations and the corresponding ligand concentrations into a four-parameter logistic equation ($Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC50} - X) * \text{Hill Slope}})$), and calculating the local affinity between the ligand and the protein, as represented by the EC50 value in the equation.

[0025] Compared to existing methods, the present invention, known as PELSA, exhibits several innovations and advantages:

[0026] (1) PELSA explores the enzymatic digestion, which is a disruptive process. By using a large amount of enzyme, proteins under a non-denaturing condition are initially disrupted at

the protein structures of high energy states by the disruptive effect of digestion, which leads to the exposure of cleavage sites at the protein structures of low energy states. In the presence of large amount of enzyme, the protein structures in the low energy states are also digested and disrupted, allowing most proteins to directly generate small peptides with a molecular weight less than 5 kDa. These peptides are then identified and quantified using mass spectrometry. The peptides identified in PELSA are generated from the cascading-like disruptive digestion process and these peptides can be directly analyzed by mass spectrometry in a bottom-up manner. When proteins undergo changes in energy state or bind with ligands, the cumulative difference in enzymatic digestion rates at each stage of the cascading digestion process result in significant responses from the peptides. Additionally, this method utilizes a large amount of enzyme, enabling the generation of a greater number of ligand-responsive peptides. For example, in Example 2, PELSA identified 2-5 times more ligand-responsive peptides compared to the LiP-MS method. Furthermore, the fold change magnitudes of these peptides measured in PELSA were 4-6 times higher than those observed in LiP-MS.

[0027] (2) Wide applicability: In comparison to traditional methods based on affinity chromatography, the method bypasses the need for chemical modification of ligand molecules when it is used to identify ligand-binding proteins. This means that the method can, in theory, be applied to any ligand. Additionally, the identification of ligand-binding target proteins is not dependent on the strength of their binding affinity, making it suitable for the identification of target proteins with low ligand affinity. For instance, successful identification of the target proteins of metabolites such as folate, leucine, and α -ketoglutarate (α KG) is demonstrated in Examples 4-6. Notably, Example 6 represents the largest number of known α KG target proteins identified in a single experiment to date.

[0028] (3) Determination of protein regions with different energy states or binding regions of ligands: In comparison to the widely-applied Thermal Proteome Profiling (TPP), this method enables the identification of regions within proteins with changes in energy state or regions involved in ligand binding. This determination is achieved by analyzing the positions of the peptides with changes within the proteins. As demonstrated in Example 3, this method successfully identified the binding regions of the kinase inhibitor staurosporine on 154 kinases.

[0029] (4) High sensitivity: Unlike that local differences caused by protein energy states or binding to ligand are averaged across the entire protein when digestion differences is captured at the protein level, this method directly captures the local differences induced by protein energy states or binding to ligand at the peptide level, making it more sensitive in detecting subtle

changes. Additionally, the determination of ligand-binding regions by the present method does not rely on identifying peptides with specific characteristics such as those containing non-specific cleaved sites as required in LiP-MS. This makes it easier to identify differential peptides. For instance, in Example 3, PELSA identified a 2.1-fold increase in the number of kinase target proteins for the broad-spectrum kinase inhibitor staurosporine compared to TPP, and a 12.3-fold increase compared to LiP-Quant.

[0030] (5) Evaluation of local ligand-protein binding affinity: The protein samples are divided into multiple groups, and different concentrations of ligands are added to each group respectively. The resistance of the target protein to the digestion changes as the ligand concentration varies, when the saturation concentration is not reached. By fitting a four-parameter logistic equation to the data, with ligand concentration as the x-axis and the fold change in abundance of cleaved peptides as the y-axis, the affinity information can be obtained. A single protein may be identified with multiple peptides, and the affinity curves of each peptide represent the local binding affinity of the ligand to that specific region. For example, example 11 demonstrated that the local affinity between the ligand and the protein measured by PELSA were very close to those determined using microscale thermophoresis assay.

[0031] (6) Broad application prospects: Protein-ligand binding, protein post-translational modifications, protein structural changes, or protein-protein interactions can all induce changes in protein energy states. Therefore, this method can be applied to investigate where external perturbations cause changes in protein energy states, providing a powerful tool for addressing a variety of biological questions. Example 7 demonstrates the application of PELSA in analyzing changes in protein energy states caused by protein-protein interactions. Example 8 showcases the application of PELSA in analyzing changes in protein energy states resulting from interactions between proteins and post-translationally modified peptides. Example 9 illustrates the application of PELSA in analyzing changes in protein energy states induced by interactions between metal ions and proteins.

BRIEF DESCRIPTION OF THE FIGURES

[0032] FIG. 1 Flowchart of PELSA: Proteins with different energy states (e.g., a protein in the ligand-bound state and the same protein in the ligand-unbound state) undergo disruptive digestion in a specific ratio of enzyme to protein. Due to the binding of the ligand to the protein in the ligand treated group, the binding region becomes more stable, resulting in less

susceptibility to unfolding and thus generation of less amount of cleaved peptides compared to the control group. The digestion products are subjected to denaturation and alkylation, followed by ultrafiltration to isolate the cleaved peptides, which are then subjected for quantitative mass spectrometry analysis.

[0033] FIG. 2 Application of PELSA in analyzing changes in protein energy states in cell lysates treated with the anti-breast cancer drug lapatinib. (A) Analysis of the protein secondary structures at PELSA cleaved sites: PELSA cleaved sites refer to the N- and C-terminal residues of all identified peptides in the PELSA experiment. Protein secondary structure information is sourced from AlphaFold, and the classification of secondary structures is based on the literature (Bludau I, et al, PLoS Biol, 2022, 20(5): e3001636). HELIX represents low-energy state helical structures, STRAND represents folded structures, BEND represents bent structures, TURN represents turn structures, and unstructured represents high-energy disordered structures. (B) Protein-level volcano plot corresponding to BT474 cell lysates treated with 100 nM lapatinib: peptide quantitative values were obtained from BT474 cell lysates treated with 100 nM lapatinib or DMSO (in four replicates). Fold change and P value for each peptide was calculated using Empirical Bayes t-test. The peptide with the smallest P value per protein was used to represent the corresponding protein, and the $\log_2$ fold change and $-\log_{10}$ P value of the protein are plotted as x- and y- axis, respectively. (C) Two-dimensional local stability profiles of ERBB2: $\log_2$ fold change ($\log_2$ FC) of quantified peptides of ERBB2 and their corresponding positions on the two-dimensional sequence of ERBB2. (D) Local affinity profile of ERBB2: $\log_2$ fold change of ERBB2 peptides at different concentrations of lapatinib. (E) Protein-level volcano plot corresponding to BT474 cell lysates treated with 1 μ M lapatinib. (F) Two-dimensional local stability profiles of off-target kinases identified for lapatinib, using CHEK2, SLK, RIPK2, or YES1 as examples. (G) Western blotting confirms the stabilization of PTGES2 upon lapatinib treatment.

[0034] Unless otherwise stated, the terms "fold change", "FC" (short for fold change), or "ratio" in this specification refer to the ratio of peptide abundance between the experimental and control groups.

[0035] FIG. 3 Comparison of PELSA and LiP-MS for the identification of proteins with changes in energy state in HeLa cell lysates treated with methotrexate (MTX) or SHP099. (A) (Top) Peptide-level volcano plots obtained from LiP-MS/PELSA for HeLa cell lysates treated with 10 μ M MTX. (Bottom) Peptide-level volcano plots obtained from LiP-MS/PELSA for HeLa cell lysates treated with 10 μ M SHP099. (B) In the MTX or SHP099-treated system, the

number of ligand-responsive peptides of the target proteins identified by PELSA was 2-fold and 5.25-fold higher, respectively, compared to those by LiP-MS. (C) The fold changes of ligand-responsive peptide of the target proteins DHFR and PTPN11 identified by PELSA were 4.3-fold and 6.4-fold higher, respectively, compared to those by LiP-MS. (D) The PDB structure of DHFR bound to MTX, with an arrow indicating a peptide located at the MTX-binding site within a low-energy state helical region. This peptide exhibited significant fold changes in PELSA but remained unchanged in LiP-MS; (E) The PDB structure of PTPN11 bound to SHP099, with an arrow indicating a peptide situated at the SHP099-binding site and embedded within the protein structure. Similarly, this peptide showed significant fold changes in PELSA but no fold changes in LiP-MS.

[0036] FIG. 4 shows the high sensitivity of PELSA in identifying proteins with changes in energy state, for example, in screening for target proteins of a broad-spectrum kinase inhibitor staurosporine. (A) Protein-level volcano plots corresponding to- K562 cell lysates (left) and HeLa cell lysates (right) treated with 20 μ M staurosporine. (B) A comparison of the number of staurosporine target proteins identified by PELSA in K562 cell lysates, PELSA in HeLa cell lysates, LiP-Quant in HeLa cell lysates as previously reported, and TPP in K562 cell lysates as previously reported. The x-axis represents the total number of identified target proteins, and the y-axis represents the number of kinases among the identified target proteins. (C) The number of proteins and peptides, and the final number of kinase targets identified by LiP-Quant (HeLa), TPP (K562), PELSA (K562), and PELSA (HeLa). (D) A comparison of protein sequence coverages for all proteins identified by PELSA (HeLa) and LiP-Quant (HeLa) (left), and the comparison of protein sequence coverages for kinase targets identified by PELSA (HeLa) and LiP-Quant (HeLa) (right). This graph indicates that PELSA can identify target proteins with lower protein sequence coverages. (E) Split violin plots illustrating the distribution of thermal melting points for kinase targets identified by PELSA (HeLa), PELSA (K562), or TPP (K562), and for all quantified kinase proteins in each dataset. This graph demonstrates that PELSA is effective in identifying both thermo-resistant and thermo-sensitive target proteins. (F) Overlap of kinase targets identified by PELSA (K562) and PELSA (HeLa). (G) Density plots showing the significance of stability changes in peptides located within the kinase domain versus other peptides identified by PELSA in K562 (left) and in HeLa (right). The x-axis represents the significance of change, i.e., $-\log_{10}P$ value, calculated using the Empirical Bayes t-test, and the y-axis represents the density. The circular plot shows the proportions of significantly changed peptides that are located within the kinase domain, that are very close to the kinase domain (within ± 10 amino acid residues away from the kinase domain), that belong to proteins without

a kinase domain, and that are located outside the kinase domain. It can be seen that, the majority of peptides with significant fold changes are located within the kinase domains.

[0037] FIG. 5 illustrates the application of PELSA in analyzing proteins with local energy state changes in HeLa cell lysate upon metabolite treatment. (A) Protein-level volcano plot corresponding to K562 cell lysate treated with 50 μ M folate. (B) Three-dimensional structure plot demonstrating stability changes of DHFR upon folate binding as measured by PELSA, with an arrow indicating the protein regions with the largest stability change. (C) Three-dimensional structure plot demonstrating stability changes of ATIC upon addition of 50 μ M folate as measured by PELSA, with an arrow indicating the protein fragments with the largest stability change. (D) Two-dimensional plot demonstrating stability changes of MTHFR upon addition of 50 μ M folate as measured by PELSA. (E) Three-dimensional structure plot demonstrating stability changes of GART upon addition of 50 μ M folate as measured by PELSA, with an arrow indicating the protein fragments with the largest stability change. (F) Two-dimensional plot demonstrating stability changes of P3H1 upon addition of 50 μ M folate as measured by PELSA. (G) Protein-level volcano plot corresponding to K562 cell lysates treated with 5 mM leucine. (H) Two-dimensional plot demonstrating stability changes of LARS1 upon addition of 5 mM leucine as measured by PELSA. (I) Topological structure diagram of the membrane protein SLC1A5, with the start and end positions of three quantified peptides (190-212, 493-502, and 523-541) indicated, along with their corresponding $|\log_2FC|$ values labeled beside the respective peptides. (J) Two-dimensional plot demonstrating stability changes of PPIP5K1 and PPIP5K2 upon addition of 5 mM leucine as measured by PELSA. (K) Protein-level volcano plot corresponding to HeLa cell lysates treated with 2 mM α -ketoglutarate (α KG), with the dashed lines representing $-\log_{10}P$ value = 3.4 and $\log_2FC$ = -0.5, respectively. 30 out of the 40 proteins meeting the criteria of $-\log_{10}P$ value > 3.4 and $\log_2FC$ < -0.5 are known target proteins for α KG. (L) Two-dimensional plot demonstrating stability changes of EGLN1, RSN1L, or KDM3B determined by PELSA in HeLa cell lysates treated with 2 mM α KG. The protein fragments detected to have altered energy states by PELSA are known α KG-binding regions.

[0038] FIG. 6 depicts the application of PELSA in analyzing changes in local energy states of proteins due to protein-protein interactions (illustrated by antibody-antigen binding) in HeLa cell lysates. (A) Schematic representation of PELSA identifying antibody-binding epitopes in cell lysate. (B) (Left) Protein-level volcano plot corresponding to DHFR antibody-treated HeLa cell lysate. (Right) Protein-level volcano plot corresponding to CDK9 antibody-treated HeLa cell lysates. (C) Three-dimensional structure plot demonstrating stability changes of DHFR

upon addition of DHFR antibody as measured by PELSA (PDB:1BOZ), with shaded spheres indicating the antibody binding epitope, and the arrow indicating the protein fragments with the greatest stability change. (D) (Top) Two-dimensional plot demonstrating stability changes of DHFR upon addition of DHFR antibody as measured by PELSA. (Bottom) Two-dimensional plot demonstrating stability changes of CDK9 upon addition of CDK9 antibody as measured by PELSA. (E) Abundance value changes of the two peptides, NPATTNQTEFERVF and NPATTNQTEFER, of CDK9 upon addition of CDK9 antibody.

[0039] FIG. 7 depicts the application of PELSA in analyzing changes in local energy states of proteins in BT474 cell lysates resulting from post-translational modifications and protein interactions (illustrated by identifying the recognition domains of a phosphorylated tyrosine). (A) Schematic representation of PELSA identifying recognition domains of a post-translational modification in cell lysates. (B) Protein-level scatter plot in the PELSA experiment, where pSEEI and YEEI are used as controls, $-\log_{10}$ P-value (pYEEI/pSEEI) and $-\log_{10}$ P-value (pYEEI/YEEI) are calculated for each peptide, and the peptide with the highest sum of $-\log_{10}$ P-value (pYEEI/pSEEI) and $-\log_{10}$ P-value (pYEEI/YEEI) is used to represent the corresponding protein. (C) Protein-level scatter plot in Pulldown experiment, where pSEEI and YEEI are used as controls, and $-\log_{10}$ P-value (pYEEI/pSEEI) and $-\log_{10}$ P-value (pYEEI/YEEI) are calculated for each protein. (D) Violin plots showing the $\log_2$ FC distribution of peptides within and outside the SH2 domains of 9 SH2 domain-containing target proteins identified by PELSA. (E) Two-dimensional plots demonstrating stability changes of SH2 domain-containing proteins identified by PELSA, showcasing examples such as YES1, TNS3, PLCG1, and GRB10. (F) Two-dimensional plots demonstrating stability changes of calcium-binding proteins identified by PELSA, showcasing examples such as EFHD1 and CALM1.

[0040] FIG. 8 depicts the application of PELSA in analyzing protein with changes in energy state in HeLa cell lysates treated with metal ions (illustrated with zinc ions) (A) Protein-level volcano plot corresponding to HeLa cell lysates treated with 30 μ M ZnCl₂. (B) Proportion of metal ion-binding proteins among all identified proteins (left) and proportion of metal ion-binding proteins among the 280 significantly stabilized proteins (right). The pie chart on the right illustrates the ratio of zinc ion-binding proteins among the stabilized metal ion-binding proteins. (C) Bar graph showing the number of various metal ion-binding proteins among the significantly stabilized metal ion-binding proteins. (D) Distribution of $\log_2$ fold change for peptides derived from 60 PELSA-identified proteins containing zinc finger motifs, grouped based on peptides located within and outside the zinc finger motifs. (E) Two-dimensional plots demonstrating stability changes of PELSA-identified proteins containing zinc finger motifs,

showcasing examples such as SQSTM1, TRAD1, CHAMP1, and YY1. (F) Two-dimensional plots demonstrating stability changes of a zinc ion-binding protein LIMA1, which lacks zinc finger motifs, after treatment with 30 μM ZnCl_2 . Three peptides within the LIM domain are labeled as 1, 2, and 3, respectively. (G) Three-dimensional structure of LIM domain of LIMA1, with spheres representing zinc ions. The three identified peptides of LIM domain are labeled in the same order as in (F). (H) Distribution of $\log_2$ fold changes for peptides derived from 20 PELSA-identified proteins containing EF-hand/EH motifs, grouped based on peptides located within and outside the EF-hand/EH motifs. (I) Distribution of $\log_2$ fold changes for peptides derived from 9 PELSA-identified target proteins containing Fe^{2+} -binding domains, grouped based on peptides located within and outside the Fe^{2+} -binding domains. (J) Zoom-in view of protein-level volcano plot corresponding to HeLa cell lysate treated with 30 μM ZnCl_2 , retaining only proteins with increased energy states ($-\log_{10}\text{P-value} > 6$, $\log_2$ fold change > 0). (K) Two-dimensional plots demonstrating stability changes of proteins containing IQ motifs (UBE3C, MYO1B, MYO1C) and HNRNPU containing B30.2SPRY domains after the addition of 30 μM ZnCl_2 . (L) Distribution of $\log_2$ fold changes for peptides derived from PSMC1-6, grouped based on peptides located within and outside the P-loop-NTPase domains, after the addition of 30 μM ZnCl_2 .

[0041] FIG. 9 Comparison of the protein coverages and identification of proteins with changes in energy state based on digestions with different enzymes by PELSA. (A) Comparison of numbers of peptides identified by PELSA based on Trypsin, Chymotrypsin, and Proteinase K (hereafter abbreviated as Trypsin-PELSA, Chymotrypsin-PELSA, and Proteinase K-PELSA, respectively). (B) Venn diagram showing the overlap of identified proteins between Trypsin-PELSA, Chymotrypsin-PELSA, and Proteinase K-PELSA. (C) Protein-level volcano plots for Trypsin-PELSA (left), Chymotrypsin-PELSA (middle), and Proteinase K-PELSA (right) in HeLa cell lysate treated with 20 μM methotrexate (MTX).

[0042] FIG. 10 Identification of proteins with changes in energy state in HeLa cell lysates treated with three heat shock protein (Hsp) inhibitors using dimethyl labeling-based PELSA. (A) Structures of the three heat shock protein inhibitors: geldanamycin (left), tanespimycin (middle), and ganetespib (right). (B) The experimental groups were treated with 100 μM geldanamycin, 100 μM tanespimycin, or 100 μM ganetespib and the control group was treated with an equal volume of blank solvent. A scatter plot was generated, where the peptides with the second-largest change represent the protein, and the x-axis and y-axis of the plots depict the $\log_2$ fold changes obtained from two replicates for mass spectrometry analysis. (C) Fold changes in the abundance of peptides from different domains of HSP90AA1, HSP90AB1,

HSP90B1, and HSP90AB2P after treatment with geldanamycin. (D) Validation of the interactions between MAT2A and ganetespib, AKR1C2 and ganetespib, and AKR1C2 and geldanamycin using thermal shift assay.

[0043] FIG. 11 shows the local affinity between ligands and proteins evaluated using PELSA. (A) Fold changes in peptide abundance (ganetespib-treated vs. vehicle-treated) for heat shock proteins HSP90AA1, HSP90AB1, HSP90B1, and TRAP1 at different concentrations of ganetespib. (B) Affinity measurements between the proteins of the heat shock protein family and three inhibitors calculated by PELSA. The x-axis represents different inhibitor concentrations, increasing from left to right, and the y-axis represents the fold change in peptide abundance upon drug treatment. A fold change of 1 indicates no abundance change. The annotated values indicate the half-maximal inhibitory concentrations of the three inhibitors for HSP90AA1 obtained from PELSA calculations. (C) Affinity characterization of the three heat shock protein inhibitors with purified HSP90AA1 using microscale thermophoresis (MST). The annotated values in the figure represent the dissociation constants between HSP90AA1 and the three heat shock protein inhibitors determined by MST.

EXAMPLES

[0044] To provide a clearer explanation of the technical solutions and highlight the advantages of the present invention, the following detailed description is presented in conjunction with specific examples. It should be noted that these examples are not intended to limit the scope of the invention.

[0045] In the following examples: Example 1 and Examples 4-9 illustrate the method can be used to identify proteins and protein regions whose energy states are altered in a cell lysate upon treatment with anticancer drugs, metabolites, antibodies, peptides with post-translational modifications, or metal ions. Example 2 validates that compared with the comparative method LiP-MS, PELSA generates more peptides responsive to changes in energy state or responsive to ligand binding, and the magnitude of change in these peptides in PELSA is significantly greater. Example 3 establishes that PELSA is currently the most sensitive method available for the identification of proteins and protein regions with altered energy states. Example 10 illustrates the utilization of other non-specific or specific enzymes for digestion, by detecting peptides containing two cleaved sites both capable of reflecting protein local stability, also enable the identification of proteins with changes in energy state. Example 11 demonstrates the peptide quantification by PELSA can also be performed using a dimethyl labeling-based quantification. Example 12 demonstrates that PELSA can be used in the determination of the

affinity between ligands and their binding regions. Additionally, Examples 1-9 illustrate that PELSA can be used in identifying proteins and corresponding binding regions thereof that binds to drugs, broad-spectrum kinase inhibitors, metabolites, antibodies, peptides with post-translational modifications, and metal ions. Furthermore, the binding of ligands to target proteins demonstrated in Examples 1-9 can induce conformational changes in the target proteins, thereby highlighting the applicability of the method in studying the conformational changes of proteins.

[0046] In Example 1, treating BT474 cell lysate with 100 nM lapatinib led to remarkable energy state changes ($-\log_{10}P$ value > 5) specifically in the known target protein of lapatinib, ERBB2. The energy state change precisely occurred in the region corresponding to the kinase domain, confirming that PELSA is capable of identifying proteins and regions with energy state changes, or the proteins binding to the ligand or binding regions of ligands thereof. Furthermore, based on the PELSA results, it was observed that when the lapatinib concentration was increased to 1 μ M, a higher number of off-target kinase proteins were identified.

[0047] In Example 2, a comparison between LiP-MS and PELSA was conducted for the identification of proteins with energy state changes induced by treated with MTX and SHP099 in HeLa cell lysate. The results demonstrated that PELSA was able to identify a greater number of peptides responsive to changes in protein energy states or ligand binding compared to LiP-MS (2-5 times more than those identified by LiP-MS). Additionally, the amplitude of these peptides in response to ligand binding or energy state changes was 4-6 times greater than that in LiP-MS.

[0048] In Example 3, 121 and 111 kinase target proteins for staurosporine were identified by PELSA in K562 and HeLa cell lysates, respectively. These numbers were 2.1 times greater than those reported using TPP method (53 kinase target proteins) and 12.3 times greater than that reported using the LiP-Quant method (9 kinase target proteins) when the same proportion of kinase target proteins was achieved. This result highlights that PELSA is the most sensitive for identifying proteins with changes in energy state.

[0049] Examples 4-6 demonstrated the high sensitivity of PELSA in detecting weak protein-ligand interactions and accurately locating the ligand-binding regions.

[0050] In Example 7, PELSA successfully identified the epitope of antigen binding to an antibody in HeLa cell lysate, indicating the capability of PELSA to identify other protein-protein interaction interfaces.

[0051] In Example 8, PELSA successfully identified the recognition domains for tyrosine phosphorylation (pYEEI) in BT474 cell lysates, providing a powerful tool for identifying recognition domains for other post-translational modifications. In Example 9, PELSA successfully identified 112 Zn^{2+} -binding proteins and accurately located the Zn^{2+} -binding regions in cell lysates. This finding demonstrates that PELSA can identify ligand-protein interactions regardless of ligand size. Moreover, based on the PELSA results, the addition of zinc ions stabilized the calcium ion-binding motif (EF-hand/EH motif) while destabilizing the IQ motif, which interacts with the EF-hand motif. This suggests that zinc ion binding to the EF-hand motif leads to dissociation from the IQ motif, resulting in the destabilization of the IQ motif. These results also emphasize the potential of PELSA in studying binding interfaces of protein complexes.

[0052] Example 10 investigated the use of various enzymes in PELSA other than trypsin. The results showed that although the number of peptides or proteins identified using chymotrypsin and proteinase K was significantly lower compared that using trypsin, the identification of MTX-binding proteins can be achieved. This suggests that the utilization of enzymes other than trypsin in PELSA could enable the identification of high-abundance target proteins as well.

[0053] In Example 11, the combination of PELSA with dimethyl labeling-based quantification was explored to identify target proteins for heat shock protein inhibitors. The results showed that the combination of PELSA with dimethyl labeling-based quantification and the strategy of screening the second most significantly changed peptides enabled identification of target proteins and off-target proteins for heat shock protein inhibitors at a high specificity.

[0054] Example 12 demonstrates that PELSA can accurately determine the binding affinity between ligands and proteins.

Example 1

[0055] Application of PELSA in the identification of the proteins and protein regions with changes in energy state after treatment of BT474 cell lysate with the anticancer drug lapatinib.

[0056] (1) a dish of BT474 cells (approximately 5×10^7 cells) were resuspended in 1 mL lysis buffer (PBS supplemented with 1% (v/v) protease inhibitor (Sigma, catalog number P8340-5mL)). The mixture was subjected to freeze-thaw cycles three times (frozen in liquid nitrogen for two minutes, thawed in a 37°C water bath for two minutes, and repeated three times) to obtain a mixture of cell debris and cell lysate. The mixture was centrifuged at 500g and 4°C for 10 minutes, and the supernatant was collected to obtain the cell lysate. The protein concentration

was determined using the Pierce™ 660 nm Protein Assay (Thermo, USA).

[0057] (2) The protein concentration of the extracted cell lysate was adjusted to 1 mg/mL using cell lysis buffer (PBS buffer containing 1% (v/v) protease inhibitor, Sigma, Cat. No. P8340-5mL). Four aliquots of the cell lysate were transferred to EP tubes. As experimental groups, to 50 µL each aliquot, 0.5 µL of lapatinib (Selleck, Cat. No. S2111) stock solution of different concentrations was added to achieve final concentrations of 100 nM, 1 µM, 10 µM, and 100 µM (corresponding to lapatinib stock concentrations of 10 µM, 100 µM, 1 mM, and 10 mM, respectively; lapatinib was dissolved in DMSO). As a control, 0.5 µL of DMSO was added into 50 µL of cell lysate. Both experimental and control groups were performed in quadruplicate and incubated at room temperature for 30 minutes.

[0058] (3) Trypsin (Sigma, Cat. No. T1426) was added to the experimental and control groups at a ratio of enzyme to protein of 1 : 2 (by weight). The samples were digested at 37°C and 1000 rpm on a shaker for 1 minute. The digestion was terminated by adding 165 µL of pH 8.2 HEPES buffer (Sigma, Cat. No. H3375) containing 8 M guanidine hydrochloride (Sigma, Cat. No. G3272), resulting in the digested samples.

[0059] (4) TCEP (Sigma, Cat. No. C4706) and CAA (Sigma, Cat. No. 22790) were added to the digested samples to final concentrations of 10 mM and 40 mM, respectively. The digested samples were heated at 95 °C for 5 minutes and then cooled to room temperature. The samples were further transferred to a 10 kDa ultrafiltration tube (Sartorius, Cat. No. VN01H02) and centrifuged at 14,000 g for 50 minutes. The digested peptides were collected by filtering through a membrane, and the ultrafiltration membrane was washed with 200 µL of pH 8.2 HEPES buffer. The peptides from both ultrafiltration steps were combined.

[0060] (5) The peptide solution was desalted using a 200 µL tip column filled with 2 mg of Oasis HLB filler (Waters, USA). The desalted peptides were lyophilized to obtain the peptide mixture.

[0061] (6) The above-mentioned peptide mixture was reconstituted in 20 µL of 0.1% (v/v) formic acid (Sigma, Catalog No. V900803) and subjected to LC-MS/MS analysis. Each sample was analyzed once using data-independent acquisition (DIA) mass spectrometry. The acquired spectra based on Spectronaut software (Biognosys, Switzerland) was used to identify and quantify the peptides, providing information on the corresponding protein, the peptide's location within the protein, and its abundance in each sample. In this example, we first analyzed the secondary structure at the PELSA cleaved sites. The results showed that 59% of the cleaved

sites in PELSA experiments were located within the helical regions of proteins (FIG. 2A), suggesting that the protein structures were disrupted during PELSA, resulting in the digestion of protein regions, even in stable, low-energy states.

[0062] (7) The abundance of peptides in the lapatinib-treated and untreated groups was statistically evaluated using Empirical Bayes t-test to determine the P-value and fold change for each peptide, representing the significance and magnitude of the peptide abundance change upon lapatinib treatment. For each protein, the peptide with the lowest P value (highest significance) among all its peptides was chosen to represent the protein for screening the ligand-binding protein.

[0063] In this study, proteins with a $-\log_{10}P$ value > 5 were defined as proteins with changes in energy state. Figure 2B shows a volcano plot with $-\log_{10}P$ value on the y-axis and $\log_2$ fold change ($\log_2FC$) on the x-axis for all quantified proteins. In FIG. 2B, among the 5,774 quantified proteins, only the known target protein of lapatinib, ERBB2, exhibited significant fold changes under treatment with 100 nM lapatinib.

[0064] ERBB2 is a receptor tyrosine kinase located on the cell membrane. It consists of extracellular, transmembrane, and intracellular regions, including a kinase domain of ERBB2 on which lapatinib acts. In FIG. 2C, x-axis denotes the protein sequence from the N-terminus to the C-terminus with square representing the kinase domain on which lapatinib acts, and the y-axis represents the fold change in abundance of ERBB2 peptides under 100 nM lapatinib treatment. Analysis of the identified ERBB2 peptides (Figure 2C) showed that only peptides located at or adjacent to the kinase domain exhibited significant changes ($|\log_2FC| > 0.3$ and $-\log_{10}P$ value > 2). This result demonstrates the PELSA's ability to determine the drug-binding region.

[0065] Furthermore, it was observed that at higher concentrations of lapatinib (100 μ M, 10 μ M, and 1 μ M), the peptides within the kinase domain of ERBB2 were more resistant to digestion (FIG. 2D). Additionally, it was noted that while only ERBB2 exhibited changes in energy state at a lapatinib concentration of 100 nM, increasing the lapatinib concentration led to energy state changes in several other kinases such as CHEK2, SLK, RIPK2, and YES1 (FIG. 2E). Most of the peptides exhibited changes in these kinases were also located within the respective kinase domains of the proteins (FIG. 2F). These findings indicate that lapatinib, at higher doses, exhibits off-target interactions with other kinases, suggesting that PELSA can be used to assess drug promiscuity and guide drug design and synthesis.

[0066] Furthermore, we also observed that the non-kinase protein PTGES2 exhibited changes in its energy state at high concentrations of lapatinib (FIG. 2E). To validate the binding of PTGES2 and lapatinib, we performed Western blotting using a similar procedure and conditions as mentioned above, with the following modifications: a large dish of BT474 cells (cell count approximately 5×10^7) was suspended in PBS, and cell lysis and protein concentration determination were performed as described. The resulting cell lysates were divided into 8 portions and treated with equal volumes of lapatinib at different final concentrations (lapatinib dissolved in DMSO), or treated with DMSO, resulting in final lapatinib concentrations of 100 μ M, 50 μ M, 10 μ M, 1 μ M, 0.1 μ M, 0.01 μ M, 0.001 μ M, and 0. The mixtures were incubated at room temperature (25°C) for 30 minutes. After incubation, trypsin was added at a 1:40 protease-to-protein weight ratio, and the mixture was subjected to digestion at 37°C for 1 minute. The digestion was terminated by heating the samples at 95°C, followed by the addition of 5 x loading buffer at 1/4 volume of the protein solution, and heating at 95°C for another 5 minutes. Western blotting was employed to detect the undigested proteins. The samples were run in the concentrated gel at 80 V for 20 minutes, and then in separated gel at 120 V for 60 minutes. Transfer blotting was conducted at a constant current of 250 mA for 40 minutes. After blocking, the primary antibody against PTGES2 (Proteintech, USA) was added at a 1:800 dilution and incubated at room temperature for 1 hour, followed by incubation with a goat anti-rabbit HRP-IgG secondary antibody (Abcam, UK) at room temperature for 1 hour. Chemiluminescent detection was performed using ECL reagent (Thermo Fisher Scientific, America) and the Fusion FX5 chemiluminescence system (Vilber Infnit, France) were used for imaging of the undigested proteins. The undigested protein bands were quantified based on their intensities. GAPDH was used as a reference protein, incubated with a primary antibody against GAPDH overnight at 4 °C, and then incubated with a rabbit anti-mouse HRP-IgG secondary antibody (Abcam, UK) at room temperature for 1 hour, and chemiluminescent detection was performed. As shown in FIG. 2G, with increasing concentrations of lapatinib, the bands became progressively darker, indicating an increasing amount of PTGES2 protein protected from digestion. The maximum value was reached at a lapatinib concentration of 50 μ M. These results suggest that the resistance of PTGES2 to digestion depends on the lapatinib concentration and thus provides further evidence supporting the notion that PTGES2 may be an off-target protein of lapatinib.

[0067] The above analysis results demonstrate that PELSA can identify ligand-binding proteins with high specificity and determine the ligand-binding regions based on the peptides with changed abundance. This unbiased, omics-level screening method also facilitates

identifying off-target proteins, such as other kinases and PTGES2, as identified in this example. This indicates that the method can evaluate drug promiscuity and provide guidance for drug design and development. Furthermore, only peptides within the ligand-binding regions showed significant abundance fold changes, indicating that the method can reveal the drug acting regions. The dose-dependent changes in abundance of the target proteins upon treatment with ligands at different concentrations suggest the capacity of PELSA to determine the binding affinities between ligand and the target proteins.

Example 2

[0068] Comparison between LiP-MS and PELSA for the identification of proteins with changes in energy state upon treatment with methotrexate (MTX) and SHP099 in HeLa cell lysates.

[0069] To showcase the advantages of PELSA in identifying proteins with changes in energy state, in this example, the existing method LiP-MS was compared with the present invention (PELSA) for identifying proteins exhibiting energy state changes based on digestion after MTX and SHP099 treatment in HeLa cell lysates. DHFR is the target protein of MTX, and PTPN11 is the target protein of SHP099. In this example, the PELSA procedure and experimental conditions were the same as in Example 1, except for the following differences: HeLa cell samples were used, and the ligands were MTX (Selleck, catalog number S1210) at a final concentration of 10 μ M or SHP099 (Selleck, catalog number S8278) at a final concentration of 10 μ M. The LiP-MS procedure adheres to the protocol described in the literature by Piazza et al. (Nature Communication, 2020, 11(1):4200), with the following steps (The following describes the procedures and experimental conditions that differ from those in Example 1):

[0070] (1) The cell lysis buffer used was composed of 60 mM HEPES pH 7.5, 150 mM KCl, and 1 mM $MgCl_2$ (Piazza et al., Nature Communication, 2020, 11(1):4200).

[0071] (2) After incubation of the cell lysate with the drug, Proteinase K (Sigma, catalog number P2308) was added at a 1:100 (wt/wt) ratio of protease to protein. The mixture was incubated at 25°C, 1000 rpm on a shaker for 4 minutes, heated at 98°C for 1 minute, and then an equal volume of 10% by volume sodium deoxycholate (Sigma, catalog number D6750) was added. The mixture was heated at 98°C for another 4 minutes to denature the protein fragments to obtain a protein solution.

[0072] (3) After denaturation, to the aforementioned protein solution, TCEP (Sigma, Cat. No. C4706) and CAA (Sigma, Cat. No. 22790) was added to a final concentration of 10 mM and 40

mM, respectively. The sample was heated at 98°C for 5 minutes, cooled to room temperature, and subsequently diluted with a four-fold volume of pH 8.2 60 mM HEPES buffer to attain sodium deoxycholate at a final concentration of 1%.

[0073] (4) Lys-C (Wako chemicals) was added at a 1:100 (wt/wt) ratio of protease to protein and incubated for 4 hours. Subsequently, trypsin (Promega) was added at a 1:50 (wt/wt) ratio of protease to protein and incubated for 16 hours.

[0074] (5) After the digestion, formic acid (FA) was added to achieve a final volume of 1.5%. The sample was left to settle for 10 minutes, and once the sodium deoxycholate precipitate had achieved equilibrium, the solution was centrifuged at room temperature for 10 minutes at 20,000 g (twice) to remove the sodium deoxycholate precipitate.

[0075] (6) The peptide desalting, mass spectrometry quantification, software searching, and data analysis processes were the same as in Example 1, except when searching with Spectronaut, enzyme was set as “trypsin” and digest type was set as “semi-tryptic”.

[0076] In FIG. 3A and 3B, the peptides that meet the criteria of $-\log_{10}P$ value > 2 (above the horizontal dashed line in the figure) and $|\log_2$ fold change $| > 0.3$ (outside the vertical dashed line in the figure) are defined as ligand-responsive peptides. In the MTX-DHFR system, PELSA identified 2 times more MTX-responsive DHFR peptides than those identified by LiP-MS. In the SHP099-PTPN11 system, PELSA identified 5.25 times more SHP099-responsive PTPN11 peptides than those identified by LiP-MS. Furthermore, the response magnitude of DHFR peptides to MTX in PELSA was 4.3 times higher than in LiP-MS (FIG. 3C), and the response magnitude of PTPN11 peptides to SHP099 in PELSA was 6.4 times higher than in LiP-MS (FIG. 3C). For example, in the MTX-DHFR system, a peptide located at the MTX-binding site displayed significant fold changes in PELSA experiments, whereas no fold change was detected in the same peptide in LiP-MS experiments (FIG. 3D). This peptide in DHFR is in a low-energy state helical structure, which is difficult to access with mild digestion in LiP-MS. However, the disruptive digestion in PELSA allows regions of the protein with low-energy states to be cleaved, enabling the detection of changes in this peptide. Similarly, in the SHP099-PTPN11 interaction system, change of the peptides located at the SHP099 binding site and within the internal structure of the protein was detected only in the PELSA experiments (FIG. 3E). This finding further demonstrates that PELSA can disrupt internal, stable structures of the protein, thereby capturing changes in energy state that occur within these regions. These findings suggest that PELSA surpasses LiP-MS in identifying a larger number of ligand-responsive peptides in the target proteins. Moreover, the ligand-responsive peptides identified by PELSA

exhibit significantly higher response magnitudes compared to those identified by LiP-MS. This also makes PELSA more sensitive in identifying proteins with changes in the energy states. For example, in this example, GART, which is involved in the pharmacokinetics of MTX (Mikkelsen T S et al, Pharmacogenet Genomics, 2011, 21(10): 679-686), was identified as a protein with changes in the energy states only in PELSA, but not in LiP-MS (Figure 3A).

[0077] The example demonstrates that the present invention (PELSA), compared to the existing LiP-MS method, can generate more ligand-responsive peptides and these ligand-responsive peptides display large magnitudes of fold changes in response to ligand binding. Consequently, PELSA enables the identification of a larger number of ligand-binding proteins. This partly explains the exceptionally high sensitivity of the present invention in the identification of ligand-binding proteins.

Example 3

[0078] PELSA identification of the proteins and protein regions in HeLa and K562 cell lysates that with changes in energy state upon treatment with a broad-spectrum kinase inhibitor staurosporine.

[0079] This example demonstrated the high sensitivity of PELSA in identifying proteins with changes in energy state, through the identification of staurosporine-binding proteins and comparison with results reported in the literature.

[0080] The experimental procedure and conditions were similar to Example 1, with the exception of using K562 and HeLa cell samples. The ligand molecules used in the experimental group was 20 μ M staurosporine (final concentration, Selleck, catalog number S1421). As depicted in FIG. 4A, PELSA revealed that a large number of kinases were stabilized in both HeLa and K562 lysates upon staurosporine treatment. By setting cutoff of kinase proportion among the identified target proteins to 80%, PELSA identified 121 kinase targets (out of a total of 143 target proteins) stabilized in K562 cell lysate and 111 kinase targets (out of a total of 135 target proteins) stabilized in HeLa cell lysate. In contrast, according to published literature, LiP-Quant only identified 9 kinase targets responsive to staurosporine under the same cutoff of kinase proportion (i.e., $\geq 80\%$ of target proteins being kinases), while TPP identified 53 kinase targets responsive to staurosporine (out of a total of 60 target proteins) (FIG. 4B). These findings unequivocally demonstrate the exceptional sensitivity of PELSA compared to existing methods.

[0081] We further compared the protein and peptide coverage depths of LiP-Quant, TPP, and

PELSA. We observed that although in LiP-Quant experiment, a greater number of peptides were identified (FIG. 4C), and LiP-Quant achieved higher overall protein sequence coverages compared to PELSA (FIG. 4D), PELSA identified a significantly larger number of kinase responsive to staurosporine (i.e., kinases identified as target proteins) than LiP-Quant (111 kinases versus 9 kinases). Further analysis revealed that the sequence coverages of kinase targets identified by PELSA were lower than those identified by LiP-Quant in LiP-Quant experiment (FIG. 4D). This suggests that PELSA is capable of identifying binding proteins even with lower sequence coverages as target proteins. In contrast, LiP-Quant, due to the presence of many irrelevant peptides resulting from complete digestion by trypsin, requires higher protein sequence coverages to accurately identify target proteins.

[0082] Although TPP identified more proteins (7673) compared to PELSA (6310) in -K562 (FIG. 4C), PELSA ultimately identified 2.28 times more kinase targets than TPP, further highlighting the high sensitivity of PELSA in target protein identification. Analysis of the melting points of kinase targets identified by TPP and PELSA revealed that TPP has a limited capacity in identifying kinase targets with very high or very low melting temperatures. In contrast, PELSA effectively identifies kinase targets having a wide range of melting temperature points (FIG. 4E).

[0083] PELSA identified a total of 192 target proteins for staurosporine in the two cell lines, of which 154 target proteins were kinases, accounting for 80% of all target proteins (FIG. 4F). Additionally, PELSA is capable of identifying the regions binding to staurosporine, as shown in FIG. 4G. Within the significantly changed peptides identified by PELSA, over 92% of the peptides were located within or in close proximity (within 10 amino acid residues) to kinase domains in both K562 and HeLa lysates.

[0084] The experimental results indicate that PELSA not only exhibits extremely high sensitivity in the identification of ligand-binding proteins but also accurately identifies the regions of proteins binding to the ligands.

Example 4

[0085] PELSA identification of proteins and protein regions with changes in energy state upon treatment with the metabolite folate in K562 cell lysate.

[0086] Unlike the strong interaction between lapatinib and ERBB2 (with an affinity of approximately 9 nM), folate exhibits weaker binding affinity to its binding proteins. For instance, previous research indicates that folate binds to its target protein DHFR with a

dissociation constant ranging from 3 to 60 μM (Ozaki Y et al., *Biochemistry*, 1981, 20(11): 3219-3225). Therefore, we employed folate to investigate whether PELSA is capable of analyzing the changes in protein energy state induced by binding of a ligand to a protein at a low affinity.

[0087] Most experimental procedures and conditions were the same to Example 1, except the followings: K562 cell samples were used. After subjecting the cell samples to three rounds of freeze-thaw cycles, the supernatant was obtained by centrifugation at 500 g, 4°C for 10 minutes. To remove endogenous folate, a protein desalting step was performed using Zeba Spin desalting columns (Thermo Fisher Scientific). The protein concentration of the desalted lysates was determined using the Pierce™ 660nm Protein Assay (Thermo, USA). The protein concentration was adjusted to 1 mg/mL using cell lysis buffer, i.e. a PBS buffer containing 1% (v/v) protease inhibitor (i.e. cocktail; Sigma, Cat. No. P8340-5mL). The ligand used in this experiment was folate (Sigma, catalog number F7879) at a final concentration of 50 μM . The subsequent steps were carried out as described in Example 1.

[0088] As shown in FIG. 5A, the stability of DHFR, a known protein to which folate targets, exhibited the most significant changes. By mapping the DHFR peptides with changes onto its protein structure (FIG. 5B), we observed that the regions experiencing the most notable stability changes corresponded to the binding site of folate. Furthermore, we identified stability changes in three proteins (ATIC, MTHFR, and GART) which interact with with folate analogs. The stabilized regions of these proteins coincided with the sites binding to the folate analogs (FIG. 5C-5E). P3H1, is a prolyl 3-hydroxylase involved in collagen prolyl hydroxylation, and literature suggested that folate can participate in proline hydroxylation in collagen (Haustvast J, et al., *British Journal of Nutrition*, 1974, 32(2): 457-469). Our experimental results revealed that the hydroxylase domain of P3H1 was stabilized upon the addition of folate (FIG. 5F), providing evidence for folate's involvement in collagen prolyl hydroxylation.

Example 5

[0089] PELSA identification of proteins and protein regions with changes in energy state upon treatment with the metabolite leucine in K562 cell lysate.

[0090] The dissociation constants of Leucine with its target proteins, LARS1 and SESN2 are 95 μM (Kim S, et al., *Cell Reports*, 2021, 35(4): 109301) and 20 μM (Wolfson RL, et al., *Science*, 2016, 351(6268): 43-48), respectively. Therefore, we also used leucine to assess the applicability of PELSA in analyzing the changes in protein energy state induced by ligand-

protein interactions at a weak affinity.

[0091] The procedures and conditions followed those outlined in Example 4, except that leucine (Sigma, catalog number 61819) was used as the ligand with a final concentration of 5 mM. As depicted in FIG. 5G, we observed significant changes in the energy states of well-known leucine target proteins, including LARS1, LARS2, GLUD1, and SENS2. LARS1 possesses two leucine-binding sites located in the CD and CP domains, corresponding to the synthetic site and editing site, respectively. We noted that the peptides from the synthetic site of LARS1 (labeled as CP in the FIG. 5H) exhibited more pronounced change magnitude compared to those from the editing site (labeled as CD in the FIG. 5H). Moreover, peptides from the C-terminal domain, which does not contribute to leucine binding, showed no response (FIG. 5H). These findings indicate the ability of PELSA to differentiate between distinct binding sites on a single protein.

[0092] We also observed significant changes in the stability of SLC1A5, another known leucine-binding protein (FIG. 5G). SLC1A5 is a membrane protein composed of intracellular, transmembrane, and extracellular regions. The Na-dicarboxylate_symporter domain (residues 54-483), located in the extracellular region, plays a role in amino acid transport. Among the identified peptides of SLC1A5, only the peptide (residues 190-212) from Na-dicarboxylate_symporter domain showed a significant change (FIG. 5I), demonstrating that our method can be used to identify binding regions of membrane proteins and determine the binding regions. Interestingly, we also discovered that leucine stabilized PPIP5K1 and PPIP5K2 (FIG. 5G), specifically at their shared histidine phosphatase domains (FIG. 5J).

[0093] This example showcases PELSA is also highly effective in identifying weak interactions between metabolites and proteins and accurately identifying the binding regions of the metabolites. Additionally, the successful identification of SLC1A5 as a leucine target protein demonstrates ELSA performs well in identifying target membrane proteins. Several proteins that may interact with leucine were also identified by PELSA, shedding light on future investigations into the functions of leucine.

Example 6

[0094] PELSA identification of proteins and protein regions with changes in energy state upon treatment with the metabolite alpha-ketoglutarate (α KG) in HeLa cell lysate.

[0095] The procedures and conditions followed those outlined in Example 4, except that the cell lysate was derived from HeLa cells and α KG (Sigma, catalog number 75890-25g) was used

as the investigated ligand with a final concentration of 2 mM. Proteins that met the criteria of $-\log_{10}P$ value > 3.4 and $\log_2FC < -0.5$ were considered as proteins stabilized by 2 mM α KG.

[0096] As depicted in FIG. 5K, PELSA identified 40 proteins that were stabilized by 2 mM α KG in HeLa cell lysate treated with 2 mM α KG, out of which 30 proteins were already known α KG target proteins. This represents the largest number of known α KG target proteins identified in a single experiment to date. While literature reports the use of LiP-MS to identify α KG target proteins in *Escherichia coli* (Piazza I et al., Cell, 2018, 172(1-15)), only 2 out of the 34 identified target proteins were previously known α KG-binding proteins. Additionally, two-dimensional profiles showing the stability changes determined by PELSA (FIG. 5L) demonstrated that the regions of the α KG target protein with energy change precisely matched the α KG-binding regions.

[0097] In summary, this example demonstrates that PELSA exhibits extremely high sensitivity in analyzing weak interactions between metabolites and proteins, and is capable of identifying the regions binding to metabolites.

Example 7

[0098] PELSA identification of proteins and protein regions with changes in energy state upon treatment with antibodies in HeLa cell lysate.

[0099] This example demonstrates the application of PELSA in identifying antibody-binding epitopes by identifying protein regions with changes in energy state in HeLa cell lysate upon treatment with antibodies.

[0100] Most experimental procedures and conditions were the same to Example 1, except the followings: HeLa cells were used, and in the experimental groups, two commercial antibodies, DHFR antibody (Wabways, China, RRID: AB_2877179) and CDK9 antibody (Wabways, China, RRID: AB_2877178), were used as the investigated ligands with a final concentration of 2% (v/v) and 1% (v/v), respectively, while in control group, an equivalent volume of excipient was used. Proteins met a $-\log_{10}P$ value > 5 were considered as proteins with changes in energy state. FIG. 6A illustrates the schematic representation of identifying antibody-binding epitopes in cell lysate. FIG. 6B (left) depicts the volcano plot of proteins obtained from DHFR antibody-treated HeLa cell lysate, whereas FIG. 6B (right) shows the volcano plot of proteins obtained from CDK9 antibody-treated HeLa cell lysate. The addition of DHFR and CDK9 antibodies caused significant changes in the energy states of DHFR and CDK9, respectively, indicating that PELSA can directly identify antigen proteins in cell lysate. Other proteins

showing changes in energy state might be attributed to non-specific binding to antibody.

[0101] Further analysis of changes in all DHFR peptides identified by PELSA revealed that the peptides with changes in energy state precisely corresponded to the known epitopes recognized by the antibody (FIG. 6C and 6D). For CDK9, two peptides exhibited abundance changes in opposite directions. The CDK9 antibody recognized the epitope sequence PATTNQTEFERVF located at the C-terminus of CDK9. The sequence of NPATTNQTEFER contains a site within the epitope, resulting in a reduced peptide yield upon antibody addition (FIG. 6D). Conversely, the sequence of NPATTNQTEFERVF (NPxxVF), which fully encompassed the epitope sequence but had no the trypsin cleavage site located in the epitope, exhibited an increased peptide yield upon antibody addition (FIG. 6D). Further analysis revealed that the summed intensities of these two peptides were minimally affected by the addition of the antibody (FIG. 6E). Thus, the opposite changes observed in these two peptides can be attributed to the protective effect of the antibody, rendering the C-terminal R site of the NPATTNQTEFER sequence less susceptible to cleavage, resulting in a decreased yield of the corresponding peptide. As consequence, the NPATTNQTEFERVF (NPxxVF) sequence, which should remain unchanged by CDK9 antibody treatment, were left more. These results demonstrate the high resolution of PELSA in identifying antibody-binding epitopes.

[0102] This example illustrates that PELSA can effectively identify interactions between antibodies and proteins, and revealing antibody-protein binding sites. Furthermore, it indicates the potential of PELSA to be applied to various protein-protein interaction systems, or the like.

Example 8

[0103] PELSA identification of proteins and protein regions with changes in energy state upon treatment with post-translational modified peptides in BT474 cell lysate.

[0104] Post-translational modifications (PTMs) of proteins play a crucial role in various biological activities. The regulation of these activities often requires the recognition and recruitment of effector proteins by downstream proteins. Therefore, the identification of proteins that recognize PTMs is essential for understanding functions of PTMs and studying disease mechanisms. Phosphorylated tyrosine-glutamate-glutamate-isoleucine (referred to as pYEEI) is known to be recognized by proteins containing SH2 domains. In this analysis, we demonstrated that PELSA can be used to identify the PTM-recognizing domains by studying proteins and protein regions with changes in energy state upon treatment with pYEEI in BT474 cell lysates.

[0105] Most experimental procedures and conditions were the same to Example 1, except the followings: To prevent the phosphorylated peptide from being dephosphorylated by active phosphatases present in the cell lysate, an additional 2 mM phosphatase inhibitor was added to the cell lysis buffer. For parallel comparison with results of pulldown experiments, we utilized 100 μ M N-terminal biotin-conjugated pYEEI (abbreviated as Biotin-pYEEI, synthesized by Qiangyao Biotechnology) as the ligand molecules. To eliminate any potential influences arising from the phosphate group and YEEI motif, two control groups were included. One control group was treated with 100 μ M N-terminal biotinylated phosphorylated serine-glutamate-glutamate-isoleucine (abbreviated as Biotin-pSEEI, synthesized by Qiangyao Biotechnology), while the other control group was treated with 100 μ M N-terminal biotinylated tyrosine-glutamate-glutamate-isoleucine (abbreviated as Biotin-YEEI, synthesized by Qiangyao Biotechnology).

[0106] The pulldown experiment was conducted as follows: BT474 cells was used, and the cell lysis, lysate preparation, and measurement and adjustment of protein concentration were performed as described in Example 1. After adjusting the protein concentration of the cell lysate to 1 mg/mL, 100 μ L of the cell lysate was treated with Biotin-pYEEI at a final concentration of 100 μ M as the experimental group. Two additional 100 μ L of the cell lysate were treated with Biotin-pSEEI at a final concentration of 100 μ M and Biotin-YEEI at a final concentration of 100 μ M, respectively, serving as control groups. This process was repeated three times, and the cell lysate together were incubated with the added ligand molecules at room temperature for 30 minutes. After incubation, 200 μ L of avidin beads (Thermo, USA) were added to each group and incubated overnight at 4°C. Subsequently, the beads were washed four times with 400 μ L of a washing buffer (1% phosphatase inhibitor (i.e. 1% cocktail), and 0.5% NP40 in PBS), followed by four washes with 400 μ L of a cell lysis buffer (1% phosphatase inhibitor (i.e. 1% cocktail) in PBS solution). The proteins were eluted by adding 100 μ L of HEPES buffer (pH 8.2) containing 8M guanidine hydrochloride, and the elution step was repeated twice. The eluted proteins were treated with 10 mM TCEP and 40 mM CAA, and heated at 95°C for 5 minutes, to ensure complete alkylation. The eluted protein solution was transferred to an ultrafiltration tube and centrifuged at 14,000 g for 30 minutes and the filtrate was discarded. The ultrafiltration membrane was washed twice with 200 μ L of 10 mM ammonium bicarbonate (NH_4HCO_3) buffer and the filtrate was discarded. Then, 100 μ L of 10 mM NH_4HCO_3 buffer was added to resuspend the proteins retained on the membrane, and 2 μ g of trypsin (Promega) was added for overnight digestion. The next day, the ultrafiltration tube was centrifuged at 14,000 g for 50 minutes to collect the peptides obtained from digestion. The ultrafiltration

membrane was washed with 100 μ L of NH_4HCO_3 buffer and the filtrate was collected to recycle the remaining peptides. The peptides from both ultrafiltration steps were pooled and subjected to lyophilization. The procedures of mass spectrometry analysis, software analysis, and data validation were the same as described in Example 1.

[0107] FIG. 7A depicts a schematic diagram illustrating the identification of tyrosine phosphorylation recognition domains in cell lysates. Using YEEI as the control group, the Empirical Bayes t-test yielded the P value ($p_{\text{YEEI/YEEI}}$). Using pSEEI as the control group, the Empirical Bayes t-test yielded the P value ($p_{\text{YEEI/pSEEI}}$). The logarithm of both P values was plotted, and proteins meeting the criteria of $-\log_{10}P$ value ($p_{\text{YEEI/YEEI}}$) > 3.1 , $\log_2\text{FC}$ ($p_{\text{YEEI/YEEI}}$) < 0 , $-\log_{10}P$ value ($p_{\text{YEEI/pSEEI}}$) > 3.1 , and $\log_2\text{FC}$ ($p_{\text{YEEI/pSEEI}}$) < 0 were defined as proteins with a reduced energy state, i.e., proteins stabilized by treatment of pYEEI. This results in the identification of 28 proteins stabilized by pYEEI. Among these 28 proteins, 9 contained the SH2 domain, and 8 proteins were found to be calcium ion-related proteins (FIG. 7B). However, in the pulldown experiment, no SH2 domain-containing proteins were identified (FIG. 7C), possibly resulting from the high stringency of washing. PELSA also successfully identified the recognition domains for pYEEI. As shown in FIG. 7D and 7E, only peptides within the SH2 domain were stabilized upon the addition of pYEEI, while the abundance of other peptides in other regions remained unchanged. Furthermore, the identified calcium ion-related proteins were stabilized only in specific EF-hand domains (FIG. 7F).

[0108] These results demonstrate that PELSA can identify PTM-recognizing proteins and recognition regions with changes in energy state upon the treatment of cell lysates with post-translationally modified peptides.

Example 9

[0109] PELSA identification of proteins and protein regions with changes in energy state upon treatment with metal ions in HeLa cell lysate.

[0110] In this example, we investigated whether PELSA can be applied to detect changes in protein energy state induced by binding of the proteins to small-sized metal ions.

[0111] Most experimental procedures and conditions were the same to Example 1, except the followings: HeLa cells were used, and the cell lysis buffer was those in example 1 supplemented with 2 mM ethylenediaminetetraacetic acid sodium salt (EDTA, purchased from Sigma). After cell lysis following the procedure described in Example 1, the added EDTA was removed using Zeba Spin desalting columns (Thermo Fisher Scientific) through two rounds of protein

desalting. A final concentration of 30 μM zinc chloride (Sigma, catalog number 450111-10G) was added to the cell lysate, serving as the experimental group. Proteins meeting the criteria of $-\log_{10}\text{Pvalue} > 3$ and $\log_2\text{FC} < -0.5$ were considered as proteins stabilized by Zn^{2+} .

[0112] FIG. 8A depicts that PELSA identified a significant number of metal ion-binding proteins that were stabilized by treatment of Zn^{2+} . Among all 280 stabilized by treatment of Zn^{2+} , more than 66% (185 proteins) were already known metal ion-binding proteins. In contrast, within the entire identified proteome, the proportion of metal ion-binding proteins was only 19%, indicating that PELSA can successfully identify metal ion-binding proteins by detecting changes in energy state upon Zn^{2+} treatment. It has been reported that Zn^{2+} can bind to the EF-hand motifs of Ca^{2+} -binding proteins (Tsvetkov P O et al., Front Mol Neurosci, 2018, 11: 459) and can bind to Mg^{2+} -binding proteins and occupy Mg^{2+} -binding sites (Dudev T et al., Chemical Reviews, 2003, 103(3): 773-787), suggesting the promiscuous nature of these divalent metal ions in metal ion-binding proteins. Consistent with the literature, we found that among the 185 metal ion-binding proteins stabilized by zinc ions, 112 proteins are zinc ion-binding proteins, and 73 proteins were other metal ion-binding proteins (FIG. 8B). Among these 73 proteins, 25 proteins were Ca^{2+} -binding proteins, 20 proteins were known Mg^{2+} -binding proteins, 15 proteins were known iron ion-binding proteins, 9 proteins were known Mn^{2+} -binding proteins, and 6 proteins were proteins that bind to further metal ions (FIG. 8C). Within the group of 112 Zn^{2+} -binding proteins, 60 proteins contained zinc finger motifs (FIG. 8C). Analyzing the proteins with zinc finger motifs revealed that the median value of $\log_2\text{FC}$ for peptides within the zinc finger motifs was significantly lower compared to peptides outside the zinc finger motifs (FIG. 8D). Analyzing individual zinc finger-containing proteins also shows that only peptides located within the zinc finger structures were significantly stabilized (Figure 8E). These results indicate the ability of PELSA to accurately identify Zn^{2+} -binding sites. Furthermore, in the case of proteins lacking zinc finger motifs, such as LIMA1 which possesses a LIM Zn^{2+} -binding domain, three peptides from this domain were quantified using PELSA (FIG. 8F). Among these peptides, the one directly involved in Zn^{2+} binding exhibited the largest fold change, while the other two peptides not directly involved in Zn^{2+} binding showed minimal changes ($|\log_2\text{FC}| < 0.3$, FIG. 8G). These results support the high precision of PELSA in accurately localizing binding sites.

[0113] Analysis of the identified Ca^{2+} -binding proteins revealed that 20 out of 27 proteins contained EF-hand/EH motifs (Ca^{2+} -binding motifs). Similar to proteins that contains zinc finger motifs, the median of $\log_2\text{FC}$ values for peptides within the EF-hand/EH motifs were significantly lower compared to peptides outside these motifs (FIG. 8H), indicating that Zn^{2+}

can act on the EF-hand/EH motifs in these Ca^{2+} -binding proteins. Furthermore, among the 9 proteins containing iron ion-binding domains, the median of $\log_2\text{FC}$ values for peptides within the iron ion-binding domains was significantly lower compared to peptides outside these domains (FIG. 8I), indicating that Zn^{2+} can act on the iron ion-binding proteins at these iron ion-binding regions.

[0114] When a ligand binds to a protein, it can dissociate the protein from its original complex, leading to destabilization of the partner protein in complex with the protein. We observed destabilization in several proteins containing IQ motifs ($-\log_{10}P$ value > 6 and $\log_2\text{FC} > 0$) (FIG. 8J). The destabilized peptides precisely locate at the IQ motifs or were in close proximity to them (FIG. 8K). IQ motifs are known binding sites for EF-hand motifs, which can interact with Zn^{2+} (FIG. 8H). Therefore, the destabilization of IQ motifs may be attributed to the dissociation between IQ motifs and EF-hand motifs caused by interaction between the EF-hand motifs with Zn^{2+} . IQ motifs and EF-hand motifs are crucial interfaces for protein-protein interactions, suggesting that PELSA can be used to analyze changes in the energy state resulting from the association and dissociation of protein complexes in cells and reveal the binding interfaces of proteins within these complexes.

[0115] We also observed destabilization in the components of the 26S proteasome regulatory subunits, specifically PSMC1-6 (FIG. 8J). Each of the PSMC1-6 proteins contain a P-Loop-NTPase domain located at the binding interface of the complexes formed with the PSMC1-6. PELSA results demonstrated that only the peptides within this domain exhibited significant destabilization (FIG. 8L), indicating that Zn^{2+} induce the dissociation of the 26S proteasome regulatory subunits, thereby destabilizing the protein-protein interaction interface of PSMC1-6. These findings further suggest that this method can be used in studying the dynamic changes due to the association and dissociation of protein complexes.

Example 10

[0116] Analysis of proteins with changes in energy state in HeLa cell lysates upon treatment with Methotrexate (MTX) by other enzyme-Based PELSA.

[0117] To assess the applicability of PELSA using proteases other than trypsin in identifying proteins with changes in energy state, we conducted parallel comparisons using trypsin and two other proteases with different cleavage specificities: chymotrypsin (purchased from Sigma, catalog number C3142) and proteinase K (abbreviated as PK, purchased from Sigma, catalog number P2308). These enzymes were used to analyze proteins with changes in energy state in

HeLa cell lysate upon treatment with MTX. Chymotrypsin primarily cleaves at the N-terminus of aromatic amino acids, while proteinase K exhibits broad cleavage specificity.

[0118] The procedures and conditions for Trypsin-PELSA were the same to those in Example 1, except the followings: HeLa cells were used; the experimental group was treated with MTX at a final concentration of 10 μ M (dissolved in DMSO, with a stock concentration of 1 mM; purchased from Selleck, catalog number S1210) as ligand molecules.

[0119] The procedures and conditions for Chymotrypsin-PELSA were the same to those in Trypsin-PELSA, except the following: trypsin was replaced with chymotrypsin; the digestion was carried out at 25°C, 1000 rpm for 1 minute; when searching with Spectronaut, the cleavage sites for the digestion were set as F, W, Y, L, and M.

[0120] The procedures and conditions for Proteinase K-PELSA were the same to those in Trypsin-PELSA, except the following: trypsin was replaced with proteinase K; the digestion was carried out at 25°C, 1000 rpm for 1 minute; when searching with Spectronaut, the cleavage sites for the digestion were set as trypsin, unspecific.

[0121] FIG. 9A shows that Trypsin-PELSA identified a total of 69,245 peptides, while Chymotrypsin-PELSA and Proteinase K-PELSA identified significantly fewer peptides (18,027 and 28,702 peptides, respectively) compared to Trypsin-PELSA. Correspondingly, Trypsin-PELSA identified a larger number of proteins (5,487) compared to Chymotrypsin-PELSA (2,710) and Proteinase K-PELSA (1,937) (FIG. 9B). Furthermore, the proteins identified by the Trypsin-PELSA covered the majority of proteins identified by the Proteinase K-PELSA and Chymotrypsin-PELSA (FIG. 9B). This observation can be attributed to the fact that tryptic peptides generated by trypsin are more favorable for mass spectrometry identification compared to the peptides generated by other enzymes. In terms of identifying proteins with changes in energy state, since DHFR, a known MTX-binding protein, is relatively abundant, all three proteases (trypsin, chymotrypsin, and proteinase K) used in PELSA can detect this protein and distinguished it from the background proteins (FIG. 9C). This example illustrates that PELSA is not limited to the use of trypsin and can also utilize other specific and unspecific proteases to identify proteins with changes in energy state. However, it may encounter the issue of a lower number of protein and peptide for identifications.

Example 11

[0122] PELSA coupled with dimethyl labeling quantification to identify proteins and protein regions with changes in energy state in HeLa cell lysates upon treatment with three heat shock

protein inhibitors.

[0123] The procedure was as follows:

[0124] (1) HeLa cells were used. The process of cell lysis, protein extraction, and protein concentration determination followed the protocol described in in Example 1. Six 50 μ L aliquots of cell lysate were prepared in Eppendorf tubes. Three aliquots were treated with 100 μ M geldanamycin, 100 μ M tanespimycin, and 100 μ M ganetespib, respectively (stock concentration: 10 mM; all dissolved in DMSO and purchased from Selleck). The remaining three aliquots were added with an equal volume of DMSO. The samples were then incubated at room temperature (25 $^{\circ}$ C) for 30 minutes. After the incubation, trypsin was added to each sample at a trypsin-to-protein ratio of 1:2 (wt/wt), and the samples were incubated at 37 $^{\circ}$ C for 1 minute. The digestion was terminated by heating the samples at 100 $^{\circ}$ C for 5 minutes.

[0125] (2) To each above protein solution, sodium dihydrogen phosphate buffer (pH 6.5) containing 8 M guanidine hydrochloride at three times volume of the sample was added. The TCEP and CAA were added with final concentrations of 10 mM and 40 mM, respectively. The samples were heated at 95 $^{\circ}$ C for another 5 minutes and then cooled to room temperature. Subsequently, the samples were transferred to 10 kDa ultrafiltration tubes and centrifuged at 14,000 g for 50 minutes to collect the peptides obtained from digestion. The ultrafiltration membranes were washed twice with 200 μ L sodium dihydrogen phosphate buffer (pH 6.5), and the filtrates from both ultrafiltration steps were pooled.

[0126] (3) Dimethyl labeling: The peptides in drug-treated group were labeled with the medium reagent, i.e., 16 μ L of 4% by volume deuterated formaldehyde (Sigma, Cat. No. 596388) and 16 μ L of 0.6 M cyanoborohydride (Sigma, Cat. No. 156159). For the control group, 16 μ L of 4% by volume formaldehyde (Sigma, Cat. No. 252549) and 16 μ L of 0.6 M cyanoborohydride were added as the light labeling. The labeling reaction was carried out at 30 $^{\circ}$ C for 1 hour. After the reaction, 10 μ L of 10% by volume ammonium hydroxide (Sigma, Cat. No. 338818) was added and incubated for an additional 30 minutes. Finally, the peptides from the drug-treated and control groups were mixed in equal masses for further analysis.

[0127] (4) The dimethyl-labeled samples were acidified by adding 4.5 μ L of trifluoroacetic acid (Sigma, catalog number T6508). The solution was desalted using a tip column (200 μ L capacity) packed with 2 mg of HLB resin (Waters, USA), and then lyophilized to obtain a peptide mixture.

[0128] (5) The above peptide mixture was reconstituted in 30 μ L of 0.1% by volume formic

acid. Then, 1 μ g of peptide was injected and subjected to LC-MS/MS analysis in data-dependent acquisition mode (DDA). Each sample was analyzed twice by mass spectrometer to obtain duplicate quantitative results for each peptide.

[0129] (6) The spectra obtained in DDA mode were analyzed using MaxQuant software (Cox, Germany) to identify the proteins to which the peptides belong, determine positions of the peptides on the proteins, and calculate the fold changes (FC) of peptide abundance between the experimental and control groups.

[0130] In this example, three representative heat shock protein inhibitors were used: geldanamycin and tanespimycin (ansamycin-class inhibitors containing a benzoquinone group) and ganetespib (a second-generation inhibitor with a novel structure) (Figure 10A). Geldanamycin and tanespimycin differ from each other only in the circled structural region. The second most significantly changed peptide (i.e., the peptide with the second-largest $|\log_2\text{FC}|$ value among all quantified peptides for a given protein) represents each protein, and the duplicate quantitative results are shown in Figure 10B. By considering indicative of proteins with changes in energy state with the duplicate quantitative result of $|\log_2\text{FC}| > 1.4$, heat shock proteins including HSP90AB1, HSP90AA1, HSP90A1, and HSP90AB2P, were successfully identified, demonstrating that PELSA based on dimethyl labeling quantification can effectively identify proteins with changes in energy state. Furthermore, it was observed that the mitochondrial heat shock protein TRAP1 was specifically identified as a protein with changes in energy state only in the ganetespib-treated group.

[0131] HSP90 proteins consist of three domains: the N-terminal ATP-binding domain, the middle domain, and the C-terminal domain. Geldanamycin, tanespimycin, and ganetespib all target the N-terminal ATP-binding domain of HSP90. As shown in FIG. 10C, taking geldanamycin as an example, analysis of the fold changes in peptide abundance across different domains revealed that only peptides from the N-terminal domain (the drug-binding region) showed significant changes in abundance. This finding further supports that PELSA can accurately identify protein regions with changes in energy state.

[0132] In addition to known target proteins, PELSA also identified several previously unknown off-target proteins. For geldanamycin, PELSA identified destabilization of several PRDX family proteins, with $\log_2\text{FC} > 1.4$ (FIG. 10B). It has been reported in the literature that geldanamycin can induce the generation of reactive oxygen species (ROS), leading to severe liver toxicity (Clark et al., Free Radical Biology & Medicine, 2009, 1440-1449). PRDX family proteins play a protective role on cells by clearing ROS under oxidative stress conditions. The

destabilization of PRDX family proteins suggests that their structures are disrupted at high concentrations of geldanamycin, which provides a possible explanation for the hepatotoxicity mechanism of geldanamycin. For tanespimycin, a structurally similar molecule to geldanamycin, PELSA identified PRDX5 and NQO1 as shared off-target proteins with geldanamycin. It has been reported that NQO1 is involved in the metabolism of ansamycin-class heat shock protein inhibitors (Reigan P D, et al., *Molecular Pharmacology*, 2011, 79(5): 823-832). Additionally, PELSA identified two previously-unreported off-target proteins of ganetespib, namely MAT2A and AKR1C2 (FIG. 10B). To further validate the binding between these two proteins and ganetespib, both proteins were purified, and a thermal shift assay was conducted to assess their interactions with ganetespib. As depicted in FIG. 10D, the thermal melting temperatures of both MAT2A and AKR1C2 significantly increased upon the addition of ganetespib, confirming the binding of AKR1C2 and MAT2A with ganetespib. Furthermore, the thermal shift assay results demonstrate that AKR1C2 binds to geldanamycin, albeit with a lower stabilizing effect of geldanamycin compared to ganetespib (FIG. 10D). Consistently, the PELSA results also indicated a smaller stabilizing effect of geldanamycin on AKR1C2 compared to ganetespib (FIG. 10B).

[0133] These findings clearly demonstrate that PELSA coupled with dimethyl labeling can precisely determine proteins with changes in energy state and effectively distinguish target proteins among structurally-similar and distinct inhibitors. The in vitro thermal shift assays with purified proteins validated that the high reliability of PELSA-identified off-target proteins. The identification of these target proteins offers valuable insights into understanding the hepatotoxicity of ansamycin HSP90 inhibitors and exploring novel applications of ganetespib.

Example 12

[0134] PELSA evaluation of the local affinity between heat shock protein inhibitors and their target proteins.

[0135] The procedures and conditions were the same as in Example 11, with the following modifications: 14 aliquots, each containing 50 μ L HeLa cell lysate, were divided into experimental and control groups, with 7 aliquots in each group. In the experimental group, different concentrations of geldanamycin are added to the lysate to achieve final concentrations of 100 μ M, 10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM, or 0.1 nM (geldanamycin dissolved in DMSO), while the samples in control group received an equal volume of DMSO. the subsequent experimental steps, mass spectrometry quantification, and software analysis procedures were the same as in example 11. The treatment procedures for tanespimycin and ganetespib followed

the same protocol as that for geldanamycin.

[0136] FIG. 11A illustrates that, using ganetespib as an example, only the peptides within the N-terminal domain of HSP90 proteins show an increase in change of peptide abundance with increasing drug concentration, eventually reaching a plateau. The affinities between the heat shock proteins and three HSP90 protein inhibitors were then calculated based on fold change of these peptides with varying drug concentrations. The peptides of the heat shock proteins with at least 12 quantification values (a total of 14 quantification values) were fitted to a four-parameter logistic equation using Prism software (GraphPad): $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC50} - X) * \text{HillSlope}})$. The quality of fit between the raw data to this equation is evaluated using the Pearson correlation coefficient (R^2), and peptides with a high fit quality ($R^2 > 0.9$) were selected as candidate peptides. Additionally, the abundance change magnitude of candidate peptides at the highest ligand concentration should be at least 30% or higher. The median fold change of all qualified peptides from the same protein is considered as the fold change of protein at a given ligand concentration, corresponding to the Y value in the four-parameter logistic equation. X still represents the ligand concentration. The four-parameter logistic equation was refitted using Prism software to determine the EC50 (FIG. 11B). As shown in Figure 11C, the proteins were purified, and the affinity (K_d) between the three inhibitors and HSP90AA1 was measured using microscale thermophoresis (MST). The affinity values K_d obtained through MST align closely with the EC50s calculated using PELSA.

[0137] These findings demonstrate that the affinity values determined by PELSA are in agreement with conventional methods for determining affinity, such as microscale thermophoresis (MST) employed in this example. Moreover, this approach enables the acquisition of affinity data at the peptide level, which will contribute to understanding the interaction between ligands and proteins.

What is claimed:

1. A method for detecting a protein with change in energy state, comprising steps of:
 - A. mixing and incubating two or more groups of protein solutions, each representing different energy states (one group serving as a control and being a protein solution in original energy state, and the others as experimental groups and being a protein solution with altered energy states), with a protease at a weight ratio of protease to total protein range from 1:1 to 1:50, for 0.5-60 minutes to enable digesting even a low-energy protein structure (i.e., digestion of a protein in a disruptive manner), which results in the direct generation of small peptides (having a molecular weight < 5 kDa) containing two cleaved sites reflecting local stability of the protein and suitable for bottom-up (identifying a protein with the peptides) mass spectrometry analysis,
 - B. isolating the small peptides (having a molecular weight < 5 -10 kDa) from the larger protein fragments (having a molecular weight ≥ 10 kDa),
 - C. determining abundance of the small peptides (having a molecular weight < 5 kDa), the protein to which the small peptides belong, or position of the small peptides on the protein by quantitative proteomic techniques and software analysis (i.e., mass spectrometry quantification and analysis),
 - D. determining the protein with change in energy state by analyzing abundance differences of the small peptides (having a molecular weight < 5 kDa) between the experimental and control samples, and
 - E. determining the protein region with change in energy state by analyzing the position of the small peptides (having a molecular weight < 5 kDa) with abundance change in the protein to which the small peptides belong.
2. The method of claim 1, wherein different energy states of the protein include but are not limited to changes in energy state caused by one or more of the following:
 - A. change in protein energy state resulting from interaction of a ligand (including one or more of a drug molecule, a metabolite in humans or animal, a plant extract, a nucleic acid, a metal ion, a peptide, an antibody, and a protein) with protein,
 - B. change in protein energy state resulting from alteration of post-translational modification of the protein, or
 - C. change in protein energy state resulting from one or more of thermal stimulation, osmotic

pressure changes, denaturing agent stimulation, oxidative stress, or disease.

3. A method for detecting affinity between a ligand and a protein, comprising steps of:

A. mixing and incubating a protein solution with a ligand at a series of (or multiple) different final concentrations (one of the concentrations may serve as a blank control without the ligand as a control group, and the others as experimental groups), respectively;

B. after incubation, subjecting the samples to digestion at a protease-to-protein mass ratio of 1:1 to 1:50 under a non-denaturing condition for 0.5–60 minutes, ensuring that even a low-energy protein structure are disrupted by enzymatic cleavage (i.e., inducing destructive enzymatic cleavage of proteins) and directly generating a large number of small peptides (having a molecular weight < 5 kDa) containing two cleaved sites capable of reflecting local stability of the protein and suitable for bottom-up mass spectrometry analysis;

C. isolating the small peptides (having a molecular weight < 5-10 kDa) suitable for mass spectrometry from large protein fragments (having a molecular weight ≥ 10 kDa).

D. determining abundance of the small peptides (having a molecular weight < 5 kDa), the protein to which the small peptides belong, or position of the small peptides on the protein by quantitative proteomic techniques and software analysis (i.e., mass spectrometry quantification and analysis); and

E. calculating the local affinity between the protein and the ligand, represented as the half-maximal effective concentration (EC₅₀), according to abundance change of the small peptides in samples treated with different ligand concentrations, wherein the calculation is performed based on a four-parameter logarithmic equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{(\text{LogEC}_{50} - X) * \text{Hill Slope}})$, where Y (vertical axis) represents the fold change in peptide abundance between the experimental and control groups, X (horizontal axis) represents the different ligand concentrations, Bottom and Top represent the plateau values at the lower and upper ends of the four-parameter logarithmic equation curve, respectively (in the same unit as Y), Hill Slope represents the absolute value of the maximum slope of the curve at the midpoint, and EC₅₀ is a half-maximal effective concentration to be calculated; a smaller EC₅₀ indicates a stronger affinity between the ligand and a protein region to which the peptide belongs to; and "a series of different final concentrations" represents at least 3 or more final concentrations, preferably 5 or more final concentrations.

4. The method of claim 3, wherein the ligand may be one or more of a drug molecule, a

metabolite in animal or plant, a plant extract, a nucleic acid, a metal ion, a peptide, an antibody, and a protein, or any other substances capable of interacting with a protein.

5. The method of claim 1 or 3, wherein the protein solution comprises a single-protein solution containing one protein or a mixed protein solution containing two or more proteins; the mixed protein solution comprises a cell or a tissue extract derived from humans, animals, plants, or bacteria; and gentle extraction methods, including but not limited to liquid nitrogen freeze-thaw extraction, liquid nitrogen grinding extraction, or homogenization extraction, are used for protein extraction; if an extraction buffer is used during the extraction process, surfactants or denaturants at a concentration that disrupt protein conformation are avoided to ensure that the protein retain its conformation to be tested; and the protein to be tested may be presented in the form of a free protein and/or a immobilized protein.

6. The method of claim 1, wherein the method comprises subjecting protein solutions in different energy states under a conformation to be tested to digestion with an protease added according to the mass ratio of protease to protein under the same digestion condition, such that protein structure in high-energy state is exposed to the protease, disrupted and unfolded by digestion, thereby inducing exposure of a low-energy state cleavage site of the protein, and in the presence of a relatively large amount of protease, the low-energy state structure is disrupted by digestion, directly generating small peptides (having a molecular weight < 5 kDa) suitable for identification by bottom-up mass spectrometry (identifying a protein with the peptides), and resulting in varying digestion degrees of proteins in different energy states; and

an optional protease comprises one or more selected from trypsin, glutamyl endopeptidase, thermolysin, proteinase K, or other site-specific or non-site-specific proteases, a the mass ratio of protease to protein is between 1:1 and 1:50, preferably between 1:1 and 1:10 in the presence of 1%–2% by volume fraction of a protease inhibitor, a digestion duration is in a range of 0.5–60 minutes, and a digestion temperature is determined based on the optimal temperatures required to maintain activity of the protease.

7. The method of claim 3, wherein the method comprises subjecting a ligand-treated group treated with various ligand concentrations, and the control group treated with a blank solvent to digestion with an protease added according to the mass ratio of protease to protein, such that protein structure in high-energy state was exposed to the protease, disrupted and unfolded by digestion, thereby inducing exposure of a low-energy state cleavage site of the protein, and in the presence of a relatively large amount of protease, the low-energy state structure is disrupted by digestion, directly generating small peptides (having a molecular weight < 5 kDa) suitable

for identification by bottom-up mass spectrometry (identifying a protein with the peptides), and resulting in varying digestion degrees of ligand-bound protein from ligand-unbound protein; and

an optional protease comprises one or more selected from trypsin, glutamyl endopeptidase, thermolysin, proteinase K, or other site-specific or non-site-specific proteases, a the mass ratio of protease to protein is between 1:1 and 1:50, preferably between 1:1 and 1:10 in the presence of 1%–2% by volume fraction of a protease inhibitor, a digestion duration is in a range of 0.5–60 minutes, and a digestion temperature is determined based on the optimal temperatures required to maintain activity of the protease.

8. The method of claim 1 or 3, wherein separating small peptides from larger protein fragments comprises but is not limited to utilizing the differences in one or more of molecular weight, hydrophobicity, or thermal stability.

9. The method of claim 1 or 3, wherein the quantitative proteomics technique refers to the quantitative analysis of the cleaved peptides in a bottom-up mass spectrometry analysis mode (identifying proteins with the peptides); the mass spectrometry data acquisition comprises data-dependent acquisition (DDA) and data-independent acquisition (DIA); the quantification method comprises label-free quantification and/or labeling-based quantification, (for example, dimethyl labeling quantification, iTRAQ labeling quantification, TMT labeling quantification, TMTpro labeling quantification, or a combination thereof), and

software analysis refers to importing the raw spectral files generated by mass spectrometry into database search software to obtain quantitative information of small peptides, information about the protein to which the small peptides belong to, and information about position of the small peptides on the protein; and the software comprises Maxquant (Cox, Germany), MSFragger (Alexey, USA), Spectronaut (Biognosys, Switzerland), or a combination thereof.

10. The method of claim 1 or 3, wherein the method for determination of proteins with change in energy state is based on one of or any combination of the following factors: significance of differential peptide differences represented by $-\log_{10}P$ values of the peptides, magnitude of the fold change of peptides represented by $|\log_2 \text{ fold change}|$ of the peptides, the number of differential peptides (N), whether the fold change of peptides shows dose-dependent changes with the ligand concentrations, or a combination thereof;

determining the significance of difference in differential peptides comprises performing significance tests, such as Empirical Bayes t-test or Student's t-test on the quantitative values

of peptides in the experimental and control groups, where the obtained P values is used as the index for significance of peptide abundance difference between control and experimental groups; and a protein containing peptides with $-\log_{10}P$ value $> 2\sim 5$ is considered as indicative of the protein with changes in altered energy state;

determining the fold change of differential peptides comprises determining directly from the database search software or through data validation methods such as Empirical Bayes t-test or Student's t-test, a protein containing peptides with $|\log_2 \text{ fold change}| > 0.3\sim 3$ is considered as a protein with changes in energy state;

processing of the number of differential peptides comprises ranking all peptides within each protein based on their significance of difference or fold change; the peptide with the highest significance of difference or fold change is defined as the top-ranked changed peptide for that protein; the second-ranked peptide represents peptide with the second highest significance of difference or fold change, followed by the third and fourth-ranked peptides; the $-\log_{10}P$ values and $\log_2$ fold change of the top-ranked, second-ranked, or third and fourth-ranked peptide is defined as the quantitative value of the protein; a protein with $-\log_{10}P$ value $> 2\sim 5$ or $|\log_2 \text{ fold change}| > 0.3\sim 3$ are defined as a protein with changes in energy state; and the protein with changes in energy state identified in there mode contain one, two, three, or four peptides that meet the criteria of $-\log_{10}P$ value $> 2\sim 5$ or $|\log_2 \text{ fold change}| > 0.3\sim 3$;

determining whether the fold change of peptide abundance exhibits a dose-dependent response to ligand concentration comprises fitting the fold change of peptides at different ligand concentrations and the ligand concentrations as variates to a four-parameter logistic equation, where the quality of fitting between the raw data to this equation is evaluated using the Pearson correlation coefficient (R^2), and if $R^2 > 0.75\sim 0.95$, the peptide is considered as exhibiting a dose-dependent response to ligand concentration, and the protein to which the peptide belongs is defined as a target protein for the ligand, and

any combination of the factors comprises meeting any two or more of the aforementioned factors, or combining $-\log_{10}P$ value, $|\log_2 \text{ fold change}|$, N (number of differential peptides), and R^2 in any proportion to generate a comprehensive score to determine the protein with changes in energy state.