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Optimization of Enzyme Dilution Buffer and Reaction Buffer for Vaccinia H-1 Related Wild Enzyme Assay and Its Stability at 4°C

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Abstract

A human dual specific phosphates (DUSP), Vaccinia H-1 related wild enzyme (VHR), dephosphorylates both phosphotyrosine and phosphoserine/phosphothreonine residue of a protein. VHR is considered as a promising therapeutic target for cancer because the cells lacking VHR are arrested at the G1-S and G2-M transitions of the cell cycle with a decreased telomerase activity. VHR being a therapeutic target for cancer is crucial to know about its stability and its assay procedure conditions. This study was conducted to verify the viability of VHR enzyme at 4°C. Protein concentration and specific activity were calculated from Bradford method and p-nitrophenylphosphate (*p*NPP) assay by measuring the absorbance at 595 nm and 405 nm respectively in each respective day. The absorbance showed invariable difference in protein concentration and specific activity from starting to final days. Buffers like enzyme dilution and reaction buffers played significant role in VHR enzyme stability and activity. To find out the correct buffer components for carrying out the VHR enzyme assay, several experiments were carried out by using variable constituents in enzyme dilution buffer and reaction buffer. The present study revealed that 3-component buffer system without thiol i.e. Dithiothreitol (DTT) or β-Mercaptoethanol (β-ME) as a reaction buffer and 2-*N*-morpholino ethanesulfonic acid (MES) buffer with DTT as an enzyme dilution buffer demonstrated invariably different in the stability throughout the experiment.

Key words: dilution buffer, dual specific phosphatase, reaction buffer, stability, and VHR enzyme

Introduction

A variety of cellular functions is governed by a process called protein tyrosine phosphorylation (Kuban *et al.* 2010, Walton & Dixon 1993). Due to the interaction between Protein Tyrosine Phosphatases (PTP) and Protein Tyrosine Kinases (PTK), the dynamic balance of tyrosine phosphorylation is possible (Hunter 1989, Alonso *et al.* 2004, Nicholas 2003). Disturbance in maintaining the dynamic balance can result in cancer and other diseases like diabetes etc (Hunter 1989, Charbonneau & Tonka 1992). The role of PTP and PTK in the process of cell growth, proliferation, oncogenic transformation, and cell cycle regulation is remarkable. The prototypical member of the dual specific phosphatase (DUSP) group (Amand *et al.* 2014), VHR dephosphorylates preferably phosphotyrosine residue (Xiao *et al.* 2013). Positively charged crevices near the active site may explain the enzyme's preference for substrates with two phosphorylated residues. The lack of specific contacts to the aromatic moiety of the phosphotyrosine as well as the shallower depth of VHR's active site likely explains VHR's weak activity toward dephosphorylating phosphothreonine residues (Maria *et al.* 2002). DUSPs regulate mitogenic signal transduction and control the cell cycle. VHR is isolated from human fibroblasts by Ishibashi *et al*. (1992). Over expression of VHR enzyme localized in the nucleus brings a key role in cellular proliferation and senescence triggering for the onset of human cervical cancer cells (Panico & Forti 2013, Wu *et al*. 2009).

Correct selection of the required ingredients in the reaction buffer and enzyme dilution buffer is really meaningful to the *p*NPP and Bradford assay. Wrong choice of these constituents in the buffers causes drastic diminishing of specific activity. In this work several trials were performed by taking MES buffer with DTT or *β*-ME as enzyme dilution buffer and 3-component buffer with DTT or *β*-ME as reaction buffer and MES buffer with or without thiol (DTT, *β*-ME) as an enzyme dilution buffer as well as reaction buffer. Storing VHR enzyme mandatorily at -20 ˚C (Rahmouni *et al*. 2006) is cumbersome for those labs lacking -20 ˚C refrigerators. If the enzyme can be stored at higher temperature like 4 ˚C, the enzyme related works make easier and also becomes a novelty of this research article.

Methodology

The strain BL21DE3 (isolated from human fibroblast) of VHR gene was inserted in *E. Coli* by the pET-21A vector. A loopful of *E. coli* strain harbouring pET-21A-VHR(from glycerol stock) was transferred into a hard glass test tube containing 6 ml of sterilized LB medium containing 30 µl of Ampicillin (10 mg/ml) was added to the LB medium. The culture medium was incubated in a shaking incubator at 37 ˚C, and at 250 rotations per minute (rpm). Two ml of the overnight cultured medium was added to 200 ml of LB medium containing 400 µl of Ampicillin (50 mg/ml) and incubated in shaking incubator at 37 ˚C and 250 rpm. Optical density (OD) was checked at absorbance 590 nm at different intervals of time till OD becomes 0.6. Then 200 µl of 950 mg/ml Isopropyl *β*-D-1-thiogalactopyranoside (IPTG) was added to the inoculated solution and incubated at 37 ˚C in shaking incubator with agitation of 250 rpm for about 6 hours. Then the culture solution was kept on ice for 15 mins. The cold solution was centrifuged at 4˚C and 1000 rpm for 15 mins. Supernatant was discarded and 2.4 ml of the buffer A (50 mM HEPES, 5.0 mM EDTA, pH 7.0) and 130 µl of 95mg/ml Lysozyme were added to the cell pellets. Freezing in liquid nitrogen and thawing in a palm were performed three times. 13.3 µl of 1M $MgCl_2$ and 7 µl of 16mg/ml DNase I were added and incubated in a shaking incubator at 30 ˚C with constant agitation (250 rpm) for 30 mins. To this solution, 67 µl of 0.4 M EDTA (pH 8) and 160 µl of 10% Triton × 100 were added and centrifuged at 4 ˚C, 1000 rpm for 30 mins. Finally, the supernatant was collected in an eppendorf tube (500 µL in each tube), frozen in liq. nitrogen and stored at -50 ˚C. Thus obtained solution was used as a crude VHR enzyme. Protein concentration was calculated from Bradford assay graph and then specific activity was computed.

Purification of crude VHR enzyme

The crude VHR enzyme was purified by a column chromatography. The column was loaded up to 8 cm by resin (SP Sephadex C-50, Pharmacia LKB, Biotechnology AB, and Uppsala, Sweden) in MES buffer. The crude VHR enzyme was diluted in 10 times in MES buffer (pH 6). The diluted VHR enzyme was loaded to the column by 1 ml each time and eluted by several concentrations (0.2 mM, 0.5 mM, 1 mM, 1.25 mM 1.5 mM, 1.75 mM, 2.0 mM, 2.25 mM, 2.5 mM, 2.75 mM and 3 mM) of NaCl in MES. Eluate obtained from different concentrated salt solution was collected separately in a tube. Protein concentration and specific activity of each tube were computed. The tubes containing comparatively high specific activity were loaded in Millipore minicolumn (10,000MWCO, 500 µl, Millipore Corporation, Bedford, MA) and centrifuged at 4 ˚C, 4000 rpm, and 10 mins to reduce volume. Eventually, protein concentration and specific activity of the concentrated enzyme were measured by Bradford and *p*NPP assays respectively.

Protein estimation

Protein concentration was measured by Bradford method (Noble & Bailey 2009). 1 ml of Bradford reagent was taken in two different eppendorf tube and 4 µl and 5 µl of E/10 D (E/10D is the 10-fold diluted crude lysate in buffer A: 50 mM Hepes, 5 mM EDTA, 2mM DTT, pH 7.0) crude lysate (diluted in buffer A) was added in two different tubes. The mixture of E/10 D crude enzyme (diluted in buffer A) was kept at room temperature for 10 minutes. Then the absorbance was measured at 595 nm separately.

*p*NPP assay

VHR enzyme activity was determined by *p*NPP (Luechapanichkul *et al*. 2013). Each of two mixtures containing 30 µl of H_2O, 10 µl of 5 × acetate buffer, 5 µl of E/10 D (E/10D is the 10-fold diluted crude lysate in buffer A (50 mM Hepes, 5 mM EDTA, 2mM DTT, pH 7.0) crude enzyme and 5 µl of 200 mM *p*NPP was kept at 30 ˚C. One of the two mixtures was quenched by 950 µl of 0.5 M NaOH in 1 min and another in 2 mins. The absorbance was measured at 405 nm separately.

Optimization of enzyme dilution and reaction buffer

MES buffer as enzyme dil. buffer (w/ DTT) as well as reaction buffer (w/ or w/o DTT)

For this experiment**,** MES buffer pH 6 (MES 20 mM, EDTA 1 mM) was used as both enzyme dilution buffer and reaction buffer. A reaction mixture contained 525 µl of H_2O, 210 µl of reaction buffer (MES buffer pH 6 with DTT), 105 µl 2.3mg/ml of E/10 D VHR enzyme (diluted in MES buffer). Then 45 µl of aliquot of the reaction mixture was incubated at 30 ˚C for the given time periods (on the basis of X-axis) followed by the addition of 5 µl of 20 mM *p*NPP and the mixture was incubated at 30 ˚C for 3 min. The reaction mixture was quenched with 950 µl of 0.5 M NaOH and the absorbance was measured at 405 nm. The similar experiment was carried out with reaction buffer contain MES buffer pH 6 with *β*-mercaptoethanol and MES buffer pH 6 without DTT respectively.

MES buffer as enzyme dil. buffer (w/ DTT) and 3 comp. buffer (w/or w/o DTT) as reaction buffer

In this experiment, MES buffer as an enzyme dil. buffer and 3 components buffer (CH_3COONa 50 mM, Tris HCl 25 mM, bis-tris propane 25 mM) as a reaction buffer were used. The same experimental procedure was followed as mentioned above.

Stability at 4 ˚C

Generally, VHR enzyme is stored at -20 ˚C. In this study, the viability of the enzyme was determined by storing the enzyme at 4 ˚C for ten days. Then the protein concentration and specific activity of the enzyme were determined from first day of storage to the tenth day. In our attempt, the viability of the enzyme was testified by storing at 4 ˚C. The experiment was carried out for ten days. Computation of the protein concentration and specific activity were determined by Bradford and *p*NPP assays as mentioned above.

Results and Discussion

Buffers play important roles in carrying out enzyme assay. Here we tried both buffers i.e. enzyme dilution and reaction buffers varying in ingredients to check the stability of an enzyme. The first experiment was carried out by taking MES buffer with DTT as enzyme dilution buffer and MES buffer with or without thiol (DTT/ *β*-Mercaptoethanol) as reaction buffer. To find out the effect of reaction buffer, three different sets of reactions were performed. In all three experiments, MES buffer with DTT as enzyme dilution buffer and the components of reaction buffer was varied. In the first case, MES buffer with DTT was used as reaction buffer (Fig. 1 ■). In the second case, MES buffer with *â*-mercaptoethanol was used as reaction buffer (Fig. 1 ●). Consequently, MES buffer without thiol as reaction were taken to carry out the third trail (Fig. 1 ▲). Each of the above three experiments was triplicated. From Fig. 1, it was revealed that the absorbance was decreasing from zero time to 90 mins in all the experiments. Among the three, the first trail showed relatively less decrease in the absorbance than the second trial than the third trial. From the experiment, it was revealed that the selection of variable constituents in both buffers was misleading i.e. the buffer systems were not suitable for conducting the enzyme assay.

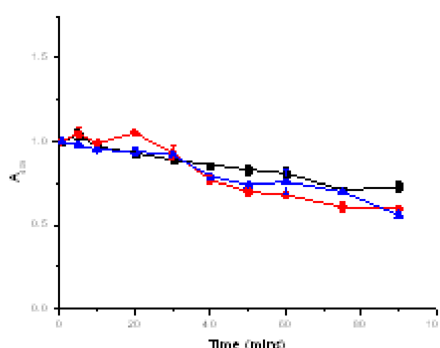

Fig. 1. Stability of VHR with DTT in enzyme dil. buffer and with or without thiol in reaction buffer.

(**Note:** A on y-axis stands for absorbance) The symbol (■ indicates DTT in both enzyme dilution buffer as well as reaction buffer, (●) symbolizes DTT in enzyme dil. buffer and â-me ptoethanol in reaction buffer and the symbol (▲ denotes DTT in enzyme dilution buffer but no DTT/ *β*-ME in reaction buffer (each experiment was carried out in triplicates).

In the second experiment (Fig. 2), a set of trails was done by taking MES buffer with DTT as enzyme dilution buffer and 3 component buffer with or without DTT as reaction buffer. For all three sets of the trails, enzyme dilution buffer contained DTT. In case of reaction buffer, DTT was added for the first trail, *β*-mercaptoethanol for the second trail, and without

thiol for the third trial. Each trail was conducted in triplicates. While comparing the absorbance of all three trails, the third trail (Fig. 2▲ showed invariably different from starting to the end. The remaining two trails indicated that the absorbance was irrelevant (variably different) with increasing time. Hence, while using VHR enzyme for a certain purpose, it is suggested that MES buffer with DTT as enzyme dilution buffer and 3-component buffer without thiol as reaction buffer are to be used.

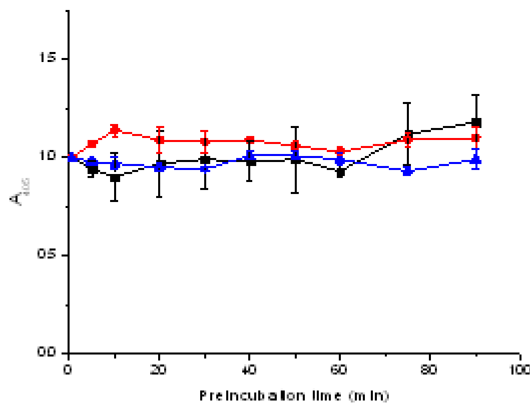

Fig. 2. Stability with MES buffer (w/ DTT) as enzyme dil. buffer and 3 component buffer (w/or w/o DTT) as reaction buffer.

(**Note:** A on y-axis s(**Note:** A on y-axis stands for absorbance) The symbol (■ indicates DTT in both enzyme dil. buffer as well as reaction buffer, (● symbolizes DTT in enzyme dil. buffer and mercaptoethanol in reaction buffer and the symbol (▲ denotes DTT in enzyme dilution buffer but no DTT/ β-ME in reaction buffer (each experiment was performed in triplicate).

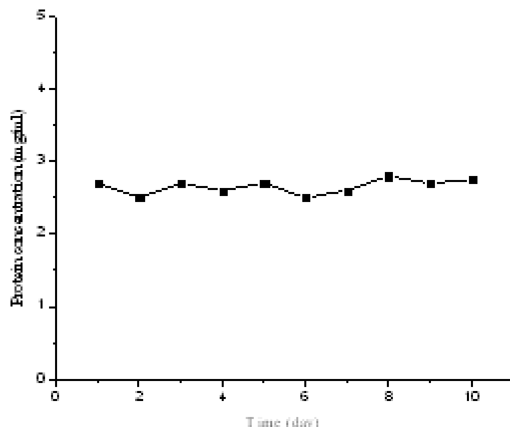

Fig.3. Protein concentration of VHR at 4 °C

An experiment was also carried out to testify the stability of VHR enzyme at 4 °C where protein concentration and specific activity were measured. For this experiment, the enzyme has been stored at 4 °C. Protein concentration and specific activity were measured every day by Bradford assay and *p*NPP as mentioned above. The experiment was continued for ten consecutive days. Fig 3 revealed that the protein concentration remained nearly consistent throughout the experiment. From Fig 4, the specific activity of the VHR enzyme looked nearly the same throughout the 10 days experiments. So, it is concluded that the VHR enzyme could be stored at 4 °C for a month.

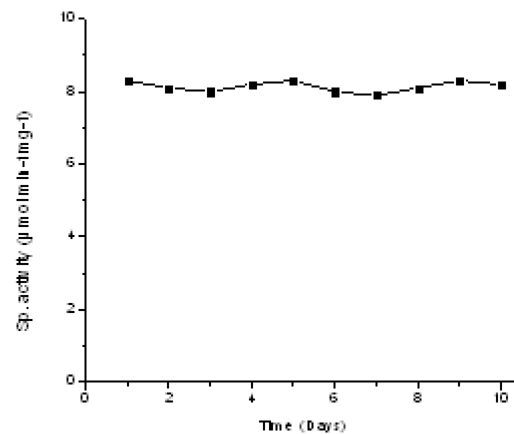

Fig.4. Specific activity of VHR at 4 ºC

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