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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 May 2006 (26.05.2006)

PCT

(10) International Publication Number
WO 2006/054164 A2

(51) International Patent Classification:
C12N 9/12 (2006.01)

(21) International Application Number:
PCT/IB2005/003462

(22) International Filing Date:
19 November 2005 (19.11.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2330/DEL/2004 19 November 2004 (19.11.2004) IN

(71) Applicant *(for all designated States except US)*: **RANBAXY LABORATORIES LIMITED** [IN/IN]; Plot No. 90, Sector - 32, Gurgaon, Haryana 122 001 (IN).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **KUNDU, Prabuddha** [IN/IN] (IN). **GULATI, Pankaj** [IN/IN] (IN). **SONI, Rajeev** [IN/IN] (IN). **SAINI, Kulvinder, Singh** [IN/IN] (IN).

(74) Common Representative: **RANBAXY LABORATORIES LIMITED**; c/o DESHMUKH, Jay R., 600 College Road East, Suite 2100, Princeton, New Jersey 08540 (US).

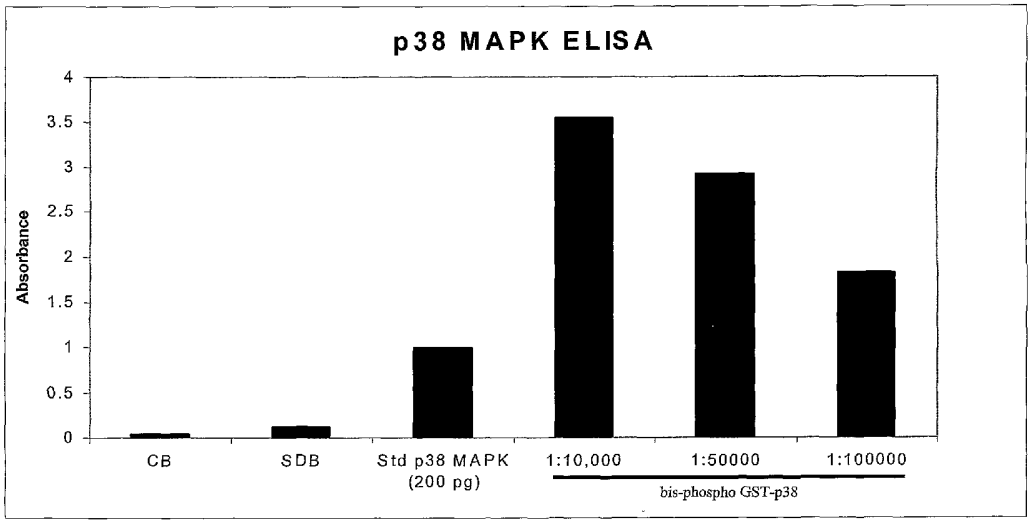
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, 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, 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*

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.

(54) **Title:** A SINGLE STEP PROCEDURE FOR THE PURIFICATION AND ACTIVATION OF TAGGED P38 MAP KINASE



(57) **Abstract:** The present invention provides a single step procedure for the purification and activation of tagged p38 MAP kinase by affinity chromatography. The tagged p38 MAP kinase is expressed in *E. coli* and subsequently activated by using differentially tagged active upstream activators.

WO 2006/054164 A2

A SINGLE STEP PROCEDURE FOR THE PURIFICATION AND ACTIVATION OF TAGGED P38 MAP KINASE

Technical Field of the Invention

The present invention provides a single step procedure for the purification and activation of tagged p38 MAP kinase by affinity chromatography. The tagged p38 MAP kinase is expressed in *E. coli* and subsequently activated by using differentially tagged active upstream activators.

Background of the Invention

MAP Kinases (MAPKs) can be activated directly by dual phosphorylation at threonine and serine residues in the activation loop by upstream dual specificity protein kinases, such as MAP kinase kinases (MAPKKs or MKKs). Many proteins that are activated by phosphorylation are artificially modified into active forms by mutating the desired amino acid to a phosphoamino acid-mimicking acidic amino acid(s). One of the obstacles in studying the biochemical properties of individual MAP kinase modules and their constituents is the difficulty in obtaining sufficient quantities in a purified form.

The other problematic area is that even after obtaining the pure form expressed as recombinant proteins in bacteria, they have to be activated *in vitro* using the individual components. The present invention now provides a simultaneous purification and activation process for appropriately tagged p38 MAP kinase.

Summary of the Invention

In one general aspect there is provided a process for the preparation of purified bisphosphorylated GST- p38 MAP Kinase. The process includes adsorbing of the impure GST- p38 MAP Kinase on a GST specific column; washing the column with a phosphate buffered saline solution; loading of the tagged upstream activator and activation buffer into the column; washing of the column with phosphate buffered saline, and eluting the bisphosphorylated GST- p38 MAP Kinase off the column with one or more elution buffers.

Embodiments of the present invention may include one or more of the following features. For example, the tagged upstream activator may include His-MKK6, MYC-MKK6, His-MKK3, MYC-MKK3, CBD-MKK6, CBD-MKK3, MBP-MKK6 or MBP-MKK3 changes.

The activation buffer may include one or more of 8-12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 8-12 mM $MgCl_2$, 0.1-0.4 mM Na_3VO_4, 0.02-0.05% Brij-35, 0.5-1.5 mM ATP and 0.5-2.0 mM DTT or 5-20 mM TRIS (tris (hydroxymethyl) aminomethane), 8-12 mM $MgCl_2$, 0.1-0.4 mM Na_3VO_4, 0.02-0.05% Brij-35, 0.5-1.5 mM ATP and 0.5-2.0 mM DTT. The activation buffer may be one or more of 10 mM HEPES, 10 mM $MgCl_2$, 0.2 mM Na_3VO_4, 0.03% Brij-35, 1 mM ATP and 1.5 mM DTT or 10 mM TRIS, 10 mM $MgCl_2$, 0.2 mM Na_3VO_4, 0.03% Brij-35, 1 mM ATP and 1.5 mM DTT.

The elution buffer may include 50 mM Tris-HCl and10 mM glutathione, 50 mM Tris-HBr and10 mM glutathione or 50 mM Tris-HI and 10 mM glutathione. The elution buffer may be adjusted to pH 8.0.

The purity of bisphosphorylated GST- p38 MAP Kinase may be greater than about 85% to about 90%. About 75% to about 90% of the GST- p38 MAP Kinase may be bisphoshorylated.

Detailed Description of the Figures

Figure 1: The ELISA test revealed the activation of GST p38 MAP Kinase was from about 75% to about 90% as compared with the standard p38 MAPK.

Figure 2: 10% SDS-PAGE gel showing the profile of bisphosphorylated GST-p38 MAPK and subsequent densitometry analysis showed purity greater than or equal to about 90%. The arrow is directed to the 70 KDa marker.

Detailed Description of the Invention

The present invention is a unique methodology for the activation of proteins. It allows for simultaneous purification and activation in a column as a single step. The activation of the protein is done by bisphosphorylation of the tagged p38 MAP Kinase with an upstream activator in the presence of a phosphate donor, such as ATP. The activated p38 MAP kinase is purified to greater than or equal to about 90% purity with about 75% to 90% activation.p38 MAPK was cloned as an appropriately tagged fusion protein, in this case GST, and expressed in *E.coli* BL21 cells as per the process described by Lisnock et. al., Biochemistry, 37 (47), 16573-81 (1998). The cells were grown in 1000 ml LB medium at 37°C with agitation at 225 rpm until the $O.D_{600}$ was 0.8. Expression

was induced with isopropylthio-,-D-galactoside (IPTG) at a final concentration of 0.25 mM for 8 to 12 hours at 18-30°C. The final cell suspension was centrifuged at 2380 *g* for 10 min.

The upstream activator (for example, MKK6 or MKK3) was cloned as a differentially tagged (e.g. His-tag, Myc-tag, Maltose binding protein (MBP) tag, cellulose binding domain (CBD)-tag) fusion protein and expressed in *E.coli* M15 cells. The cells were grown in 500 ml LB medium at 37°C with agitation at 225 rpm until the $O.D_{600}$ was 0.8. Expression was induced with isopropylthio-,-D-galactoside (IPTG) at a final concentration of 0.5 mM for 3 hours at 37°C. The final cell suspension was centrifuged at 2380 *g* for 10 min to form a cell pellet.

Cell Lysis and Solubilization

The cell pellet was resuspended in lysis buffer, containing 10 mM Tris-HCl, pH 7.4, 1 50 mM NaCl, 10 mM DTT, 10% glycerol, 1% Triton-X100 and 1X protease inhibitors. The mixture was freeze-thawed 4 times and was put on ice for 45 mins. The suspension was then centrifuged at 35,000 rpm at 4°C for 30 mins. The supernatant was collected and kept aside.

Affinity Chromatography and Activation

The differentially tagged upstream activator, wherein the upstream activator includes MKK6 or MMK3 and the tag includes His (Histidine), MYC protein, Maltose binding protein (MBP) or cellulose binding domain (CBD) (as per the process described by Lisnock et. al., Biochemistry 37(47), 16573-81 (1998)), was purified using an affinity column according to the manufacturer's instructions. The affinity column may include a HisTrap™, MYCtrap FF, Amylose-column or Calmodulin-binding resin column.

The GST-p38 MAP Kinase was loaded onto the GST column (Amersham Biosciences) with PBS (phosphate buffered saline), pH 7.3 and washed until the $O.D_{280}$ achieved a stable base line. Next, the differentially tagged upstream activator, wherein differentially tagged upstream activator is the same as defined above, was loaded along with activation buffer that includes 8-12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 8-12 mM $MgCl_2$, 0.1-0.4 mM Na_3VO_4, 0.02-0.05% Brij-35, 0.5-1.5 mM ATP and 0.5-2.0 mM DTT or 5-20 mM TRIS (tris (hydroxymethyl) aminomethane),

8-12 mM $MgCl_2$, 0.1-0.4 mM Na_3VO_4, 0.02-0.05% Brij-35, 0.5-1.5 mM ATP and 0.5-2.0 mM DTT and incubated between 15-30°C for 5-18 hrs. The tagged GST-p38 MAPK was eluted with elution buffer that includes 50 mM Tris-HCl, 10 mM glutathione, pH 8.0.

An ELISA was performed to determine whether the p38 MAP Kinase was phosphorylated. The ELISA was run using the commercial Biosource ELISA kit and protocol. The total protein was estimated by the Bradford protein estimation method (Bio-Rad). The ELISA test revealed the activation of GST p38 MAP Kinase was from about 75% to about 90%, which is shown in Fig. 1 as compared with standard p38 MAPK:

The ELISA results showed the activation or bis-phosphorylation of GST-p38 MAPK. The assay was carried out with a commercially available kit from Biosource. CB-chromogen blank, SDB – standard dilution buffer, standard p38 MAPK (200 pg) and sequentially diluted bisphosphorylated GST-p38 MAPK.

As seen in Fig. 2, the purity of the activated protein was determined by running the sample on a SDS-PAGE (10%) gel and using densitometric analysis. The densitometry test revealed that GST-p38 MAP Kinase was obtained with a purity of more than about 85% to about 90%.

While the present invention has been described in terms of its specific embodiments, certain modifications and equivalents will be apparent to those skilled in the art and are included within the scope of the present invention.

We claim:

1. A process for the preparation of purified bisphosphorylated GST- p38 MAP Kinase, the process comprising:

a) adsorbing of the impure GST- p38 MAP Kinase on a GST specific column,

b) washing the column with a phosphate buffered saline solution,

c) loading of the tagged upstream activator and activation buffer into the column,

d) washing of column with phosphate buffered saline, and

e) eluting the bisphosphorylated GST- p38 MAP Kinase off the column with one or more elution buffers.

2. The process according to claim 1, wherein the tagged upstream activator comprises His-MKK6, MYC-MKK6, His-MKK3, MYC-MKK3, CBD-MKK6, CBD-MKK3, MBP-MKK6 or MBP-MKK3 changes.

3. The process according to claim 1, wherein the activation buffer comprises one or more of 8-12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 8-12 mM $MgCl_2$, 0.1-0.4 mM Na_3VO_4, 0.02-0.05% Brij-35, 0.5-1.5 mM ATP and 0.5-2.0 mM DTT or 5-20 mM TRIS (tris (hydroxymethyl) aminomethane), 8-12 mM $MgCl_2$, 0.1-0.4 mM Na_3VO_4, 0.02-0.05% Brij-35, 0.5-1.5 mM ATP and 0.5-2.0 mM DTT.

4. The process according to claim 1, wherein the activation buffer comprises one or more of 10 mM HEPES, 10 mM $MgCl_2$, 0.2 mM Na_3VO_4, 0.03% Brij-35, 1 mM ATP and 1.5 mM DTT or 10 mM TRIS, 10 mM $MgCl_2$, 0.2 mM Na_3VO_4, 0.03% Brij-35, 1 mM ATP and 1.5 mM DTT.

5. The process according to claim 1, wherein the elution buffer comprise 50 mM Tris-HCl and10 mM glutathione, 50 mM Tris-HBr and10 mM glutathione or 50 mM Tris-HI and 10 mM glutathione.

6. The process according to claim 1, wherein the elution buffer is adjusted to pH 8.0.

7. The process according to claim 1, wherein the purity of bisphosphorylated GST-p38 MAP Kinase is greater than about 85% to about 90%.

8. A process according to claim 1, wherein about 75% to about 90% of the GST- p38 MAP Kinase is bisphoshorylated.

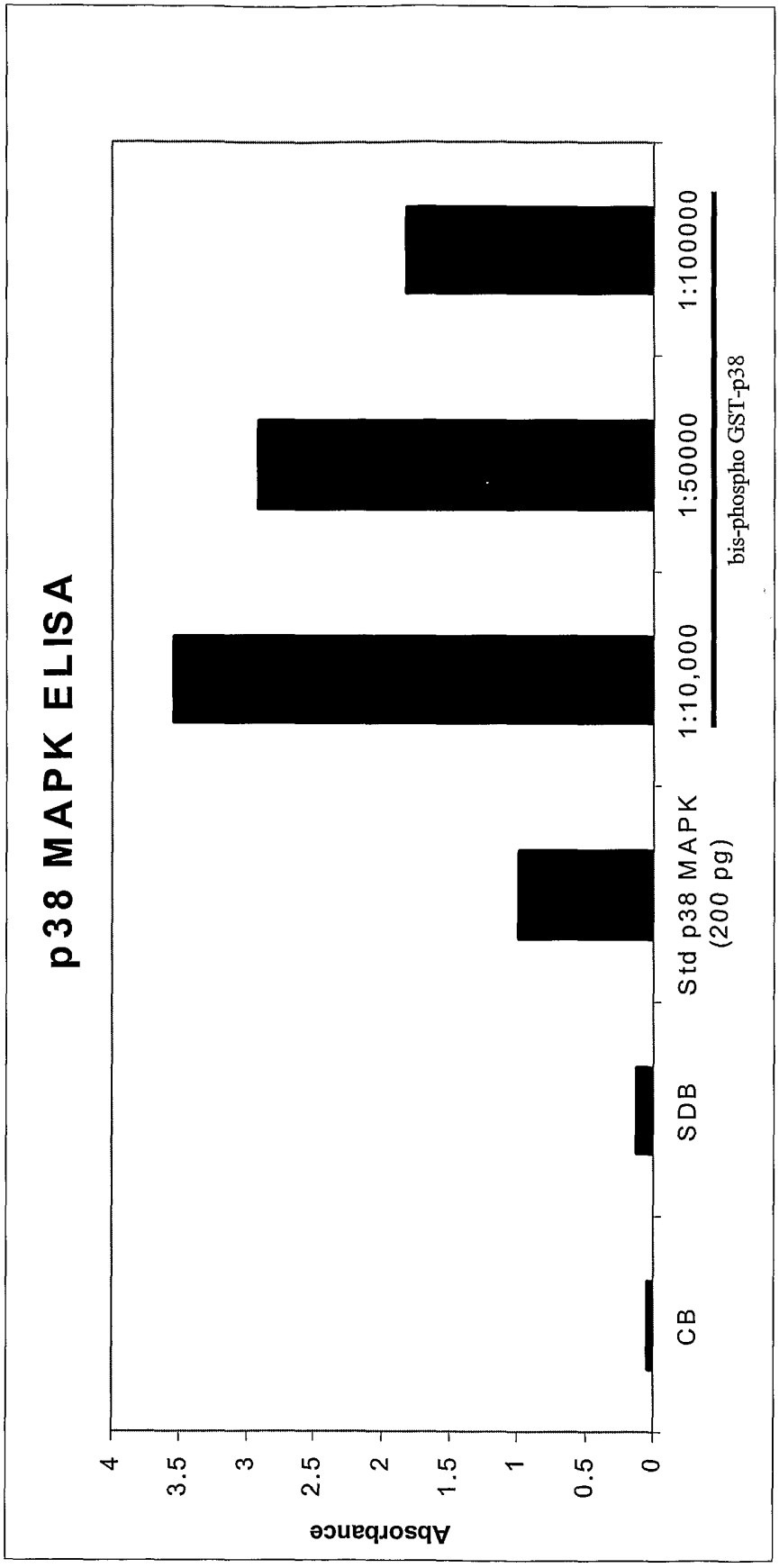

p38 MAPK ELISA
Absorbance
4
3.5
3
2.5
2
1.5
1
0.5
0
CB
SDB
Std p38 MAPK (200 pg)
1:10,000
1:50000
1:100000
bis-phospho GST-p38

Figure 1

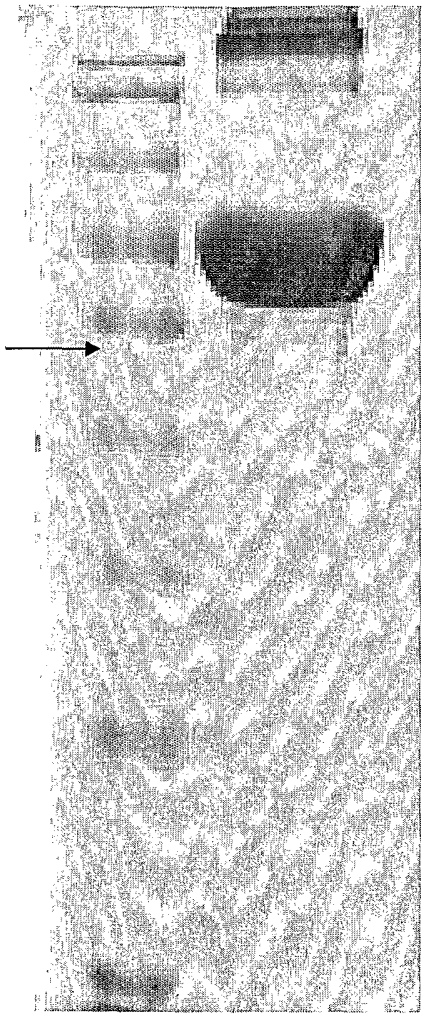


Figure 2