PURIFICATION, CHARACTERIZATION & QUANTIFICATION OF TYPE II CLASS CGMP-DEPENDENT PROTEIN KINASE II (PKG-II) FROM RAT INTESTINAL MUCOSA

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ABSTRACT

CGMP-dependent protein kinase (PKG-II) is a serine/threonine-specific protein kinase that is activated by cGMP. It phosphorylates a number of biologically important targets and is implicated in the regulation of smooth muscle relaxation, platelet function, sperm metabolism, cell division, and nucleic acid synthesis. A protein kinase type II was purified from rat intestinal mucosa using ammonium sulphate fractionation, ion exchange chromatography on DEAE and phosphocellulose and affinity chromatography on phosvitin- and casein-sepharose columns using AKTA-FPLC system. The Isolated PKG-II was characterized using western blot technique in comparison with Native form of PKG-II. Enzymatic activity was performed successfully to correlate the functioning aspects of both Native and isolated PKG-II proteins.

KEY WORDS: PKG-II, Phosphorylation, AKTA, rat intestinal mucosal cells, etc.

INTRODUCTION

In biochemistry, kinase is a type of enzyme that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecules to specific substrates. This process is
known as phosphorylation when the substrate gains a phosphate group and the high energy molecule of ATP donates a phosphate group (producing a phosphorylated substrate and ADP). Conversely, it is referred to as dephosphorylation when the phosphorylated substrate donates a phosphate group and ADP gains a phosphate group (producing a dephosphorylated substrate and the high energy molecule of ATP).\[^1\] These two processes, phosphorylation and dephosphorylation, occur four times during glycolysis. Kinases are part of the larger family of phosphotransferases. Kinases are not to be confused with phosphorylases, which catalyze the addition of inorganic phosphate groups to an acceptor, nor with phosphatases, which remove phosphate groups. The phosphorylation state of a molecule, whether it be a protein, lipid, or carbohydrate, can affect its activity, reactivity, and its ability to bind other molecules. Therefore, kinases are critical in metabolism, cell signalling, protein regulation, cellular transport, secretory processes, and many other cellular pathways. Kinases mediate the transfer of a phosphate moiety from a high energy molecule (such as ATP) to their substrate molecule.\[^2\] Kinases are needed to stabilize this reaction because the phosphoanhydride bond contains a high level of energy. Kinases properly orient their substrate and the phosphoryl group within their active sites, which increases the rate of the reaction. Additionally, they commonly use positively charged amino acid residues, which electrostatically stabilize the transition state by interacting with the negatively charged phosphate groups.\[^4\] Alternatively, some kinases utilize bound metal cofactors in their active sites to coordinate the phosphate groups.

**METHODOLOGY**

**Isolation of Rat Intestinal Mucosal smooth muscle\[^5\]**

The starting material was obtained by scraping off the mucosal tissue of rat duodenum and jejunum. Animals were treated by stomach tube test solution 15 min before removing the gut segment. The weight of tissue obtained.

**Culturing of Smooth muscle cells in growth medium\[^6\]**

The base medium for this cell line is ATCC formulated Eagle's Minimum Essential Medium, Catalog No. 302003. To make the complete growth medium, added the components to the base medium.

**Subculturing Procedure (Cell Passaging)\[^6\]**

Removed the medium, and rinsed with 0.25% trypsin, 0.53 mM EDTA solution. Removed the solution and added an additional 1 to 2 mL of trypsin-EDTA solution. Allow the flask to
sit at room temperature until the cells detach. Add fresh culture medium, aspirated and
dispensed into new culture flasks. Corning T75 flasks (catalog #430641) are recommended
for subculturing this product. Medium Renewal was recommended.

**Isolation of Protein Kinase GII (PKG-II)**\(^7\,^18\)

Placed the cell culture dish in ice and washed the cells with ice-cold PBS. Drained the PBS,
then added ice-cold lysis buffer (1 ml per \(10^7\) cells/100 mm dish/150 cm\(^2\) flask; 0.5ml per
5\(\times\)\(10^6\) cells/60 mm dish/75 cm\(^2\) flask). Scraped adherent cells off the dish using a cold plastic
cell scraper, then gently transferred the cell suspension into a pre-cooled microfuge
tube. Maintained constant agitation for 30 minutes at 4°C. Centrifuged the cells in a
microcentrifuge at 4°C. the centrifugation force and time depending on the cell type; a
guideline is 20 minutes at 12,000 rpm but this must be determined by the end-user. Gently
remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a
fresh tube keet on ice, and discard the pellet.

**Protein purification**\(^20\)

For the purification of a protein it is generally necessary to use more than one step. Each step
of the purification should be evaluated by measuring the concentration and the activity of the
protein. The purification of a protein can be based on either its physical or biological
properties. Physical properties used for separation are size and shape, charge, hydrophobicity
and solubility. Biological properties could for example be specific interactions with other
molecules. One should take advantage of properties, which distinguish the protein of interest
from the other proteins present.

**Characterization of PKG-II by SDS PAGE Analysis**\(^21\)

A typical gel of 7% acrylamide composition nicely separates polypeptides with molecular
mass between 45 and 200 kDa. Polypeptides below the cutoff of around 45 kDa do not
resolve. A denser gel, say 14%T, usually resolves all of the smallest polypeptides in a mix.
Such a gel would be needed to resolve hemoglobin, for example. It would be useless for
resolving bands much above 60 kDa, though. To analyze the entire profile of a fraction that
contains heavy and light polypeptides, one should usually run two gels. In the teaching lab
we recommend that alternate teams prepare low or high percent gels, with each team
exchanging samples with a team that prepared the other type gel. Each team, then, would load
its set of samples, appropriate standards, and another team's samples on its gel, and have its
samples loaded onto another percent gel as well.
Preparing Protein Samples for Electrophoresis\(^{[22]}\)
A polypeptide is a macromolecule consisting of a non-branching sequence of amino acids, each connected to the next by a single peptide bond. A protein consists of one or more polypeptides and/or additional types of molecules, held together by any of a number of molecular interactions often including covalent bonds. Such interactions result in several levels of organization, which we call primary, secondary, tertiary, and quaternary structures. Intact proteins are notoriously difficult to separate reproducibly. Patterns of bands vary depending on temperature, buffer, variations in pH, quality of a preparation.

Determination of concentration of Native PKG-II\(^{[22]}\)
The microplate supplied with the kit contains 96 wells which permits the analysis of 42 samples in duplicate (84 wells) and 6 calibrant dilutions in duplicate. Standards Added 5 µL of each standard in duplicate to microplate wells. Serum Added 100 µL of sample in duplicate to microplate wells. Incubated the samples for 30 minutes at 30\(^{\circ}\)C. Added 100 µL of HRP conjugated anti-phospho-specific antibody. Incubated for 1 hr at room temp. Washed the wells Add 100 µL of Substrate Reagent and Added 100 µL of Stop Solution was measured the absorbance at 450 nm.

RESULTS AND DISCUSSION
Native PKG-II from rat smooth muscle was purified from the cytoplasmic fraction of homogenized Cells using procedure ammonium sulfate fractionation and a series of IMA chromatography steps by AKTA Purifier. The first IMA chromatography column in the purification resolved Na.PKG-II into two peaks of activity.

Which shows two major immune reactive peaks with apparent Molecular weight values of 120,000 Da and 95,000 Da. that eluted from the column in regions corresponding to the PKG-II activity. The lower Peak, which had a molecular weight consistent with Native cGMP PKG-II, also eluted broadly but peaked late in the gradient. The inability to
completely resolve the PKG-II peaks from one another on this column may reflect structural or other heterogeneity in the two PKG forms.

After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane, visualized by Coomassie Blue staining, and subjected to N-terminal sequence analysis. The biological specific activity of the purified PKG species is reported. Mucosal PKG-II purified 2,000-fold with an overall yield of 55%. The biological specific activity was 33,000 units/mg, as determined in a Pi-release assay using N-ras-bound GTP as the PKG substrate. Na. PKG was purified 700-fold with an overall yield of 11%. Since Na. PKG was recovered from the AKTA FPLC step at a purity of only 27% (determined by scanning densitometry of a Coomassie Blue stained SDS-polyacrylamide gel), the specific activity of this PKG was estimated to be -39,000 units/mg, essentially equivalent to that of Na. PKG.

![Western blot of Na.PKG-II & Rat Mucosal PKG-II by fractions of AKTA IMAC.](image)

**Characterization of PKG-II by SDS PHAGE &Immunoglobin technique**

IMAC chromatography of crude or Na.PKG-II and Rat mucosal PKG-II preparation. A mucosal layer extract was fractionated by ammonium sulfate precipitation followed by chromatographed on IMAC column. Upper, absorbance at 280 nm and PKG activity assayed by the immunoprecipitation method showing the conversion of ras-bound GTP to GDP plotted as the ratio of GDP to GDP + GTP; lower Western blot analysis of the column factions using the anti-rGAP antibody (described below) and protein A. The two major PKG bands are indicated. Fractions 72-82 were pooled for peak I PKG, and fractions 108-120 for peak II PKG. Analysis also revealed several other bands with smaller molecular weights than
either of the major PKG forms. These may represent proteolytic fragments of peak II PKG since they appear to co-elute with the second peak of PKG activity.

**PKG activity assay**

Cells were harvested and homogenized. Supernatants were assayed for PKG activity by measuring the incorporation of p32 from ATP in a specific PKG substrate. Briefly, 10 µl of extract was assayed for PKG activity in 100 µl assay buffer with 0.2 mM ATP, 100 µM of a PDEtide, and 1µM protein kinase A (PKA) inhibitor. Assays were conducted at 30°C for 5 min in the presence or absence of 10 µM cGMP. In the absence of cGMP, the constitutive PKG activity is measured; the addition of cGMP allows measuring cGMP-stimulated PKG activity. The reaction was terminated by aliquoting samples to P81 phosphocellulose paper and washing in 75 mM phosphoric acid. Next, the paper was dried and counted in 10 ml scintillation fluid. PKG activity was expressed as nanomoles of peptide phosphorylated per minute per milligram of cell extract obtained was 35 g tissue containing 5.2 g of PKG-II.

**CONCLUSION**

The purified c GMP dependent PKG-II from intestinal mucosal layer of cells cytoplasmic extract and determined in approximately equal amounts with Native form of PKG-II. The larger molecular weight form has been previously shown to be type II PKG-II and represents the predominant PKG species detected in the mammalian tissues that have been examined. A smaller molecular weight form of PKG-II was purified and was shown to type II PKG-II. The shorter rat mucosal type II PKG was not simply a truncated form of type II PKG of native form because its N-terminal sequence is not present anywhere in the Na. type II PKG sequence. Both PKG species had similar biological specific activities when assayed in vitro.

The specific activity that we obtained for purified mucosal type II PKG was apparently 10-fold higher than that reported for Native PKG -II. This may be a reflection of the different affinities of both proteins, differences in the individual PKG preparations, or other differences in the assays themselves. Here finally I concluded that the methods followed to isolate and purify rat mucosal cGMP dependent PKG-II were highly enzyme specific. And the isolated protein was characterized using western blot analysis and that was showed almost equivalent characteristics as with native form of PKG-II. Up to the current knowledge the work is higly novel to carryout isolation, purification and characterization of cGMP dependent PKG-II.
REFERENCES


