

A novel assay method for calcium calmodulin dependent phosphatase from bovine brain extract

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Calcium calmodulin dependent protein ser/thr phosphatase, also referred to as protein phosphatase 2B (PP2B), is rich in neural tissue, and plays an important role in the overall function of the nervous system. Routinely phosphatase assay employs, *para*-Nitrophenylphosphate (p-NPP), as a substrate, is also extended to assay PP2B. However, in the present study, the differential spectral characteristic property of tyrosine and phosphotyrosine has been exploited to employ the latter as a candidate substrate for the PP2B assay. The specific activity of PP2B using phosphotyrosine in bovine *Bos Taurus indicus* brain extract (*Bos Taurus indicus*), was measured in presence of different metal ions like Ca²⁺, Mn²⁺ and Mg²⁺. Further modulators like dithiothreitol (DTT), calmodulin (CaM) and metal chelators such as EGTA and EDTA were applied to confirm the role of divalent cations and to determine calcium calmodulin dependent phosphatase activity. PP2B activity was higher with phosphotyrosine in presence of Ca²⁺ than with p-NPP. Further experiments, involving calmodulin as a modulator, confirmed phosphotyrosine as a better substrate over p-NPP. Calmodulin further enhanced the effect of phosphotyrosine as a potential substrate confirming calcium calmodulin dependent phosphatase activity. Phosphotyrosine is proposed as a better substrate in assaying calcium dependent phosphatase activity when compared to *para*-nitrophenylphosphate.

Keywords: Calmodulin, Dithiothreitol, Para-nitrophenylphosphate, Phosphatase, Phosphotyrosine

Many recent studies have underscored the importance of protein phosphatases in health and diseases¹. In particular, there is accumulating evidence to demonstrate the importance of Ca²⁺/calmodulin-regulated serine/threonine protein phosphatase type 2B (PP2B)² in various biological events such as signal transduction^{3,4}, learning and memory³, immunity⁴, apoptosis⁵ and hypertrophy of the heart⁶. However, very little of this knowledge has percolated to laboratory medicine and the patho-physiological significance of protein phosphatases as a diagnostic tool is yet to be established. One of the main reasons for this slow pace translation of the research findings to clinical situation is the lack of appropriate assay methods that can be easily adoptable in the laboratories. Earlier investigators working on protein

phosphatases have employed endogenous substrates such as protein kinase regulatory subunit type II, which was P³² radiolabelled before application as a substrate^{7,8} and hence, required elaborate preparative steps prior to the assay. A non-radioactive enzyme assay method was also introduced based on the measurement of both the substrate (phosphoprotein substrate) and the product (dephosphorylated protein), by reverse phase HPLC analysis with UV detection⁹. In the same line of research, peptide analogues or protein kinase regulatory subunit type II was also synthesized to employ as a substrate for calcium calmodulin dependent phosphatase enzyme¹⁰.

Though, PP2B activity *in vivo* is dependent on Ca²⁺ and calmodulin, it is activated *in vitro* by other divalent cations such as Ni²⁺ and Mn²⁺¹¹⁻¹². This observation has led to the introduction of the assay method comprising of the unnatural substrate, *para*-nitrophenylphosphate (p-NPP) and the metal activator, Mn²⁺¹³ using different inhibitors for the assay¹⁴. Furthermore, PP2B, being a serine/threonine protein phosphatase, was also seen to act upon phospho-tyrosyl residues of proteins; hence,

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substrates that have tyrosyl and/or tyrosyl-related residues, such as p-NPP, were also used to assay the enzyme activity.

The specificity of the assay method for protein phosphatase was addressed by calculating the difference between the total phosphatase and non-specific phosphatase fraction in the presence and absence of known protein phosphatase inhibitor such as trifluoperazine (TFZ). Thus, the relative simplicity, if not the absolute specificity, of the method, based on *para*-nitrophenylphosphate (p-NPP) as a substrate, has encouraged many investigators to employ this method to assay the PP2B activity. The present study has elaborated the significance of *ortho*-phospho-L-tyrosine as a better substrate over *para*-nitrophenylphosphate.

Materials and Methods

Chemicals—Tris buffer, ethylenediaminetetraacetic acid (EDTA), Triton X-100, Phenylmethanesulfonyl fluoride (PMSF), ethylene glycol tetraacetic acid (EGTA), sodium orthovanadate, *O*-phospho-L-tyrosine and *para*-nitrophenylphosphate were purchased from Sigma Aldrich, USA. Dialysis tubing and cellulose membrane used for filtering brain homogenate, was procured from Sigma, St. Louis. All other chemicals were of the highest purity and commercially available were purchased from either Merck or BDH, unless otherwise specified.

Solutions—The enzyme, phosphatase was prepared from bovine brain as homogenate. The stock solutions (10 mM) of substrates, *para*-nitrophenylphosphate and *O*-phospho-L-tyrosine, were prepared separately in double distilled water. Modulators, such as ethylene glycol tetra acetic acid (EGTA), ethylene diamine tetra acetic acid EDTA, calcium (Ca^{2+}), magnesium (Mg^{2+}) and manganese (Mn^{2+}) were prepared in double distilled water, separately, to obtain 10 mM concentration. The stock solution (200 mM) of phosphatase inhibitor, sodium orthovanadate, was depolymerized for activation¹⁵. All reagents, substrates and enzyme extracts were aliquoted and stored in deep freeze at $-80\text{ }^{\circ}\text{C}$, till further use.

Preparation of phosphatase homogenate—All procedures were in strict accordance with the guidelines of the Central Animal Research Facility (CARF) and were approved by the National Institute of Mental Health and Neurosciences (NIMHANS),

Bangalore. The bovine brain was collected in ice cold ($4\text{ }^{\circ}\text{C}$) normal saline (0.9 g NaCl/100 mL water; pH 7.4), in an ice bucket. Blood vessels and meninges were removed from the tissue. Brain tissue (25 g), suspended in extraction buffer in 1:10 (w/v) [100 mM Tris buffer saline, 1 mM EDTA, 1% Triton X-100 (v/v); pH 7.4], was homogenized by adding 1 mM PMSF. Aailed homogenate was stirred for 2-3h at $4\text{ }^{\circ}\text{C}$ and centrifuged at $75,000g$ for 90 min. The supernatant collected was dialyzed using dialysis kit (Sigma, USA), aliquoted and stored in deep freeze at $-80\text{ }^{\circ}\text{C}$ till further use. There was no significant change in enzyme activity after freezing and thawing. The standard assay measured 50 μg of protein unless otherwise specified.

Isolation and purification of calmodulin—Calmodulin (CaM) from bovine brain was prepared as described by Buccigross *et al.*¹⁶. Purity of the calmodulin was assessed by SDS PAGE; both in presence and in absence of Ca^{2+} (see also Fig. 5). Calmodulin preparation had electrophoretic mobility according to their relative molecular weight on SDS PAGE run with and without Ca^{2+} as 14 kDa and 20 kDa mass of calmodulin protein¹⁷.

HPLC analysis—Shimadzu HPLC RF-530 series equipped with degasser, quaternary pump (Shimadzu 10AD) was used for solvent, 20 μL injection loop and C18 column (25 cm), fitted with guard column (1 cm) and housed in an incubator oven (Mayoora Analyticals Pvt. Ltd, India) set at $40\text{ }^{\circ}\text{C}$ along with a UV-Fluorescence detector (Shimadzu 5301 PC) system. Analyst software Shimadzu CLASS-VP (V6.12SP5) series was used for HPLC system control, data acquisition and processing. Analysis of L-tyrosine, *O*-phospho-L-tyrosine was carried out by precolumn *ortho*-phthalaldehyde (OPA) derivatisation¹⁸, and the purity of substrate was verified by reverse phase HPLC.

Briefly, OPA-derivatised amino acids were injected into the HPLC system and individual amino acids were separated by reverse phase gradient of 0.05 M acetate buffer, pH 6.8 (solvent A) and methanol (solvent B) with the flow rate of 1.0 mL/min. Resolution of amino acid derivatives were monitored through a fluorescence detector, with excitation and emission set at 330 nm and 450 nm, respectively.

Enzyme assay system—All the assays for phosphatase activity were measured in UV-visible Shimadzu Spectrophotometer (UV-2450) equipped

with a thermostat and two cell holders with 1 cm path length quartz cuvettes, were employed for the assay. The activity was measured against different substrates in 100 mM Tris buffer (pH 7.4), with different metal ions like Ca^{2+} , Mg^{2+} and Mn^{2+} (1 mM each). The study was also extended with the addition of modulators like EGTA (2.5 mM), EDTA (2.5 mM), DTT (1 mM) and calmodulin. The enzyme solution was pre-incubated at 37 °C for 10 min. The enzyme reaction was initiated by adding a substrate, and the activity was monitored by measuring the increase in absorbance at a corresponding wavelength depending on the nature of the product formed. The absorbance for p-NPP (1 mM) and phosphotyrosine (100 μM) were measured at 405 and 280 nm respectively.

Data presentation and statistical analysis—The specific activity for phosphotyrosine phosphatase was calculated by employing molar absorption coefficient (ϵ) = $1.25 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as (release of nanomoles of tyrosyl residues/mg protein/minute); while the specific activity for *para*-Nitrophenylphosphatase was calculated by employing $\epsilon = 15.65 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as (release of micromoles of nitrophenyl residues/mg protein/min). All the experiments were repeated 4-5 times and the results are reported as mean \pm SD. Comparisons between groups were analyzed and plotted using SIGMA Plot 10 (Systat Software Inc., USA). $P < 0.005$ was considered statistically significant using one way analysis of variance (ANOVA).

Results and Discussions

The present study aims at validating the methodological aspects to be employed for monitoring the protein phosphatases 2B assay *per se*, while emphasizing more on the use of natural substrates and modulators in the assay system. One of the main approaches was to establish differences with respect to the spectral characteristics of the potential substrate(s) and the products that are expected to be formed of phosphatase activity. Hence, the spectral characteristics of phosphotyrosine and tyrosine, the prospective substrate and the expected product of phosphatase were studied.

Absorption spectral characteristics of tyrosine and phosphotyrosine—Considering the proposed study of employing phosphotyrosine as a substrate, molar absorption coefficient (ϵ) of tyrosine and phosphotyrosine was determined. The spectral

characteristics and molar absorption coefficient of tyrosine and phosphotyrosine in 0.1 M tris buffer (pH 7.2) exhibits identical pattern at $\lambda_{\text{max}} = 278 \text{ nm}$, and has shown a distinct difference in molar absorption coefficient (ϵ). Thus, tyrosine was found to is ϵ of $6.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and phosphotyrosine is $2.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, a significantly lower ϵ as compared to the former (Fig. 1).

It is possible that the observed identical spectral characteristics with respect to $\lambda_{\text{max}} = 278 \text{ nm}$, is due to the differential ϵ for both tyrosine and phosphotyrosine. This could also be due to the presence of tyrosine in phosphotyrosine solution as an impurity and/or as a consequence of degradation of phosphotyrosine, giving rise to its free tyrosine. Alternatively, the observed low ϵ of phosphotyrosine at 280 nm, as compared to tyrosine, could be due to the quenching of absorbance as a consequence of the presence of the phospho-ester bond in the phenolic group of the former. Accordingly, the purity of the tyrosine and phosphotyrosine were assessed by HPLC analysis (Fig. 2). Both phosphotyrosine and tyrosine were found to have single peaks with retention time of 9.8 and 26.5 min, respectively; thereby confirming the purity. Thus, the possibility that the presence of tyrosine due to degradation or as an associated impurity in phosphotyrosine was ruled out by confirming the purity of the same by HPLC analysis. These observations on the purity of both tyrosine and

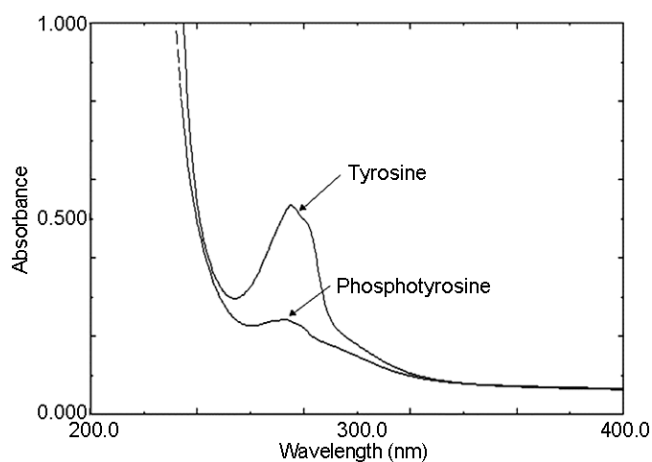


Fig. 1—Spectral characteristics and molar absorption coefficient of tyrosine and phosphotyrosine. The absorption spectral characteristics of an equimolar concentration (50 μM) of tyrosine and phosphotyrosine. Note the identical spectral characteristics ($\lambda_{\text{max}} = 278 \text{ nm}$) with differential molar absorption co-efficient of $\epsilon = 6.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon = 2.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, respectively, for tyrosine and phosphotyrosine.

phosphotyrosine further confirm the established molar absorption coefficient of the amino acids and underscores feasibility of its application to monitor the phosphatase activity. Accordingly, subsequent studies on phosphatase activity using phosphotyrosine as substrate were monitored by measuring the increase in absorbance at 280 nm.

The present study tries to show that phosphotyrosine as a potential substrate compared with p-NPP, a routinely used substrate, in PP2B assay. Although the above observation that molar absorption coefficient of free phenolic group of tyrosine can be used to monitor the phosphatase activity, it is fraught with interference by the presence of UV absorbing modulators in the assay system. To this effect, one needs to establish spectral characteristics and the interference, if any, of TFZ a known inhibitor of PP2B¹⁹. TFZ had spectral characteristics with $\lambda_{\text{max}} = 256$ nm with significant overlapping of absorbance at 280 nm (data unpublished). Further, TFZ was also found to interact with protein solution, resulting in turbidity and rendering it incompatible for monitoring the rise in

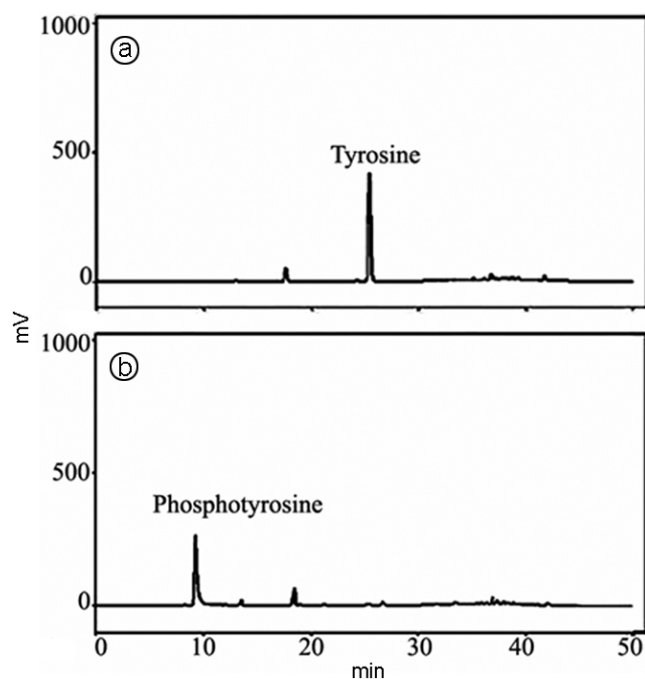


Fig. 2—HPLC pattern of tyrosine (a) and phosphotyrosine (b) to demonstrate the purity of the amino acids used in the study. Note the corresponding retention time of 26.5 and 9.8 minutes. Also note the absence of any peak corresponding to tyrosine in phosphotyrosine chromatogram (b). The minor peaks seen (possible impurities with different retention time) are common to the chromatograms of both the tyrosine and phosphotyrosine.

absorbance at 280 nm as a function of phosphatase activity. Hence, if increase in absorbance at 280 nm could be effectively used to monitor phosphatase activity, one needs to have cognizance of modulators that adversely affect the measurement of enzyme activity.

Effect of metal ions on phosphatase activity—The basal activity of phosphatase on substrates alone was minimal. Hence metal ions were introduced as cofactors for assaying the enzyme activity. Ca^{2+} , Mn^{2+} and Mg^{2+} were used. Presence of Ca^{2+} , but not Mn^{2+} and Mg^{2+} , was found to be a better metal activator for phosphatase activity against phosphotyrosine. However, when p-NPP was used as a substrate, Mn^{2+} proved to be a better activator than Ca^{2+} and Mg^{2+} (Fig. 3). The relative low affinity of Ca^{2+} , in spite of being the native activator of calcineurin to phosphatase activity against p-NPP, suggested what is being measured as phosphatase with p-NPP is unlikely to represent Ca^{2+} dependent phosphatase activity¹³. On the other hand, there is no report on Mn^{2+} dependent protein phosphatases activity in eukaryotes, though there are reports to suggest that Mn^{2+} activates phosphatases in prokaryotes²⁰. Both substrates, in the presence of Mg^{2+} , showed low enzyme activity.

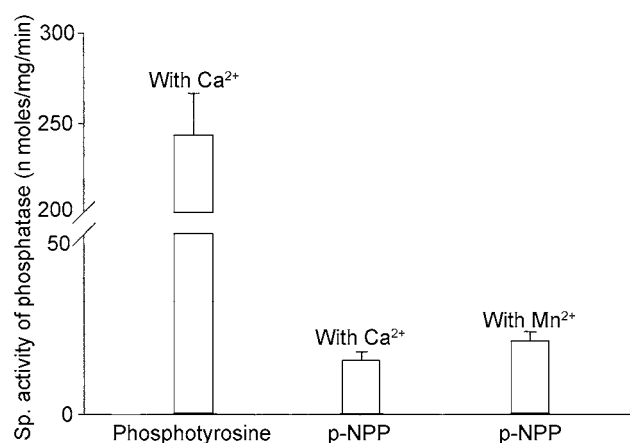


Fig. 3—Phosphatase activity against phosphotyrosine and p-NPP as respective substrates in the presence of different metal ions: Ca^{2+} , Mn^{2+} and Mg^{2+} (data not shown). Increase in release of phenolic tyrosyl residues and phenolate ion as a function of Phosphatase activity was monitored at 280 nm and 405 nm respectively. Phosphotyrosine (100 μM) with Ca^{2+} showed maximum enzymatic activity compared to p-NPP (1 mM) with Ca^{2+} or Mn^{2+} ; thereby confirming phosphotyrosine as a better substrate. Note the relatively higher phosphatases activity against p-NPP in presence of Mn^{2+} (12.7 $\mu\text{mol/mg/min}$) as compared to Ca^{2+} (6.36 $\mu\text{mol/mg/min}$) and Mg^{2+} (3.02 $\mu\text{mol/mg/min}$).

The present study estimated a tenfold increase in the Ca^{2+} activated phosphatase activity against phosphotyrosine when compared to Mn^{2+} activated phosphatase activity against p-NPP. Thus, introducing phosphotyrosine as a potential substrate over p-NPP in measuring the phosphatase activity is crucial.

Effect of modulators on phosphatase activity—The effect of dithiothreitol (DTT) was studied on phosphatase activity against p-NPP in presence of different metal ions. The addition of DTT (1mM) in the assay system has shown increased the phosphatase(s) activity against p-NPP in presence of all the metal activators (Fig. 4A). The positive effect of DTT on phosphatase activity, irrespective of the metal activators added confer with the known role of the -SH groups in phosphatases activity²¹.

Phosphatase activity against p-NPP, in presence of Mn^{2+} and DTT was found to be inhibited in presence of metal chelaters such as EGTA and EDTA (Fig. 4B). This supports the fact that the enzyme activity is dependent on metal activators. On the other hand, the observed total inhibition of phosphatase activity with p-NPP by vanadate appears to be in agreement with the known property of vanadate as a general inhibitor of phosphatase activity.

Effect of calmodulin on phosphatase activity—Calmodulin, a calcium binding protein, in association with Ca^{2+} plays an important role in the regulation of several enzymes including calcineurin². Hence its positive regulatory function was assessed on the phosphatase activity in presence of phosphotyrosine and p-NPP as substrate(s). Calmodulin, for the present study, was prepared in-house from bovine brain, using phenyl sepharose Cl 4B column¹⁵. Purity of the calmodulin was assessed by SDS PAGE, both in presence and absence of Ca^{2+} . Thus, calmodulin preparation had electrophoretic mobility characterized by molecular weight corresponding to 14 kDA in SDS PAGE run with Ca^{2+} and 20 kDA mass of protein in absence of Ca^{2+} (Fig. 5).

The inclusion of calmodulin in the phosphatase assay systems, comprising of different substrates, in presence of Ca^{2+} as the metal activator, clearly demonstrates the increase of phosphatase activity against phosphotyrosine and p-NPP (Fig. 6). However, it is observed that exogenous calmodulin (50 $\mu\text{g}/\text{mL}$) could enhance the phosphatase activity marginally and not several fold increase as one would expect. This discrepancy between what is observed to that of expected value may be due to endogenous calmodulin present in the crude extract of

brain tissue contributing to the basal level; hence, limiting the scope for further activation of phosphatase activity by the exogenous CaM. Possibly, similar studies on a purified calcineurin (free of calmodulin) would provide more confirmatory evidence to suggest the substrate specificity. Nevertheless, these observations suggest that the relative specificity of substrates in the presence of calcium and calmodulin. Thus, the observed findings indicate the use of phosphotyrosine as a better substrate over p-NPP for calcium calmodulin dependent phosphatase assay.

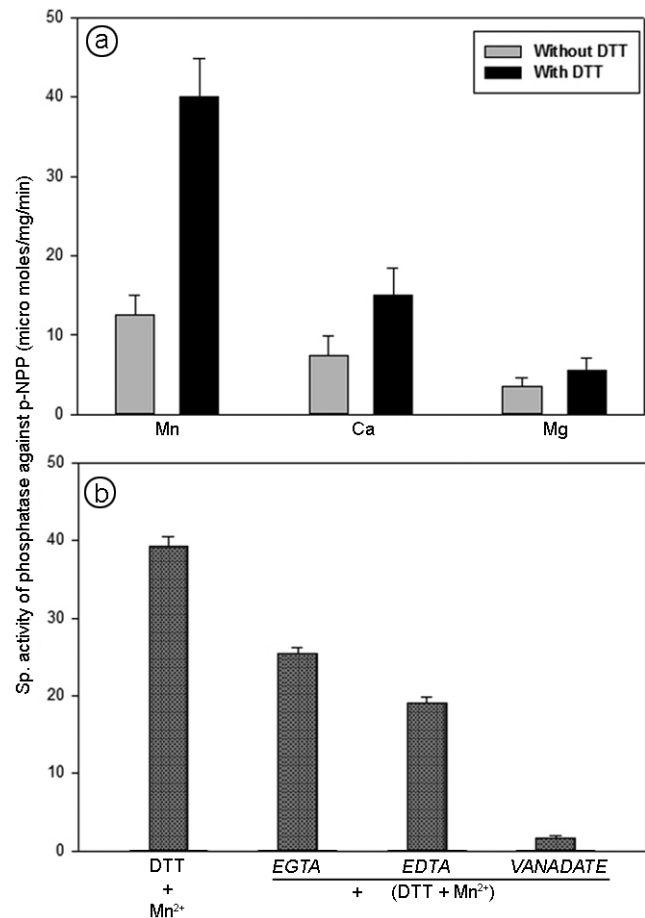


Fig. 4— a: DTT showed a positive increase in phosphatase activity against p-NPP in the presence of Manganese (Mn), Calcium (Ca) and Magnesium (Mg). Note the relatively higher phosphatases activity against p-NPP in presence of DTT, irrespective of the metal ions used in assay system. b: the effect of modulators like EGTA (2.5 mM); EDTA (2.5 mM) and Vanadate (1 mM) on phosphatase activity against p-NPP, in presence of Mn. DTT showed a positive rise in phosphates activity against p-NPP. However, EGTA and EDTA showed a partial inhibition of enzyme activity (25.448, and 19.1 $\mu\text{mol}/\text{mg}/\text{min}$ respectively) by chelating divalent ions. Note the total inhibition of enzyme activity by Vanadate (1.6 $\mu\text{mol}/\text{mg}/\text{min}$), a known inhibitor of protein phosphatases.

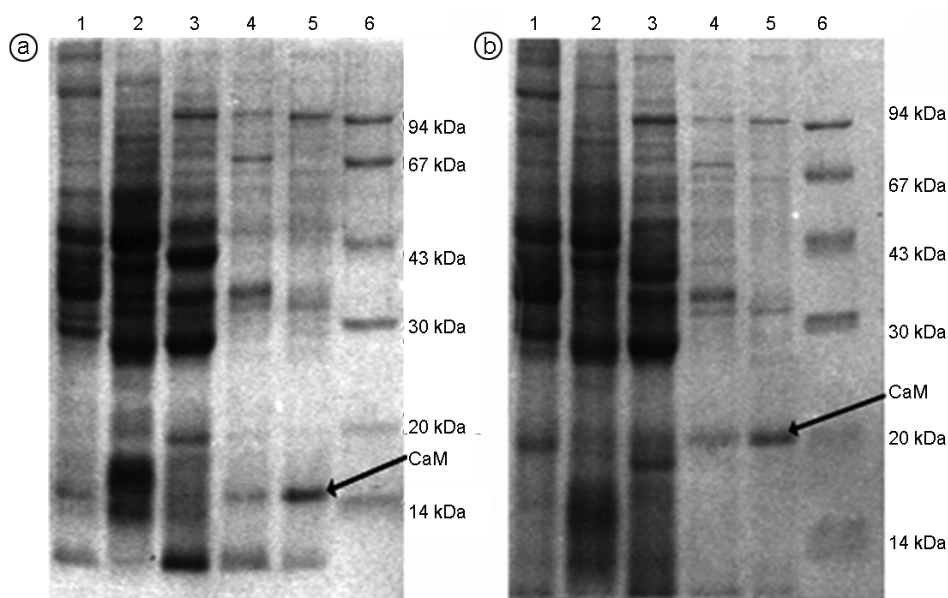


Fig. 5—SDS PAGE profile of Calmodulin (CaM) without (a) and with (b) calcium. [Lane #1: Crude preparation of (P^1 precipitate) CaM; Lanes 2, 3 and 4 represents: Fraction A, B and C; Lane 5: purified CaM; Lane 6: low molecular weight markers (Pharmacia Biotech). The difference in molecular mass of CaM in presence (15 kDa) and absence (20 kDa) of Ca^{2+} is discernible].

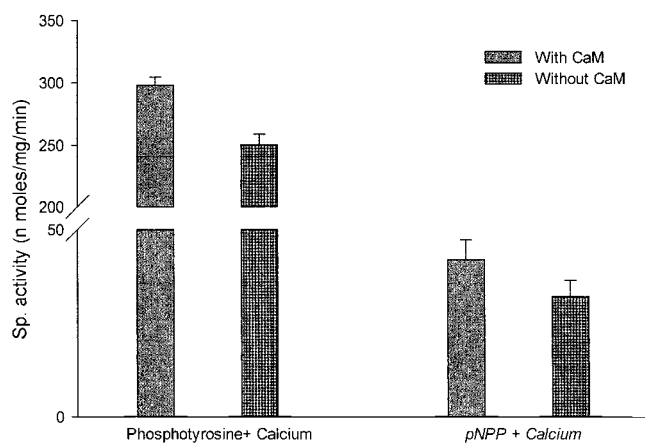


Fig. 6—Effect of calmodulin on phosphatase activity against different substrates: phosphotyrosine and p-NPP in presence of calcium. Phosphotyrosine with Ca^{2+} and calmodulin served as better substrate at lower concentration of 100 μ M than p-NPP at 1 mM in a similar combination. Note the relative increase in the phosphatase activity against all the substrates in presence of CaM.

Conclusion

The present study was successful in finding out that phosphotyrosine as a better substrate over p-NPP for assaying calcium calmodulin dependent phosphatase. There was no considerable increase in phosphatase activity with p-NPP up to 1 mM. A new candidate substrate molecule phosphotyrosine considered for the present study, exhibited significant increase in the activity with 100 μ M. Further, addition of Ca^{2+} and CaM has shown increased phosphatase activity. In

conclusion, phosphotyrosine along with Ca^{2+} and CaM can be adopted in assaying PP2B activity in diagnostic laboratories.

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