(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 February 2007 (08.02.2007)

PCT

(10) International Publication Number
WO 2007/015697 A2

(51) International Patent Classification:
*G01N 33/53* (2006.01)

(21) International Application Number:
PCT/US2005/029107

(22) International Filing Date: 16 August 2005 (16.08.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/601,791 16 August 2004 (16.08.2004) US

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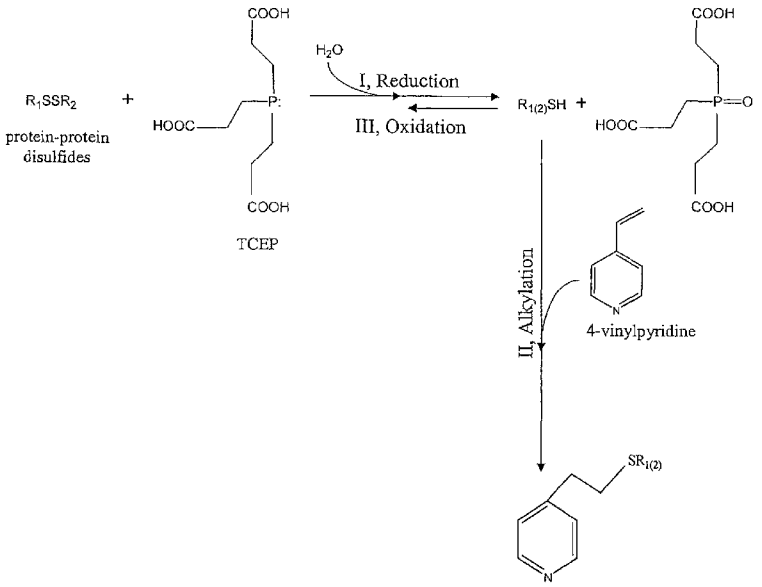
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT,

*[Continued on next page]*

(54) Title: METHOD FOR ENHANCING TWO-DIMENSIONAL GEL ELECTROPHORESIS RESOLUTION




(57) Abstract: One aspect of the present disclosure is directed to compositions and methods to decrease streaking on two-dimensional gels and enhance the resolution of proteins in the gel. In accordance with one embodiment the polypeptides are contacted with a trialkylphosphine to reduce the polypeptides, followed by alkylation with a compound that provides greater than 95% alkylation of the polypeptides. The resulting alkylated polypeptides are then subjected to two-dimensional gel analysis.

WO 2007/015697 A2

RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished upon receipt of that report*

**(48) Date of publication of this corrected version:**
19 April 2007

**(15) Information about Correction:**
see PCT Gazette No. 16/2007 of 19 April 2007

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

# METHOD FOR ENHANCING TWO-DIMENSIONAL GEL ELECTROPHORESIS RESOLUTION

## RELATED APPLICATIONS

This application claims priority under 35 USC §119(e) to US Provisional Application Serial No. 60/601,791, filed August 16, 2004, the disclosure of which is incorporated herein by reference.

## US GOVERNMENT RIGHTS

This invention was made with United States Government support under F4962-03-1-0089, awarded by the US Air Force Office of Scientific Research (AFOSR). The United States Government has certain rights in the invention.

## BACKGROUND

Two-dimensional electrophoresis (2-DE) is a powerful technique which resolves complex protein mixtures in the first dimension by pI and in the second dimension by molecular weight. The introduction of immobilized pH gradient (IPG) strips has significantly improved 2-DE separation in a reproducible manner. However, the number of the proteins that can be resolved and visualized by 2-DE using currently available techniques is limited. Large format gels (20 cm x 25 cm) can only clearly resolve approximately 1500-2000 protein spots of a sample prepared from a mammalian cell, whereas a mammalian cell contains more than 20,000 protein species and a single mammalian tissue may represent a mixture of more than 50,000 protein species. The limitations in protein detection and quantification are due in part to the presence of poorly resolved protein smears or streaks across the basic pH (>7) range in the gel.

The major cause for smears and streaks of protein samples is protein aggregation during electrophoresis through the formation of intra- and inter-molecular disulfide bridges ($R_1$-S-S-$R_2$) following the oxidation of the cysteinyl thiol groups (-SH). This is primarily due to the depletion of the reducing agent, such as dithiothriotol (DTT) or its isomer dithioerythritol (DTE) in the basic pH range during the first dimension isoelectrofocusing (IEF). DTT and DTE contained in the lysis and rehydration buffers are weak acids (pK = 8-9), that migrate toward the anode during

IEF, leading to the loss of the reducing agent from the basic portion of the IPG strip. In an environment lacking a reducing agent, the cysteinyl thiol groups of protein samples tend to crosslink, leading to formations of the disulfide bridges. This in turn results in the streaking patterns observed for proteins migrating in the basic pH (>7) range of the gel.

Optimal resolution from IEF electrophoresis in the alkaline region remains a challenge, although various attempts have been made to reduce the streaks. Strategies that have been used for streak reduction include decreasing the protein sample concentration, anodic cup-loading, shortening IEF duration, addition of a DTT wick to replenish DTT at cathode, or using an alternative reducing agent such as hydroxyethyldisulphide (HED) to form mixed disulfides with cysteinyl thiols. Some of these techniques require special apparatus and most of the time these techniques need to be combined to achieve a better result. Most importantly, those techniques for reducing streaks are not 100% effective, alone or in combination.

An alternate approach is to prepare samples in a way to prevent generation of the cross-links between cysteinyl thiols by a permanent modification of the free thiol groups through alkylation. This modification is commonly accomplished through a reduction and alkylation process using DTT to reduce the disulfide bonds to free thiol groups (Fig. 1, reaction I) and iodoacetamide or non-charged acrylamide derivatives (such as N,N-dimehtylacrylamide, DMA) to alkylate free thiols (Fig. 1, reaction II). Unfortunately, both iodoacetamide and acrylamide have an undesired effect to change the gel patterns or generate extra artifactual spots in 2-DE maps (Olsson et al., (2002) Proteomics 2:1630-2). In addition, either iodoacetamide or acrylamide cannot completely eliminate streaks. The problem concerns the incomplete alkylation of the cysteinyl thiols. Both iodoacetamide and non-charged acrylamide derivatives will react with DTT (Fig. 1, reaction III) and thus can convert no more than 80% of all the thiol groups in the protein when used in conjunction with DTT. The formation of intra- and inter-molecular disulfides between these residual thiol groups is still significant to the development of protein aggregation during IEF. The primary reason for an incomplete alkylation is the excess amount of DTT (~20-fold molar excess) that is needed to achieve a complete reduction. However, when an alkylating agent is added to the reaction, the remaining DTT in the sample will compete with the

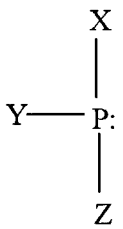
cysteinyl thiols to react with the alkylating agent (Fig. 1, reaction III), driving the reaction toward the regeneration of disulfide cross links (Fig. 1, reaction IV).

One embodiment of the present disclosure is directed to a new method for preparing polypeptides for two-dimensional gel analysis that greatly enhances the resolution of proteins migrating across the basic portion of the gel.

SUMMARY

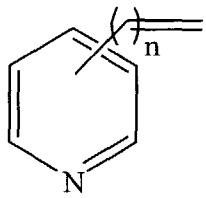
One aspect of the present invention is directed to an improved method for separating polypeptides using two-dimensional electrophoresis of protein samples. In one embodiment, the separation of polypeptides, and more particularly those polypeptides that migrate into the basic portion of the isoelectric focusing strip, is improved by eliminating streaks created during electrophoresis. In accordance with one embodiment, the number and/or size of streaks appearing in the second dimension is substantially reduced due in part to a step of processing the polypeptides prior to separating the polypeptides using 2DE, wherein the polypeptides are first substantially completely reduced and then substantially completely alkylated.

In one embodiment, the polypeptides are first reduced with a reducing agent that does not react with the alkylating agent used in the subsequent akylating step. The reduced polypeptide is then contacted with an alkylating agent, wherein the alkylating agent is a compound that is not inhibited or destroyed by neutral or zwitterionic surfactants and urea/thiourea. Examples of neutral or zwitterionic surfactants include but are not limited to CHAPS, NP-40, and HEPES. In one embodiment the reducing agent is a trialkylphosphine, and more particularly, a compound of the general structure:



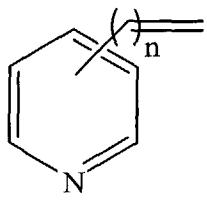

wherein X, Y and Z are independently selected from the group consisting of $C_1$-$C_4$ alkyl and $C_1$-$C_4$ carboxyalkyl, or salts thereof, and the alkylating

agent is selected from the group consisting of $CH_2$=CHCONR$_1$R$_2$ and a compound of the general structure:




wherein R is selected from the group consisting of H and $CH_3$ and n is an integer selected from 1-3. In one embodiment the reducing agent is tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) or tributylphosphine (TBP), and the alkylating agent is vinylpyridine.

In another embodiment, a kit is provided, comprising a first solution comprising a trialkylphosphine, a second solution comprising a compound selected from the group consisting of $CH_2$=CHCONR$_1$R$_2$ and




, and an IPG strip. In another embodiment, the kit comprises a composition comprising TCEP, a composition comprising vinylpyridine and an IPG strip. In another embodiment the kit further comprises a pre-cast gel.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the reduction and alkylation of a polypeptide using DTT and DMA or iodoacetymide.

Fig. 2 depicts the reduction and alkylation of a polypeptide using TCEP and vinylpyridine.

Fig. 3A-3F represent 2-DE patterns for various tissue protein lysates. Fig3A represents a 2-DE of total mouse brain lysate, Fig3B represents a 2-DE of total mouse brain lysate wherein the isolated polypeptide were alkylated with vinylpyridine, Fig3C represents a 2-DE of human liver cytosolic proteins, Fig3D represents a 2-DE of human liver cytosolic proteins, wherein the isolated polypeptide were alkylated with vinylpyridine, Fig3E represents a 2-DE of mouse brain protein lysate enriched in

basic proteins that had been obtained by ZOOM®IEF fractionation, and Fig3F represents a 2-DE of mouse brain protein lysate enriched in basic proteins, wherein the isolated polypeptide were alkylated with vinylpyridine.

**Detailed Description**

**Definitions**

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

As used herein, the term "halogen" or "halo" includes bromo, chloro, fluoro, and iodo.

The term "haloalkyl" as used herein refers to an alkyl radical bearing at least one halogen substituent, for example, chloromethyl, fluoroethyl or trifluoromethyl and the like.

The term "$C_1$-$C_n$ alkyl" wherein n is an integer, as used herein, represents a branched or linear alkyl group having from one to the specified number of carbon atoms. Typically $C_1$-$C_6$ alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, hexyl and the like.

The term "$C_1$-$C_n$ carboxyalkyl" as used herein refers to an alkyl group with a carboxylic acid group bound to the alkyl chain.

The term "$C_2$-$C_n$ alkenyl" wherein n is an integer, as used herein, represents an olefinically unsaturated branched or linear group having from 2 to the specified number of carbon atoms and at least one double bond. Examples of such groups include, but are not limited to, 1-propenyl, 2-propenyl, 1,3-butadienyl, 1-butenyl, hexenyl, pentenyl, and the like.

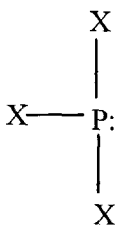
The term "$C_2$-$C_n$ alkynyl" wherein n is an integer refers to an unsaturated branched or linear group having from 2 to the specified number of carbon atoms and at least one triple bond. Examples of such groups include, but are not limited to, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, and the like.

The term "polypeptide" as used herein encompasses a sequence of 2 or more amino acids joined to each other by peptide bonds. Peptides may contain amino acids

other than the 20 gene-encoded amino acids, and includes amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts, as well as in the research literature. Modifications can occur anywhere in a peptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given peptide. Also, a given peptide may contain many types of modifications. See, for instance, Proteins-Structure and Molecular Properties, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in Posttranslational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al., "Analysis for protein modifications and nonprotein cofactors", Methods in Enzymol. 182:626-646 (1990) and Rattan et al., "Protein Synthesis: Posttranslational Modifications and Aging", Ann NY Acad Sci 663:48-62 (1992).

The term "amino acid" as used herein encompasses any molecule that comprises both amino and carboxylic acid functional groups and can be incorporated into a polypeptide structures (i.e. alpha amino acids).

The term trialkylphosphines as used herein encompasses any compound of the general structure:



wherein X is selected from the group consisting of $C_1$-$C_6$ alkyl, -($C_1$-$C_4$ alkyl)OH, -($C_1$-$C_6$ alkyl)COOH, and -($C_1$-$C_6$ alkyl)$NH_2$. In many cases, the trialkylphosphines of the present disclosure are capable of forming acid and/or base salts by virtue of the presence of amino and/or carboxyl groups in the compound.

As used herein the term "vinylpiridine" encompasses 2-vinylpyridine, 3-vinylpiridine and 4-vinylpiridine.

As used herein, the term "purified" and like terms relate to an enrichment of a molecule or compound relative to other components normally associated with the molecule or compound in a native environment. The term "purified" does not necessarily indicate that complete purity of the particular molecule has been achieved during the process.

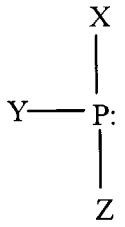
**Embodiments**

An improved method of separating complex mixtures of polypeptides is provided in the present disclosure. The method comprises modifying the polypeptides by contacting the polypeptides with a reducing agent followed by contacting the reduced polypeptides with an alkylating agent, wherein the reduction and alkylating reactions result in greater than 90%, or greater than 95%, or greater than 99%, or greater than 99.9% or all of the polypeptides that contain a cysteine residue being alkylated at the thiol group of cysteine. To obtain such high levels of alkylation of the cysteinyl thiol groups of the target polypeptides the reducing and alkylating agents are selected in part based on the reducing agent's lack of reactivity with the alkylating agent and the alkylating agents resistance to inhibition by neutral or zwitterionic surfactants and thiourea. The modified polypeptides are then subjected to two-dimensional gel electrophoresis to separate the polypeptides from one another.

The method described herein in one embodiment is used to separate polypeptides present in complex mixtures, such as polypeptides that have been purified from one or more cells. The polypeptides may be isolated from an *in situ* source of cells (e.g. a biopsy) or may be isolated from cells or cell lines cultured *in vitro*. In one embodiment the complex mixture of polypeptides comprises the total polypeptide component isolated from a particular tissue or individual cell type. Alternatively, in one embodiment the complex mixture of polypeptides consists essentially of only a fraction of the total polypeptides isolated from cells or tissues. In accordance with one embodiment the total polypeptide composition recovered from cells or tissues is fractionated based on one or more physical properties of the polypeptides (e.g. based on size, charge, isoelectric point (pI), hydrophobicity or hydrophilicity, or other physical property) using standard techniques known to those skilled in the art and a particular fraction is subjected to two-dimensional

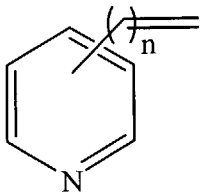
electrophoresis. In one embodiment the polypeptides are fractionated based on the basicity of the polypeptides, and a basic fraction is selected for alkylation using the method disclosed herein. The alkylated fraction of polypeptides can then be subjected to two-dimensional gel electrophoresis

In accordance with one embodiment an improved method for separating polypeptides on a two dimensional gel is provided. In one embodiment the method comprises the steps of contacting a composition comprising polypeptides with a trialkylphosphine reducing agent. In one embodiment the trialkylphosphine reducing agent has the general structure:



I

wherein X, Y and Z are independently selected from the group consisting of $C_1$-$C_4$ alkyl and $C_1$-$C_4$ carboxyalkyl, or salts thereof, to produce polypeptides that have reduced cysteinyl thiol groups. These reduced polypeptides are then contacted with an alkylating agent selected from the group consisting of $CH_2$=$CHCONR_1R_2$ and



III

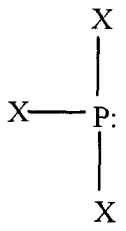
or mixtures thereof, wherein $R_1$ and $R_2$ are independently selected from the group consisting of H and $CH_3$ and n is an integer selected from 1-6 to produce alkylated polypeptides. The alkylated polypeptides are then separated in a first dimension using isoelectric focusing and then the isoelectric focused alkylated polypeptides are separated in a second dimension using gel electrophoresis.

The use of trialkylphosphines as the reducing agent is desired in one embodiment because trialkylphosphines, unlike DTT, do not react with vinylpyridine, and thus help to drive the alkylation reaction to essentially 100% completion (Fig. 2, II). Furthermore, selection of the pyridine compounds as alkylating agents is

desirable in one embodiment because their activity as alkylating agents is not inhibited or destroyed by neutral or zwitterionic surfactants and urea/thiourea, common components of the solublization cocktails for two-dimensional gel electrophoresis. Therefore, selection of vinylpyridine as the alkylating agent is particularly advantageous due to its compatibility with standard reagents used with two-dimensional gel electrophoresis. Unlike charged alkylating agents, such as iodoacetamide, alkylation using vinylpyridine does not change the pI of a protein, therefore the protein's position on a 2-DE map is not altered.

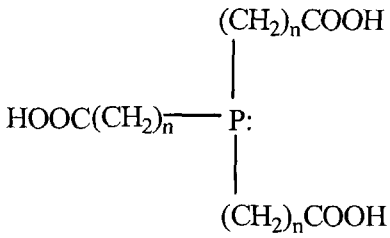
Trialkylphosphines such as tributylphosphine (TBP) and tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) are powerful reducing agents, which can readily and stoichiometrically reduce disulfides with high specificity. Unlike DTT, trialkylphosphines do not react with some alkylating reagents (Riiegg and Rudinger, (1977) Methods Enzymol. 47:111-6). It has also been shown that TBP greatly improves the protein solubility when used prior to two dimensional eletrophoresis (2-DE). Due to such issues as solubility, odor, and toxicity, reduction with TCEP is more favorable than TBP. Although TCEP is known to give essentially 100% reduction for proteins' thio groups prior to electrophoresis, protein samples still undergo oxidation during electrophoresis. Thus, alkylation is necessary to prevent oxidation of proteins' thio groups and formation of disulfide linkage during electrophoresis.

In accordance with one embodiment the reducing agent comprises a compound of the general structure:



II

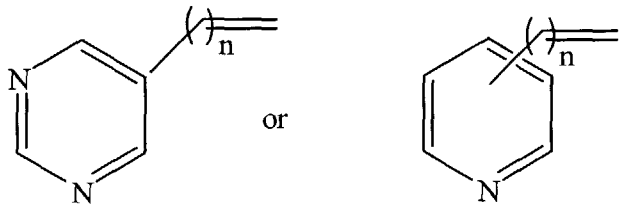
wherein X is selected from the group consisting of $C_1$-$C_4$ alkyl and $C_1$-$C_4$ carboxyalkyl, or salts thereof. In one embodiment, the reducing agent has the general structure:



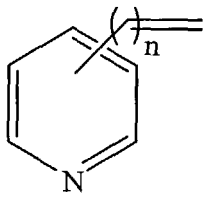
IV

wherein n is an integer selected from the range of 1-6 or a salt of such compound. In another embodiment the reducing agent has the general structure of compound **IV** wherein n is an integer selected from the range of 1-3 or a salt of such compound. In one embodiment, the reducing agent has the general structure of compound **IV** wherein n is 2 or 3 or a salt of such compound. In one embodiment, the reducing agent is tris(2-carboxyethyl)-phosphine hydrochloride.

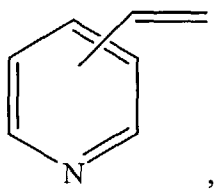
In accordance with one embodiment the alkylating agent comprises a compound of the general structure:



wherein n is an integer selected from 1-6. In one embodiment the alkylating agent comprises a compound of the general structure:



wherein n is an integer selected from 1-6, or in one embodiment n is an integer selected from 1-3. In one embodiment the alkylating agent is a compound of the general structure



, and in another embodiment the alkylating agent is 2-vinylpyridine or 4-vinylpyridine.

Vinylpyridine has been used as a protein alkylating agent for peptide mapping

and sequence analysis of proteins using mass spectrometry. It has been shown to react with the cysteinyl thiols with essentially 100% specificity and the alkylation is essentially 100% complete (Sebastiano et al., (2003) 2003;17(21):2380-6). Sebastiano and co-workers compared several alkylating agents, including acrylamide, DMA, iodoacetic acid, 2-vinylpyridine, and 4-vinylpuridine, and found that vinylpyridine was the only compound that achieved 100% alkylation. Applicants are the first to describe an improved method for separating polypeptides comprising the steps of reducing and alkylating the reduced polypeptides to prepare alkylated protein samples for 2-DE, and thus achieve the goal of reducing basic pH end streaks and improving the resolution of 2-DE.

In accordance with one embodiment a protein sample can be reduced in accordance with the present methods using TCEP at a concentration from about 1 μM to about 1 M, and in one embodiment, with a protein/TCEP weight ratio from about 0.1 to about 50. In a further embodiment, the reaction is conducted with a TCEP concentration from about 1 to about 10 mM, and in a further embodiment the reaction is conducted with a TCEP concentration of about 5 mM.

In accordance with one embodiment, protein sample alkylation can be achieved using a vinylpyridine, and more particularly 2-vinylpyridine or 4-vinylpyridine, at a concentration from about 1 μM to about 1 M. In one embodiment, the protein/vinylpyridine weight ratio is selected from about 0.1 to about 60. In one embodiment the vinylpyridine concentration is selected from about 10 to about 100 mM, and in a further embodiment the vinylpyridine concentration is about 20 mM.

The reducing and alkylation reactions are allowed to continue for a predetermined length of time to run the reaction to completion. The length of time necessary to achieve substantially complete alkylation of the polypeptides will vary depending on the conditions use and the relative concentrations of the reagents, but can be readily determined by skill practitioners using standard techniques. In one embodiment once the reactions have been conducted for the desired length of time, the polypeptides are immediately load onto the 2-DE, or alternatively frozen for later use. In another embodiment, after the polypeptides have been reacted for the desired length of time, the reaction is quenched by the addition of a second reducing agent that reacts with the alkylating agent. Suitable quenching reagents include DTT, DTE,

mercaptoethanol, glutathione, mercaptoethylamine and thioglycillic acid. In accordance with one embodiment, quenching of the reactions is achieved with DTT, at a concentration from about 1 μM to about 1 M. In one embodiment DTT is added to provide a protein/DTT weight ratio from about 0.1 to about 40. In one embodiment the DTT concentration is selected from about 10 to about 100 mM, and in a further embodiment the DTT concentration is about 20 mM.

In accordance with one embodiment, protein sample reduction and alkylation is achieved with a salt concentration lower than about 1 M, and in one embodiment, lower than about 40 mM, and in a further embodiment, with a salt concentration lower than about 10 mM. In addition, the protein sample reduction and alkylation is typically conducted at a pH selected from the range of about 5 to about 11, and in one embodiment the pH is selected from the range of about 6 to about 9, and in one embodiment the reactions are conducted at a pH of about 7.4. The protein sample reduction and alkylation reaction can be achieved at a wide range of temperatures, including any temperature ranging from about 0° to about 100°C. However, in the presence of urea, it is appreciated that the temperature should not be higher than about 40°C, and more typically is not higher than about 30°C to prevent protein carbamylation.

In accordance with one embodiment, the present method allows the preparation of a composition comprising polypeptides with reduced cysteinyl thiol groups, wherein greater than 95% of the polypeptides are reduced, and in one embodiment greater than 99% of the polypeptides are reduced and in one embodiment at least 99.9% of the polypeptides are reduced. In one embodiment this high level of reduction is achieved through the use of TCEP as the reducing agent. The reduced thiol groups of the polypeptides are then alkylated according to one embodiment through the use of vinylpyridine as the alkylating agent to produce a composition comprising polypeptides wherein greater than 95% of the polypeptides are alkylated, and in one embodiment greater than 99% of the polypeptides are alkylated, and in one embodiment at least 99.9% of the polypeptides are alkylated. Because TCEP, unlike DTT, does not react with vinylpyridine, TCEP helps to drive the alkylation reaction to substantially 100% completion (Fig. 2, II). Vinylpyridine is not inhibited or destroyed by neutral or zwitterionic surfactants and urea/thiourea, common

components of the solublization cocktails for 2-DE, therefore it is 2-DE compatible. Unlike charged alkylating agents, such as iodoacetamide, alkylation using vinylpyridine does not change the pI of a protein, therefore the protein's position on a 2-DE map would not be altered.

As discussed above, a method is provided herein for reducing and alkylating polypeptides present in a sample preparation that greatly improves the resolution of the polypeptides in 2-DE. This is particularly advantageous for polypeptides that migrate in the basic pH range of the first dimension of the 2-DE. This enhanced resolution will allow for the detection and isolation and characterization of individual proteins on 2-DE gels that were previously not distinct. The identification of such distinct protein spots will enable the analysis and identification of those proteins undergoing quantitative (up/down regulation, presence, absence, etc.) or qualitative (post-translational modification) changes. The improvement in resolution will also allow the loading of more concentrated samples, therefore enhancing the intensity of the faint protein spots and increasing the number of proteins that can be identified by peptide mass fingerprint.

In addition, since the alkylation has already been accomplished with substantially 100% effectiveness in the sample preparation step before 2-DE analysis, the reduction and alkylation steps before tryptic digestion and MS analysis is no longer required after electrophoresis. This sample preparation method is also applicable to samples subjected to microscale solution isoelectrofocusing (ZOOM®IEF) prior to narrow pH range 2-DE, in which streaking is a major problem for proteins in the basic pH fraction (Fig. 3, E and F).

This is the first application of TCEP as a reducing agent and vinylpyridine as alkylating agent to prepare protein mixtures for 2DE. Vinylpyridine is not inhibited or destroyed by neutral or zwitterionic surfactants and thiourea, common components of the solublization cocktails for 2 DE, therefore it is 2 DE compatible. This new approach features a complete alkylation of the thiol groups in the protein by vinylpyridine, which effectively reduces the streaks in the 2DE.

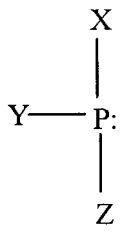
Previously reduction and alkylation modification attempts prior to 2-DE utilize DTT as reducing agent and iodoacetamide (IAA) or non-charged acrylamide derivatives (such as N,N-dimethylacrylamide, DMA) as alkylation agent.

Unfortunately, both IAA and acrylamide can change the gel patterns or generate extra artificial spots in 2-DE maps. In addition, the use of DTT and iodoacetamide (IAA) is not effective in eliminating the streaks due to the incomplete alkylation of the cysteinyl thiols by IAA. It has been reported that both iodoacetamide and non-charged acrylamide derivatives can convert no more than 80% of all the thiol groups in the protein when used in conjunction with DTT. The primary reason for an incomplete alkylation is the excess amount of DTT (~20-fold molar excess) that is needed to achieve a complete reduction. When an alkylating agent is added to the reaction, the remaining DTT in the sample will compete with the cysteinyl thiols to react with the alkylating agent (Fig.1, reaction III), driving the reaction toward the regeneration of disulfide cross links (Fig. 1, reaction IV).

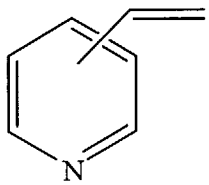
In addition to conventional 2DE, this approach can also be applied to samples subjected to microscale solution isoelectrofocusing (ZOOM®IEF) prior to narrow pH range 2DE, in which streaking is a major problem for the proteins in the basic pH fraction. Reducing sample complexity by pre-fractionation can greatly improve the efficiency of protein separation to accommodate the limitation of 2DE for the vast number of proteins and the broad dynamic range of their expression in certain proteome. In-solution IEF fractionates the proteins by their differences in isoelectric points to pI = 3-4.6, 4.6-5.4, 5.4-6.2, 6.2-7, and 7-10 fractions. The increment in the pI value is about 1 pH unit, except for the basic proteins (pI = 7-10), which are put into one combined pool. This is because the proteins in this fraction are all shown as streaks on a standard 2-DE gel.

The method disclosed herein dramatically increases the value of in-solution IEF in the proteomics studies, making it possible to further separate the pI = 7-10 fraction to pI = 7-8, 8-9, 9-10. The final impact is to be able to resolve thousands of proteins that otherwise could not be resolved and identified using previous standard 2-DE techniques. The present method is a simple approach, without need of a special instrumentation and can be easily developed to a reagent kit for the purpose of "de-streaking" in 2-DE analysis.

In accordance with one embodiment, a kit is provided for conducting two-dimensional gel analysis of compositions comprising a plurality of polypeptides. In one embodiment the kit comprises a reducing agent of the general structure:



wherein X, Y and Z are independently selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ carboxyalkyl, or salts thereof; an alkylating agent selected from the group consisting of $CH_2$=$CHCONR_1R_2$ and



or mixtures thereof, wherein $R_1$ and $R_2$ are independently selected from the group consisting of H and $CH_3$; and an ampholyte pH gradient for use in separating the polypeptides by isoelectric focusing. In one embodiment the ampholyte pH gradient is an immobilized pH gradient strip.

Traditional ampholyte pH gradients used in 2-DE analysis are formed during electrophoresis, but suffer the disadvantage of gradient drift and batch variability. These problems can be obviated by the use of Immobilines, a set of buffering acrylamide derivatives that contain either a free carboxylic acid or a tertiary amino group. Using the appropriate combination of Immobilines and a gradient maker, it is possible to generate any pH gradient in the range of pH 2.5 to 12. The Immobilines are co-polymerized with acrylamide and Bis-acrylamide so that the pH gradient exists before electrophoresis and is immobile (i.e. an Immobilized pH gradient (IPG)). Immobilized pH gradients (IPGs) ensure highly reproducible pH gradients that are insensitive to disturbances from sample components and, due to their stability, permit focusing times of sufficient duration for proteins to attain their isoelectric points. The gradients of IPGs can vary from broad range, covering 7 pH units, to ultra-narrow 0.1-pH unit gradients, allowing increased resolution of closely related proteins. The pH gradient is also stable thus allowing storage of samples separated on IPG strips at -80°C prior to running second dimension gels.

In accordance with one embodiment the kit of the present disclosure further comprises a second reducing agent that quenches the peptide alkylation reaction. In one embodiment the second reducing agent is selected from the group consisting of DTT, DTE, mercaptoethanol, glutathione, mercaptoethylamine and thioglycillic acid. In another embodiment the kit further comprises a pre-cast acrylamide gel. The reagents of the kit may also include buffers, detergents, denaturants and other reagents for use in formulating the polypeptide compositions and for conducting the 2-DE analysis. The reagents can be packaged in a variety of containers, *e.g.*, vials, tubes, bottles, and the like. Other reagents can be included in separate containers and provided with the kit; *e.g.*, positive control samples, negative control samples, buffers, etc. The kit will also include instructional materials for using the kit to prepare alkylated polypeptide compositions and for conducting the subsequent 2-DE analysis.

Protein spots are usually cut from the gel to further identify and/or sequence the protein by a mass spectrometry (MS). The procedure for the identification of gel resolved proteins includes reduction and alkylation (using DTT and iodoacetamide), in-gel tryptic digestion, peptide extraction, and MALDI or LCMS identification. Using our sample preparation method, reduction and alkylation have already been accomplished, with 100% effectiveness, in the sample preparation step before 2DE analysis. Therefore, the reduction and alkylation steps before tryptic digestion is no longer required after electrophoresis.

EXAMPLE 1

Alkylating Purified Liver and Brain Cell Polypeptides by Reacting the Polypeptides with TCEP and Vinyl Pyridine.

Fresh human liver was a generous gift from Dr. C. Max Schmidt, (Departments of Surgery and Biochemistry and Molecular Biology, Indiana University School of Medicine, Cancer Research Institute). The cytosolic fraction of liver tissue was isolated by differential centrifugation. Liver was homogenized in a buffer containing 0.25 M sucrose and 10 mM Tris-HCI, pH 7.4 and centrifuged at 100,000 x g for 45 min using a Beckman Type 45 Ti Rotor. Protein denaturization, solubilization, and reduction were performed in a portion of the supernate (cytosol) by the addition of urea, CHAPS, and DTT. Carrier ampholyte (pH 3-10) was also added.

The final concentrations for these reagents were 9M urea, 4% CHAPS, 65 mM DTT, and 0.5% pH 3-10 ampholyte.

Another portion of the supernate was subjected to the same denaturation, solubilization, and reduction process except TCEP was used instead of DTT. The final TCEP concentration was 10 mM. The sample that had been reduced by TCEP was alkylated with 1/20 volumes of VP (400 mM) for 1 h while vortexing. The reaction was quenched by the addition of the same volume of DTT (400 mM) which destroys excess vinylpyridine.

Frozen mouse brain (Harlan Sprague-Dawley, Indianapolis, IN) was minced and homogenized in 8 volumes of solubilization buffer containing 9M urea, 4% CHAPS, 65 mM DTT, and 0.5% pH3-10 ampholyte. Samples were centrifuged at 100,000 x g for 20 min using a Beckman TL-100 ultracentrifuge to remove nucleic acid and insoluble materials, and the supernate was collected. Similar to the liver cytosolic proteins, reduction and alkylation with TCEP and vinylpyridine were carried out for a portion of the brain protein lysate using the same solubilization buffer, but TCEP (10 mM) was used instead of DTT. See page 17 lines 21 to 26 details. In some experimental stages, a protein assay was performed using the RC DC Protein Assay kit (Bio-Rad, Richmond, CA) according to the manufacturer's protocol to determine protein concentration.

EXAMPLE 2

2-D Gel Analysis using TCEP reduced and Vinyl Pyridine Alkylated Polypeptides.

Some of the alkylated and unalkylated liver and brain samples (prepared as described in Example 1) were pre-fractionated by microscale solution isoelectrofocusing using the ZOOM®IEF fractionated (Invitrogen, Carlsbad, CA). The protein fractions (alkylated and unalkylated) that were enriched in basic proteins (pI = 7-10) were subjected to subsequent IEF on IPGs. In some experimental stages, a protein assay was performed using the RC DC Protein Assay kit (Bio-Rad, Richmond, CA) according to the manufacturer's protocol to determine protein concentration.

Illustratively, first dimension IEF was performed on IPG strips (pH 3-10, 24 cm, Bio-Rad, Richmond, CA). The protein lysate was diluted with rehydration buffer

(8 M urea, 2%CHAPS, 15 mM DTT, 0.2% ampholytes/pH 3-10). Rehydration of IPG strips was carried out overnight at room temperature. The proteins were focused at ≤50 μA/strip at 20°C, using progressively increasing voltage up to 10,000 V for a total of 100,000 Vh. Second dimension separation was accomplished on linear 11-19% acrylamide gradient slab gels (20 cm x 25 cm x 1.5 mm), poured and cast reproducibly using a computer-controlled gradient maker. Gels were run simultaneously for approximately 18 h at 160 V and 8°C. Slab gels were stained using a colloidal Coomassie Brilliant Blue G-250 procedure.

Alkylation with vinylpyridine was found to effectively eliminate the streaks in the basic pH range in 2-DE maps. This "de-streaking" method was efficient in various sample types, including total tissue lysate (Figure 3, A and B) and protein lysates that had been pre-fractionated, either as a subcellular fraction or a ZOOM®IEF fraction (Figure 3, C and D, E and F). The absence of streaks in the cytosolic protein fraction was shown in Figure 3, C and D.

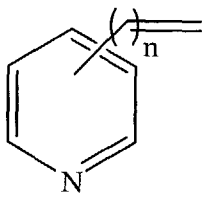
The remarkable efficiency of vinylpyridine alkylation in streak reduction was further demonstrated in more complex samples, which either contained membrane protein or was enriched in basic proteins. The streaks shown in the alkaline region of the 2-DE map for the mouse brain protein lysate (Figure 3, A) were fully eliminated in the corresponding sample that had undergone vinylpyridine alkylation (Figure 3; B). Furthermore, the basic protein enriched fraction (pI = 7-10) from ZOOM®IEF, which was shown as protein streaks in the unalkylated sample (Figure 3, E), was completely resolved in the 2-DE map of the same sample that had been alkylated with vinylpyridine (Figure 3F).

Claims:

1. An improved method for separating polypeptides on a two dimensional gel, said method comprising the steps of

contacting a composition comprising polypeptides with a composition comprising a trialkylphosphine, or corresponding salt thereof, to reduce the polypeptides;

contacting the reduced polypeptides with an alkylating agent selected from the group consisting of $CH_2$=CCONR$_1$R$_2$ and



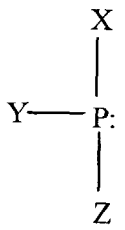

or mixtures thereof, wherein $R_1$ and $R_2$ are independently selected from the group consisting of H and $CH_3$ and n is an integer selected from 1-6;

separating the alkylated polypeptides in a first dimension using isoelectric focusing;

separating the isoelectric focused alkylated polypeptides in a second dimension using gel electrophoresis.

2. The method of claim 1 wherein the isoelectric focused alkylated polypeptides are separated by polyacryamide gel electorphoresis.

3. The method of claim 1 wherein the reducing agent has the general structure:



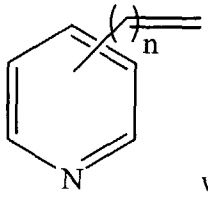

wherein X, Y and Z are independently selected from the group consisting of $C_1$-$C_6$ alkyl and $C_1$-$C_6$ carboxyalkyl, or salts thereof.

4. The method of claim 3 wherein each of X, Y and Z are either $C_1$-$C_4$ alkyl or $C_1$-$C_4$ carboxyalkyl.

5. The method of claim 1 wherein the trialkylphosphine is tris(2-carboxyethyl)-phosphine hydrochloride or tributylphosphine.

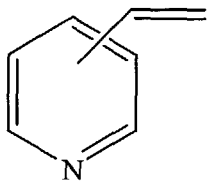
6. The method of claim 1 wherein the trialkylphosphine comprises a mixture of tris(2-carboxyethyl)-phosphine hydrochloride and tributylphosphine.

7. The method of claim 1 wherein the alkylating agent is a compound of the general structure




wherein n is an integer selected from 1-2.

8. The method of claim 3 wherein the alkylating agent is a compound of the general structure




9. The method of claim 8 wherein the alkylating agent is 2-vinylpyridine or 4-vinylpyridine.

10. The method of claim 3 wherein the alkylating agent is 3-vinylpyridine.

11. The method of claim 5 wherein the alkylating agent is 4-vinylpyrimidine.

12. The method of claim 1 wherein the isoelectric focusing is conducted over a pH range of 1 to 3 pH units in the basic pH range.

13. The method of claim 1 wherein the reducing agent is tris(2-carboxyethyl)-phosphine hydrochloride.

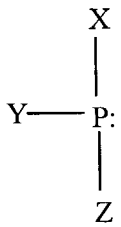
14. The method of claim 8 wherein the reducing agent is tris(2-carboxyethyl)-phosphine hydrochloride.

15. The method of claim 1 further comprising the step of quenching the alkylating reaction by contacting the polypeptides with a thio-reducing agent prior to the step of separating the polypeptides on the two-dimensional gel.

16. The method of claim 15, wherein the thio-reducing agent is dithiothriotol or dithioerythritol.

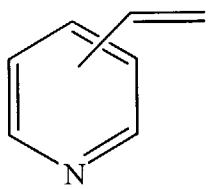
17. A kit for conducting two dimensional gel analyses of compositions comprising a plurality of polypeptides, said kit comprising

a reducing agent of the general structure:




wherein X, Y and Z are independently selected from the group consisting of $C_1$-$C_4$ alkyl and $C_1$-$C_4$ carboxyalkyl, or salts thereof;

an alkylating agent selected from the group consisting of $CH_2$=$CCONR_1R_2$ and



or mixtures thereof, wherein $R_1$ and $R_2$ are independently selected from the group consisting of H and $CH_3$ and n is an integer selected from 1-3; and

an immobilized pH gradient strip.

18. The kit of claim 17 further comprising a second reducing agent.

19. The kit of claim 18 wherein the second reducing agent is dithiothriotol or dithioerythritol

20. The kit of claim 17 further comprising a pre-cast acrylamide gel.

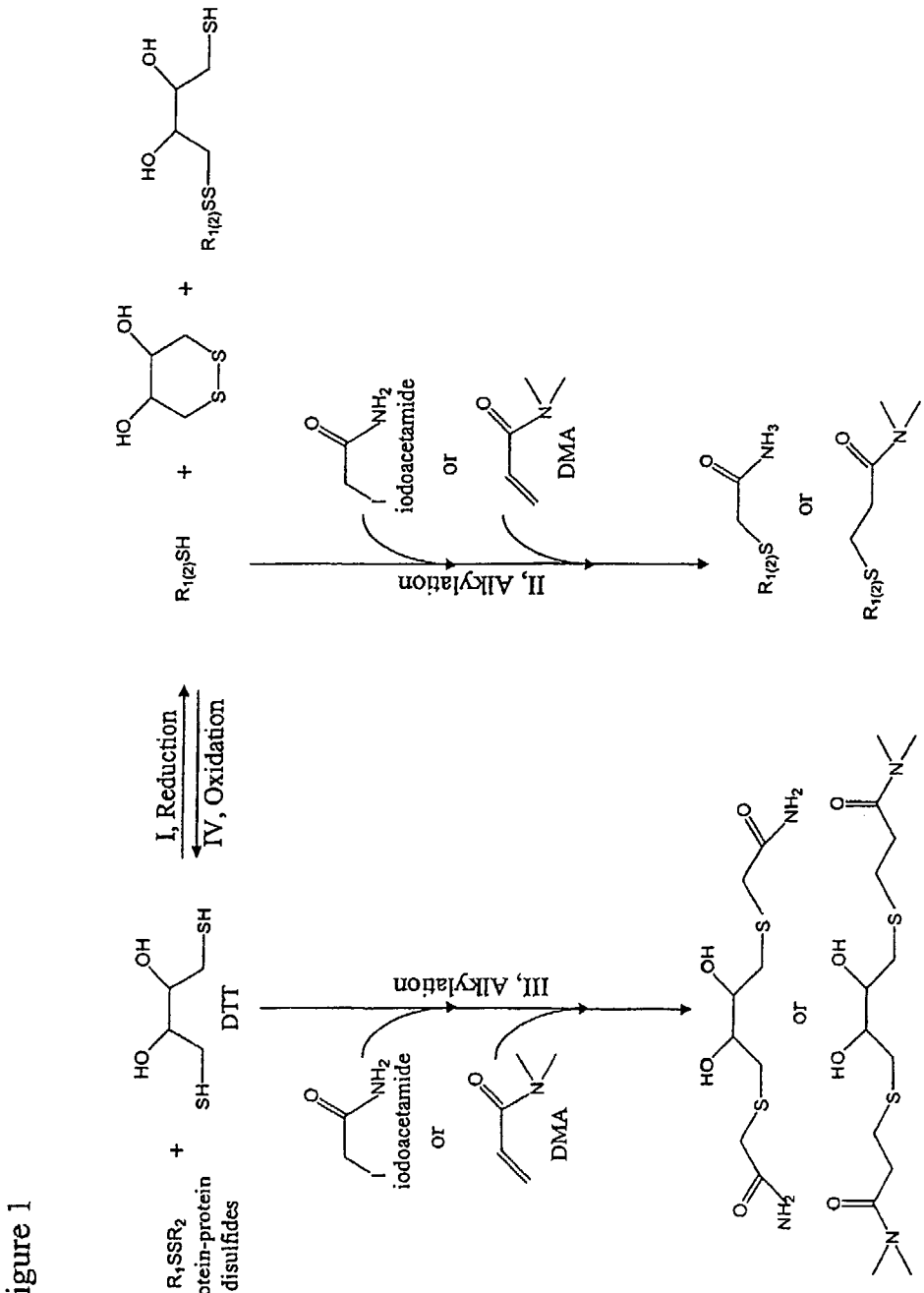

I, Reduction
IV, Oxidation
DTT
III, Alkylation
II, Alkylation
iodoacetamide
or
DMA
or
$R_1SSR_2$
protein-protein
disulfides
$R_{1(2)}SH$
$R_{1(2)}SS$

Figure 1

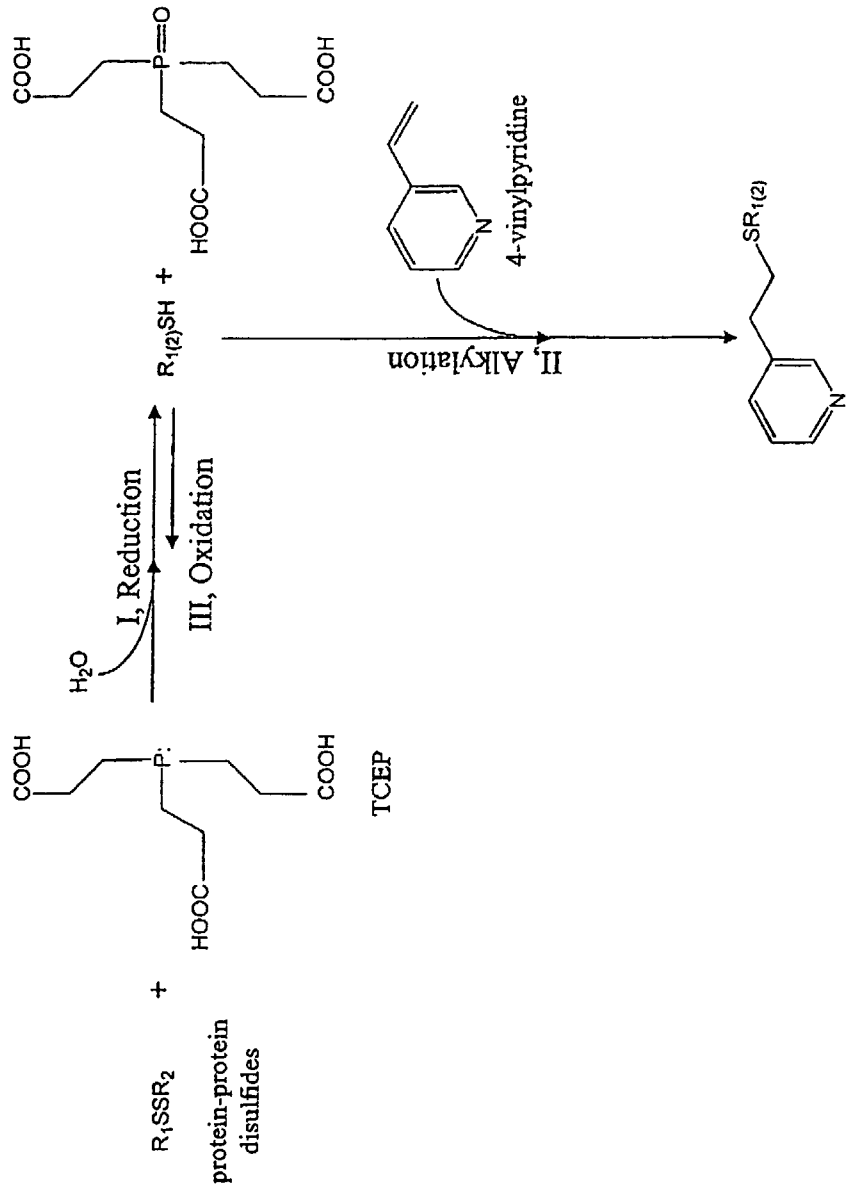

COOH
P=O
COOH
HOOC
4-vinylpyridine
N
SR1(2)
N
II, Alkylation
R1(2)SH +
I, Reduction
III, Oxidation
H2O
COOH
P:
COOH
HOOC
TCEP
R1SSR2
+
protein-protein
disulfides

Figure 2

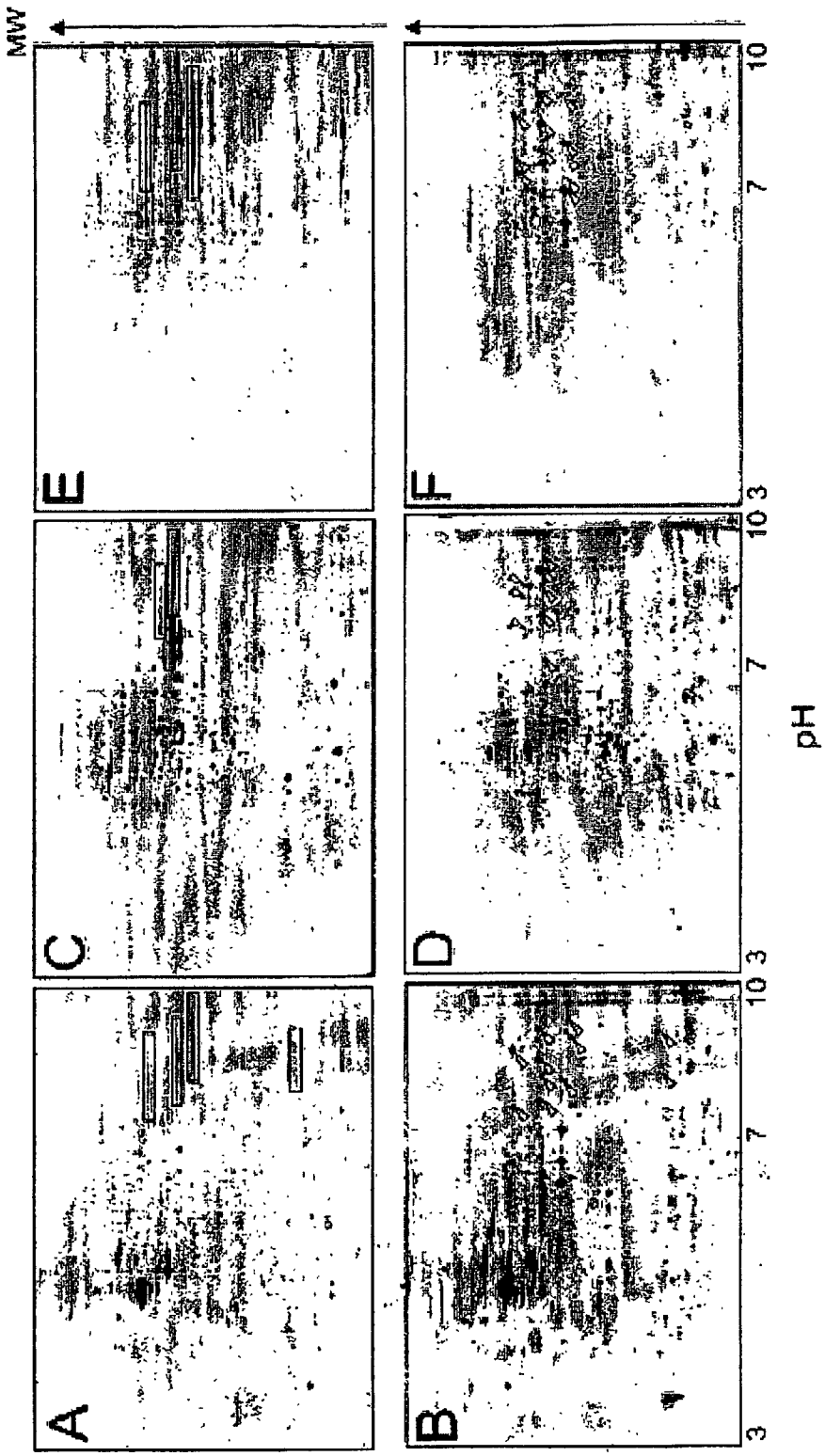



Figure 3