

A GDPase/UDPase Bifunctional Enzyme From *Candida Albicans*: Purification and Biochemical Characterization

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Research Article

Keywords: *C. albicans*, Nucleotidases, GDPase/UDPase

Posted Date: December 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1143372/v1>

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Version of Record: A version of this preprint was published at Antonie van Leeuwenhoek on February 17th, 2022. See the published version at <https://doi.org/10.1007/s10482-022-01714-y>.

Abstract

The most frequently isolated human fungal pathogen is *Candida albicans* which is responsible for about 50% of all *Candida* infections. In healthy individuals, this organism resides as a part of the normal microbiota in equilibrium with the host. However, under certain conditions, particularly in immunocompromised patients, this opportunistic pathogen adheres to host cells causing serious systemic infections. Thus, much effort has been dedicated to the study of its physiology with emphasis on factors associated to pathogenicity. A representative analysis deals with the mechanisms of glycoprotein assembly as many cell surface antigens and other macromolecules that modulate the immune system fall within this chemical category. In this regard, studies of the terminal protein glycosylation stage which occurs in Golgi vesicles has led to the identification of nucleotidases that convert glycosyltransferase-generated dinucleotides into the corresponding mononucleotides, thus playing a double function: their activity prevent inhibition of further glycosyl transfer by the accumulation of dinucleotides and the resulting mononucleotides are exchanged by specific membrane transporters for equimolecular amounts of sugar donors from the cytosol. Here, using a simple protocol for protein separation we isolated a bifunctional nucleotidase from *C. albicans* active on GDP and UDP that was characterized in terms of its molecular mass, response to bivalent ions and other factors, substrate specificity and affinity. Results are discussed in terms of the similarities and differences of this nucleotidase with similar counterparts from other organisms thus contributing to the knowledge of a bifunctional diphosphatase not described before in *C. albicans*.

Introduction

Candida species are the most frequent cause of nosocomial fungal infections in U.S. hospitals (Edmond et al. 1999; Wisplinghoff et al. 2004). *C. albicans* is by large the most frequently isolated member of the genus and lives as a commensal on healthy individuals in equilibrium with the host. However, under certain conditions, most commonly in immunocompromised individuals, this opportunistic pathogen becomes invasive causing disseminated candidiasis with a high rate of morbidity and mortality (Corner and Magee 1997; Garcia-Rubio et al. 2020; Kadosh and Mundodi 2020).

The fungal CW exhibits a characteristic composition consisting mainly of b-glucans, chitin and glycoproteins (Gow et al. 2017; Garcia-Rubio et al. 2020). These components do not exist in humans and therefore represent a potential target for antimicrobial drugs. On this basis, interest of this laboratory has dealt with the search of surface antigenic components such as glycoproteins and other components in human pathogenic fungi such as *Candida* and *Sporothrix* species. This has led us to the analysis of glycosyl transferases and glycosidases that participate in glycoprotein biosynthesis (Mora-Montes et al. 2009; López-Romero et al. 2011) and most recently on enzymes that participate in the final stages of glycoprotein assembly such as nucleoside diphosphatases (NDPases).

It is well documented that NDPases convert glycosyltransferase-generated nucleoside diphosphates into monophosphates thus preventing their accumulation and the ensuing inhibition of further sugar transfer

as well as providing substrates that exit the Golgi lumen in exchange for equimolecular amounts of nucleotide sugars from the cytosol by specific antiporters (Abeijon et al. 1989; 1993; D'Alessio et al. 2003, 2005). The presence and function of NDPases has been reported in a number of both higher and lower eukaryotes (Brandan and Fleischer 1982; Yanagisawa et al. 1990; Wang and Guidotti, 1998; Trombetta and Helenius 1999; D'Alessio et al. 2003; Uccelletti et al. 2008; López-Esparza et al. 2013). Much of our knowledge about the roles of NDPases *in vivo* has been obtained through the isolation and phenotypic characterization of mutants encoding these enzymes. For instance, a null mutation in *GDA1*, the gene encoding for a Golgi GDPase in *Saccharomyces cerevisiae*, blocked O- and N-mannosylation of chitinase and carboxypeptidase Y (Abeijon et al. 1993). In the same line, individual disruption of *Spgda1⁺* and *Spynd1⁺* genes coding for a GDPase/UDPase and an apyrase, respectively, did not affect cell viability of *Schizosaccharomyces pombe*, but the double mutant was lethal (D'Alessio et al. 2003). A null mutant of the *Kluyveromyces lactis Klgda1* gene, an ortholog of the *S. cerevisiae* gene *ScGda1* coding for a Golgi UDPase, failed to transport GDP-mannose and UDP-GlcNAc into the Golgi lumen demonstrating the lack of GMP and UMP required as antiporters (López-Avalos et al. 2001). Moreover, the ORF of the *GDA1* gene from the human pathogenic fungus *Sporothrix schenckii* fully restored the GDPase activity in a *gda1* null mutant of *S. cerevisiae* thereby proving the functionality of the ortholog (López-Esparza et al. 2013). A phenotypic analysis of a *C. albicans* mutant lacking a Golgi GDPase indicated that this NDPase is involved in cell wall composition, yeast-hypha transition and O-mannosylation (Herrero et al. 2002). To learn more about nucleotidases in this pathogen, here we report the purification to homogeneity and partial characterization of a membrane-bound enzyme whose properties are compatible with a bifunctional NDPase active on GDP and UDP.

Materials And Methods

Organism and growth conditions

The wild type strain ATCC 26555 of *Candida albicans* from the collection of the Department of Biology, University of Guanajuato, was used in this study. The organism was maintained on slants containing 0.3% yeast extract, 1.0% peptone, 2% dextrose, pH 7.8 (YPD medium) solidified with 2% agar. For propagation, *C. albicans* was first grown at 28 °C in 15-ml conical tubes containing 10 ml of liquid YPD. After 24 h, cells were counted and used to inoculate one-liter Erlenmeyer flasks containing 300 ml of liquid YPD medium at a final density of 1×10^6 cells/ml. Flasks were incubated with rotary shaking (120 rpm) at 28 °C and, unless otherwise indicated, cells were collected after 18 h and processed as described below.

Cell breakage and preparation of the enzyme source

Cells were collected in a filtration unit using MF-Millipore, pore size 0.65 µm (Merck), washed with 5 mM imidazole buffer, pH 7.2 and resuspended in the same buffer containing 2 µM E-64 and 2 mM PMSF (buffer A) to minimize cysteine and serine protease activities, respectively, during breakage. The following next steps were carried out at 4 °C. Yeast cells were disrupted mechanically with glass beads (0.45-0.50

mm in diameter) in a MSK cell homogenizer (Braun Melsungen, Germany) cooled by a stream of liquid CO₂ by alternate 5-min periods of breakage and one-min of cooling until 15 min of breakage were completed. Cell disruption (over 90%) was confirmed by phase contrast microscopy. It should be noticed that imidazole buffer was used only for cell disruption as Tris gave rise to a very foamy homogenate with reduced nucleotidase activity. The whole homogenate (WH) was aspirated with a Pasteur pipette, placed in a 50-ml conical tube and centrifuged at 10,000 x *g* for 15 min. The sediment was discarded. The supernatant was recovered and centrifuged at 25,000 x *g* for 15 min. The pellet was discarded and the supernatant was collected and centrifuged at 100,000 x *g* for 1 h. In some experiments, the supernatant was carefully decanted and kept at 4 °C. The pellet, consisting of a mixed membrane fraction (MMF), was resuspended in buffer A. NDPase activity was determined in the WH, and the soluble (SN100K) and MMF fractions.

Solubilization of nucleotidase activity

The ability of three nonionic detergents to solubilize nucleotidase activity from MMF was tested. Briefly, aliquots (2-4 ml) of the membrane fraction were gently shaken at 4 °C with either 0.5 or 1.0% (v/v) of Lubrol WX, Igepal CA-630 or Triton X-100 (all from Merck). After 30 min, samples were centrifuged at 100,000 x *g* for 1 h. The supernatant, labeled as solubilized fraction (SF), was collected and the pellet was homogenized in a small volume (1-2 ml) of 20 mM Tris/HCl buffer, pH 7.2. Nucleotidase activity was assayed in both cell fractions as described below.

Nucleotidase assay

Nucleotidase activity was determined as described by Yanagisawa et al. (1990). Accordingly, reaction mixtures containing 20 mM Tris-HCl buffer, pH 7.2, 0.1% Tritón X-100, 2.5 mM CaCl₂, 1 mM GDP or UDP and the enzyme fraction (routinely 20 µl) in a total volume of 200 µl were incubated at 37 °C. After 30 min, the reaction was stopped by adding 200 µl of 2% SDS and the amount of released inorganic phosphate was measured spectrophotometrically by the method of Ames (1966) using a standard curve prepared with sodium phosphate. Unless otherwise indicated, specific activity was described as nmol P/min/mg protein. Protein was determined by the method of Bradford (1976) using bovine serum albumin to prepare a standard curve.

Purification of solubilized nucleotidase

Nucleotidase were purified to homogeneity by a simple, two-step procedure. Briefly, a 4-ml aliquot of the SF was layered on top of a column (1 x 10 cm) of DE-52 equilibrated with 20 mM Tris-HCl buffer, pH 7.2, containing 0.05% Triton X-100 and 2.5 mM CaCl₂ (buffer B). The column was washed with the same buffer followed by a 0-2 M discontinuous gradient of NaCl in buffer B. Two-ml fractions were collected and used to measure activity and protein content. Most active fractions were mixed and the pool was subjected to size exclusion chromatography in a column (1 x 81 cm) of Bio-Gel P-100 equilibrated with buffer B. Fractions (1.6 ml) were collected and used to measure activity and protein content.

Analytical electrophoresis and gel staining

Proteins were separated by denaturing 12% polyacrylamide gel electrophoresis in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell (Bio-Rad) according to the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R-250 and silver nitrate. In the first case, gels were shaken in a staining solution containing 10% ammonium sulfate, 0.1% Coomassie Brilliant Blue R-250, 3% orthophosphoric acid and 20% ethanol for 12 h and destained for 2 h with deionized water (DW). In the second case, gels were washed thrice for 5 min with DW and fixed in a methanol:acetic acid solution (50%:15%, by vol) for 20 min. After two consecutive washings for 10 min with 50% methanol and DW, gels were immersed in 0.02% sodium thiosulfate for 90 sec. After washing twice for one min with DW, gels were incubated in darkness with 0.1% silver nitrate for 30 min at 4 °C and washed twice for one min. Protein bands were revealed by shaking the gels in a solution containing 2% sodium carbonate and 0.04% formaldehyde. Revealing was stopped by adding 5% acetic acid for 10 min and gels were placed in DW. With both staining methods, gels were analyzed in a CHEMIDOCTMMP imaging system (Bio-Rad).

Bioinformatics

Bioinformatic analysis was carried out in several platforms including the NCBI (National Center for Biotechnology Information) database, the nonredundant protein sequence database (<https://www.ncbi.nlm.nih.gov/>) and the genetic sequence database of the NIH (National Institutes of Health, USA), where a collection of DNA sequences is available to the public. The analysis of sequence homology of genes coding for guanosine diphosphatase (GDPase) from *C. albicans* was carried out in the UniProtKB/Swiss-Prot (<http://www.uniprot.org/>) database.

Protein sequence analysis

Sequence of guanosine diphosphatase (GDA1) protein for strain SC5314 of *C. albicans* was obtained from the NCBI database in FASTA format, with access numbers CAD18870.1.

Statistics

Unless otherwise indicated, results presented are the average of three independent experiments run in triplicate. The analysis of variance was carried out using the Minitab tool, version 14 for Windows and data are reported as media values. ANOVA was used to compare data and detect significant differences based on statistical degrees of freedom and an error probability of $p \geq 0.05$.

Results

Bioinformatics

A search of GDPase *in silico* as described in Materials and methods revealed a GDA1p sequence of 599 aa with a putative molecular weight of 65.89 kDa and a corresponding pI value of 5.64 (Fig. S1).

Noteworthy, *in silico* search of UDPase protein in *C. albicans* met with failure indicating that genes/proteins associated with the enzyme have not yet been annotated in these organisms.

Growth and activity of NDPases

C. albicans was grown in YPD medium at 28 °C and specific activity on GDP and UDP was determined in the whole homogenate (WH) at the indicated time points in Fig. 1a. Both GDPase and UDPase activities increased as a function of growth reaching a maximum after 18 h corresponding to the exponential phase of growth. Based on these results, further experiments were carried out in exponentially growing cells.

NDPase activity was measured in the cell fractions obtained after differential centrifugation of the WH as described in Materials and methods. As shown in Fig. 1b (inset), the highest and similar activity on both substrates was obtained in the mixed membrane fraction (MMF) isolated after centrifugation of the cell extract at 100,000 x *g* for 1 h. As expected for soluble nucleotidases, a significant difference in activity on GDP and UDP was observed in the SN100K. Average activity in the MMF was 5.6fold higher with respect to the WH.

Solubilization of NDPase activity from MMF

The ability of some non-ionic surfactants to solubilize NDPase activity was tested. Briefly, samples of the FMM (2-4 ml in 20 mM Tris/HCl, pH 7.2) were mixed with either Lubrol WX, Igepal CA-630 and Triton X-100 (all from Sigma) at 0.5 and 1% (by volume). Mixtures were gently shaken at 4 °C and after 30 min, they were centrifuged at 100,000 x *g* for 1 h and the supernatant and pellet were saved. The latter was resuspended in the same initial volume of buffer. NDPase activity was then measured in SF's and the residual MMF as described in Materials and methods. As illustrated in Fig. 2, the best results were obtained with 0.5% Lubrol WX which gave rise to a SF with comparable specific activities of GDPase and UDPase (386.3). Increase of Lubrol WX to 1% reduced soluble activity by 16.5% and 36.5%, respectively. On the other hand, treatment of the membrane fraction with Igepal CA-630 released SF's with specific activities below 75 that were slightly higher for GDPase. Likewise, incubation of MMF with 0.5% or 1% Triton X-100 yielded SF's with activities that ranged between 50 and 100. In all cases, activity in the residual MMF remained at about 60 and below. Based on these results, solubilization of NDPase activity with 0.5% Lubrol WX was chosen for further experiments.

Purification of solubilized NDPases

For isolation of the enzyme protein, the solubilized fraction enriched with NDPase activity was subjected to a simple, two-step protocol involving ion exchange and size exclusion chromatographies. Accordingly, SF (routinely 4 ml) was loaded on a column (1 x 10 cm) of DE-52 equilibrated with 20 mM Tris/HCl buffer, pH 7.2 containing 0.05% Lubrol WX (buffer B). The column was washed with the same buffer until the first protein-containing fraction emerged and the wash eluate was discarded. Then, ten 2-ml fractions were collected before applying a discontinuous gradient of 0 to 2 M NaCl in buffer B. As shown in Fig. 3a,

three peaks containing UDPase were eluted. Peak A appeared at the start of the salt gradient whereas peak B eluted at 0.5 M NaCl. Both A and B peaks co-eluted with smaller peaks of GDPase. An extended, low peak (C) of GDPase-free UDPase activity appeared between fractions 17-21 that was not considered for further analysis. Most active fractions of peaks A and B were pooled and concentrated to 1.0-1.5 ml in a 10-ml Amicon stirred cell (Merck). Further enzyme separation was carried out by size exclusion chromatography. To this purpose, the pool was placed onto a column (1 x 81 cm) of Bio-Gel P-100 equilibrated with buffer B and fractions (1.6 ml) were collected on the basis of the V_e/V_o elution quotient. To avoid contamination with spurious proteins, only the most active, single fraction was collected and used for further analysis. Representative results from several experiments are illustrated in Fig. 3b. UDPase and GDPase co-eluted with a molecular mass of 65 kDa as determined in the same column calibrated with proteins of known MW (not shown).

Electrophoresis

Purity of the enzyme protein was assessed by denaturing SDS-PAGE in 12% gels that were stained with either Coomassie Brilliant Blue or silver nitrate as described in Materials and methods. Results are illustrated in Fig. 4. Stain of the gel with either Coomassie Blue or silver nitrate revealed a single protein of 60.5 kDa. The more sensitive stain revealed two other proteins of 118.4 and 250 kDa. The bifunctional enzyme was by large the most abundant and densitometry indicated a purity of about 90% (Fig. 5).

Characterization of purified NDPase

Effect of ions on enzyme activity and substrate specificity

In most organisms, divalent cations commonly Ca^{2+} , Mg^{2+} and Mn^{2+} affect NDPase activity. For instance, a highly GDP-specific Golgi GDPase from *S. cerevisiae*, required Ca^{2+} for maximal activity and other cations such as Mn^{2+} and Mg^{2+} also activated the enzyme (Yanagisawa et al. 1990). In mammalian cells, a soluble and a membrane-bound GDPase/UDPase have been localized to the ER. The soluble enzyme is active with Ca^{2+} , Mg^{2+} or Mn^{2+} whereas the insoluble enzyme fully depends on Ca^{2+} for activity (Wang and Guidotti, 1998; Trombetta and Helenius, 1999; Failer et al. 2002). Here, activity of the enzyme on GDP increased over 40% in the presence of 5-10 mM Ca^{2+} whereas Mg^{2+} was less efficient and both were inhibitory at concentrations above 10 mM. Mn^{2+} inhibited hydrolysis of GDP at all tested concentrations (Fig. 6a). Activity on UDP was less responsive to Ca^{2+} which stimulated 17% at 5 mM and became inhibitory at 10 mM. Mg^{2+} and Mn^{2+} were inhibitory at all tested concentrations (Fig. 6b). These results led us to determine whether the substrate specificity was influenced by calcium ions. Results shown in Table 1 indicate that in the presence of Ca^{2+} the enzyme showed a relative activity of 100% on GDP and UDP and exhibited 40 and 48% activity on the corresponding monophosphates. The latter activity was not observed in the absence of Ca^{2+} while that on GDP and UDP was slightly reduced. In any case, no activity was observed on adenine or cytosine nucleotides.

Substrate affinity and effect of some phospholipids on enzyme activity

Enzyme activity was measured as a function of increasing concentrations of GDP and UDP. With any nucleotide, enzyme activity followed hyperbolic kinetics (Figs. 7a, b) that was used to estimate the K_m and V_{max} values by the double-reciprocal method of Lineweaver and Burk (Fig. 7c, d). The enzyme exhibited a slightly higher affinity for GDP over UDP as judged for the K_m values of 0.46 and 0.62 nmol, respectively. Corresponding V_{max} values were 33.5 and 25.2 nmol P/min/mg. To determine whether the enzyme required glycerophospholipids for activity, this was assayed in the absence and presence of common phospholipids, either individually or in combination. Though enzyme did not depend on exogenously added phospholipids, these stimulated both activities in comparable ranges, varying from 24.6 (PE) to 25.4 (PI + PE and PI + PE + PG) for GDPase and from 21.4 (PG) to 24.4 (PE) for UDPase (Fig. 8a-h and Table S1).

Discussion

Growth of *C. albicans* was concomitant with a parallel increase of GDPase and UDPase activities which reached maximum levels after 18 h, corresponding to the logarithmic phase of growth. Further processing of 18 h-old cells by differential centrifugation led to the isolation of a high-speed, mixed membrane fraction (MMF) enriched with very similar activities of both diphosphatases. Average enzyme enrichment was about 5.6-fold with respect to the whole homogenate. A significant difference in the activity of the enzymes was observed only in the high-speed soluble fraction. This activity may correspond to the ubiquitous, soluble nucleotidase of 45 kDa, active on GDP, UDP and IDP that prevents accumulation of UDP following reglucosylation reactions involved in glycoprotein folding and quality control in the ER lumen (Trombetta and Helenius 1999).

Non-ionic detergents commonly used to solubilize membrane-bound enzymes include Triton X-100, Igepal CA-630 and Lubrol WX. For instance, a Golgi GDPase from *S. cerevisiae* was purified and characterized after solubilization with Triton X-100 (Yanagisawa et al. 1990). Here, we used these surfactants at 0.5 and 1% to extract diphosphatase activity from the MMF. Best results were obtained with Lubrol WX at 0.5% as it yielded a soluble extract enriched with the same levels of GDPase and UDPase. Solubilization with 1% rendered a less active SF and a slightly diminished UDPase activity with respect to GDPase. These results, altogether with those of cellular distribution, particularly in the MMF, suggested that both diphosphatase activities were associated to a single, bifunctional protein.

Enzyme purification was carried out by a simple procedure involving ion exchange and size exclusion chromatographies. In the first step, enzyme activity consistently separated into two co-eluting peaks: one at the very start of the salt gradient and another at the start of 0.5 M NaCl, suggesting the presence of slightly ionic species. In both peaks, GDPase activity was lower than that of UDPase, a difference probably due to an inhibitory effect of the salt on the hydrolysis of GDP. The extended peak C showing UDPase activity only, was not considered for further analysis. In any case, most active fractions in peaks A and B were pooled and further separated by size exclusion in Bio-Gel P-100. In this case, both diphosphatase activities co-eluted in a sharp, single peak thus reinforcing the idea of a GDPase/UDPase bifunctional polypeptide whose molecular mass was calculated as 65 kDa by column chromatography.

Here, the difference in activity on GDP and UDP was not as marked as in DE-52 probably because of salt elimination. In several experiments, only the most active peak fraction was collected to avoid contamination with spurious proteins flanking the activity peak. This strategy precluded the estimation of enzyme recovery and purification. Purity of the bifunctional protein to homogeneity was assessed by denaturing, analytical electrophoresis in gels stained with either Coomassie Blue or silver nitrate. By this method, molecular mass was calculated as 60.5 kDa and densitometry after staining with silver revealed a purity of 90%. The nature of bands of 118.4 and 250 kDa remains unknown.

The molecular weight (MW) of several reported diphosphatases is variable. Accordingly, GDPase/UDPase's from *S. cerevisiae* (Abeijon et al. 1989) and *Drosophila melanogaster* (Knowles 2011) exhibited MW's of 56 kDa whereas a GDPase/ATPase from *Tetrahymena thermophila* had a molecular mass of 66 kDa (Smith et al. 1997). In *Schizosaccharomyces pombe*, two nucleoside diphosphatases were described: a GDPase/UDPase (Spgda1p) and an apyrase (Spyndp1p) of 61.6 and 64.6 kDa, respectively (Alessio et al. 2003). The molecular mass calculated here for the *C. albicans* GDPase/UDPase is well within the range reported in other organisms.

It is well documented that bivalent ions, commonly Ca^{2+} , Mg^{2+} and Mn^{2+} , are either strictly required or stimulate nucleoside diphosphatase activity. Thus, it has been described that a Golgi GDPase purified from *S. cerevisiae* requires Ca^{2+} for maximum activity whereas Mn^{2+} and Mg^{2+} activate the enzyme (Yanagisawa et al. 1990). In the same line, both a GDPase/UDPase and an apyrase from *S. pombe* required Ca^{2+} , Mg^{2+} or Mn^{2+} for activity (D'Alessio et al. 2003). Likewise, a human Golgi UDPase was stimulated by Ca^{2+} whereas Mg^{2+} and Mn^{2+} had a lower or minimal effect (Wang and Guidotti 1998). A mammalian UDPase associated with the ER and also with pre-Golgi intermediates was strongly activated by Ca^{2+} but was insensitive to Mg^{2+} (Failer et al. 2002). In a report closely related with this study, it was observed that activity of GDPase in *C. albicans* increased 100% in the presence of Mg^{2+} (Herrero et al. 2002), in contrast with *S. cerevisiae* where Ca^{2+} is the preferred cation for GDP hydrolysis (Yanagisawa et al. 1990). Taken together, these contrasting findings reveal that the response of these enzymes to bivalent ions is quite diverse. Here, though these cations were not required for activity of GDPase/UDPase, in several experiments we observed that hydrolysis of GDP was stimulated over 40% by Ca^{2+} whereas Mg^{2+} was less efficient and Mn^{2+} was inhibitory. On the other hand, hydrolysis of UDP was less responsive to Ca^{2+} and Mg^{2+} and Mn^{2+} were inhibitory. At this point, it is worth pointing out whether a bifunctional nucleotidase has a catalytic domain to interact with both GDP and UDP or it exhibits two specific sites, one for each nucleotide. In terms of the structural difference between these diphosphates at the nucleobase level, the second alternative seems more plausible. This would imply a differential response of the hydrolysis of GDP and UDP to external influencers of enzyme activity including bivalent ions and other factors.

It has been described that some NDPases are able to hydrolyze nucleoside mono-, di- and triphosphates and that some cations, commonly Ca^{2+} , may change the substrate specificity (Wang and Guidotti, 1998; Yanagisawa et al. 1990; Failer et al. 2002; D'Alessio et al. 2003). Here, maximum activity on both GDP

and UDP was observed in the presence of Ca^{2+} and a lower but significant breakdown of GMP and UMP was also detected. Hydrolysis of mononucleotides, which did not occur in the absence of the cation, is in contrast with a Golgi GDPase from *S. cerevisiae* that failed to breakdown nucleoside monophosphates in the presence of 10 mM Ca^{2+} (Yanagisawa et al. 1990) and also with the inactivity of a rat liver ER UDPase (Trombetta and Helenius 1999) and a human Golgi UDPase (Wang and Guidotti 1998) on GMP and UMP. In a previous study, we isolated a similar GDPase/UDPase bifunctional enzyme from the mycelial morphotype of *Sporothrix schenckii* that was equally active on GMP and UMP (30 and 35% relative to GDP and UDP, respectively) in the presence but not in the absence of Ca^{2+} (to be published). Here, except for a trace activity on UTP, triphosphates were not substrates for the enzyme indicating the absence of apyrase activity.

Substrate affinity values reported in the literature for NDPases are variable. For instance, UDP was a better substrate than GDP or other NDPs for a human UDPase (Wang and Guidotti, 1998) whereas a GDP/UDPase from *S. pombe* was slightly more active on UDP than on GDP (D'Alessio et al. 2003). In the same line, a bovine liver ER-UDPase acting on UDP and GDP showed an apparent K_m of 0.2-0.5 mM for both NDPs when assayed in the presence of saturating concentrations of Ca^{2+} or Mg^{2+} (Trombetta and Helenius 1999) whereas an insect Ca^{2+} -dependent enzyme and insensitive to Mg^{2+} had a K_m for UDP of 216 μM (Failer et al. 2002). A highly specific Golgi-GDPase purified from *S. cerevisiae* exhibited an apparent K_m for GDP of 0.1 mM and a V_{max} of 4.9 mmol/min/mg (Yanagisawa et al. 1990). Here, assay of enzyme activity as a function of substrate concentration showed hyperbolic kinetics for both GDP and UDP and K_m values revealed a higher affinity for GDP, namely, 0.46 vs 0.62. Corresponding values of V_{max} were 33.5 and 25.2 nmol Pi/min/mg. These values are significantly lower than other reported findings indicating a higher affinity of the enzyme purified in this study for both NDPs.

Finally, the membrane-bound enzyme isolated in this study did not depend on glycerophospholipids for activity, though these increased hydrolysis of GDP and UDP to 21-25% either alone or in combination. This may indicate either that the enzyme is embedded in the membrane with the catalytic site oriented to the lumen of the organelle as it is the case for other NDPases or that its activity does not depend on hydrophobic effectors.

Declarations

Funding Part of this study was supported by project "Análisis comparativo de nucleosido difosfatasa (UDPasa y GDPasa) en *Candida albicans* y *C. parapsilosis*. Convocatoria Institucional de Investigación Científica-2018, DAIP, Universidad de Guanajuato, México.

Acknowledgements Thanks are given to Dirección de Apoyo a la Investigación y al Posgrado, Universidad de Guanajuato for financial support. II) - Apoyo a Niveles II, III y Eméritos en el Sistema Nacional de Investigadores (S.N.I). Authors wish to thank LCAFyS. Francisco J. Alcantar-Aranda for its help in bioinformatic analysis.

Author's contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jaime A. Bibián-García, Lilia M. Almanza-Villegas, Jorge A. Ortiz-Ramírez and C. Cano-Canchola. M. Cuéllar-Cruz and Everardo López-Romero wrote the paper. All authors read and approved the final manuscript.

Availability of data and materials All data generated or analyzed during this study are included in this published article.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The authors confirm that not ethical approval was required as the research in this article involved micro-organisms only.

Consent for publication All authors read and consent to publish this manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Fig. 1 Bibián-García et al. 2021

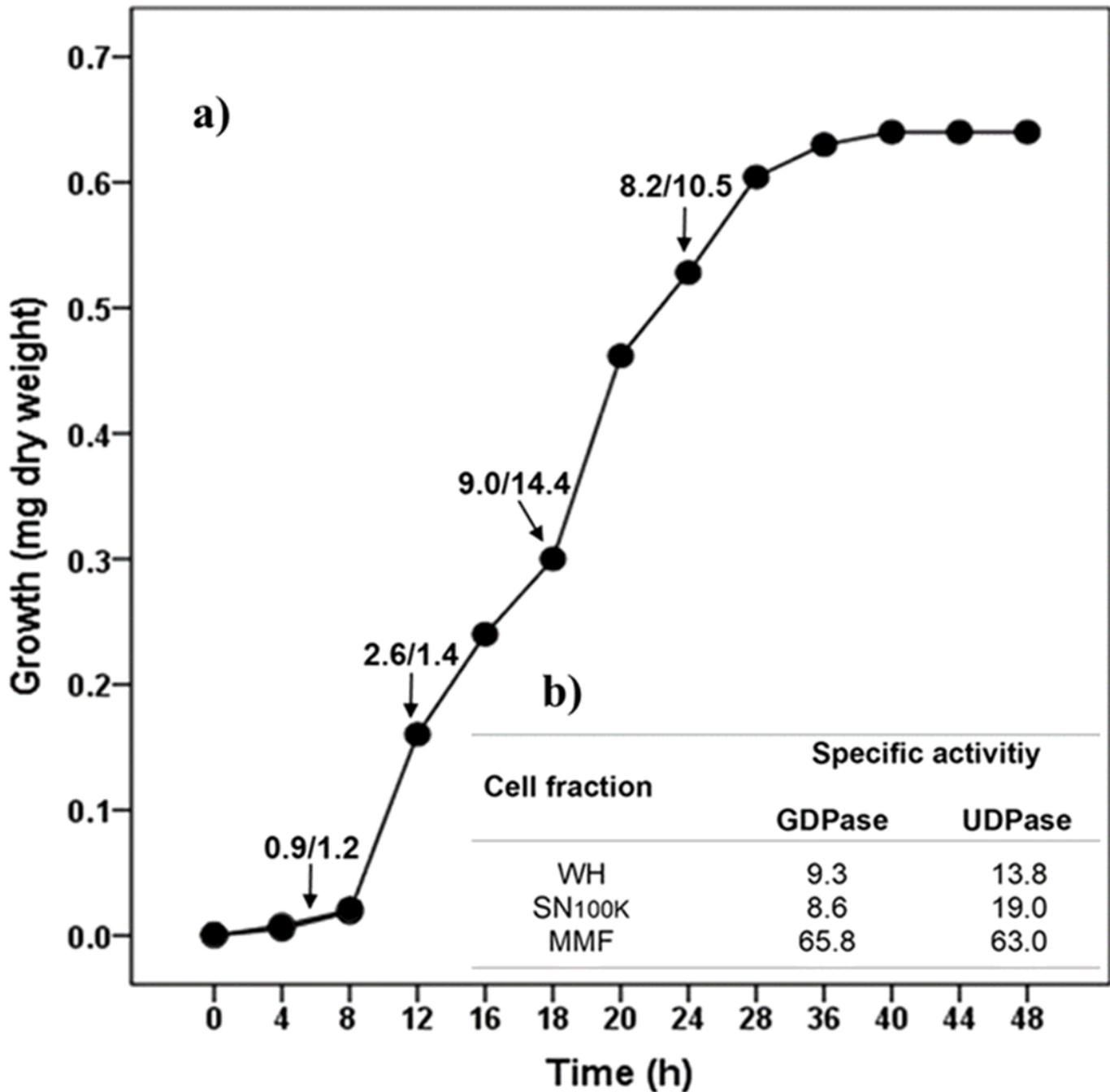


Figure 1

Growth of *C. albicans* ATCC 26555 in YPD medium and nucleotidase activity. One-liter Erlenmeyer flasks containing 300 ml of YPD medium were inoculated with *C. albicans* at a final density of 10⁶ cells/ml and shaken (120 rpm) at 28 °C. Growth was measured at the indicated times. At the indicated time points, cultures were processed as described in Materials and methods to determine specific activity of GDPase/UDPase in the whole cell homogenate (a). In addition, the whole cell extract was subjected to

differential centrifugation and the obtained cell fractions were used to measure protein and hydrolysis of GDP and UDP (b).

Fig. 2 Bibián-García et al. 2021

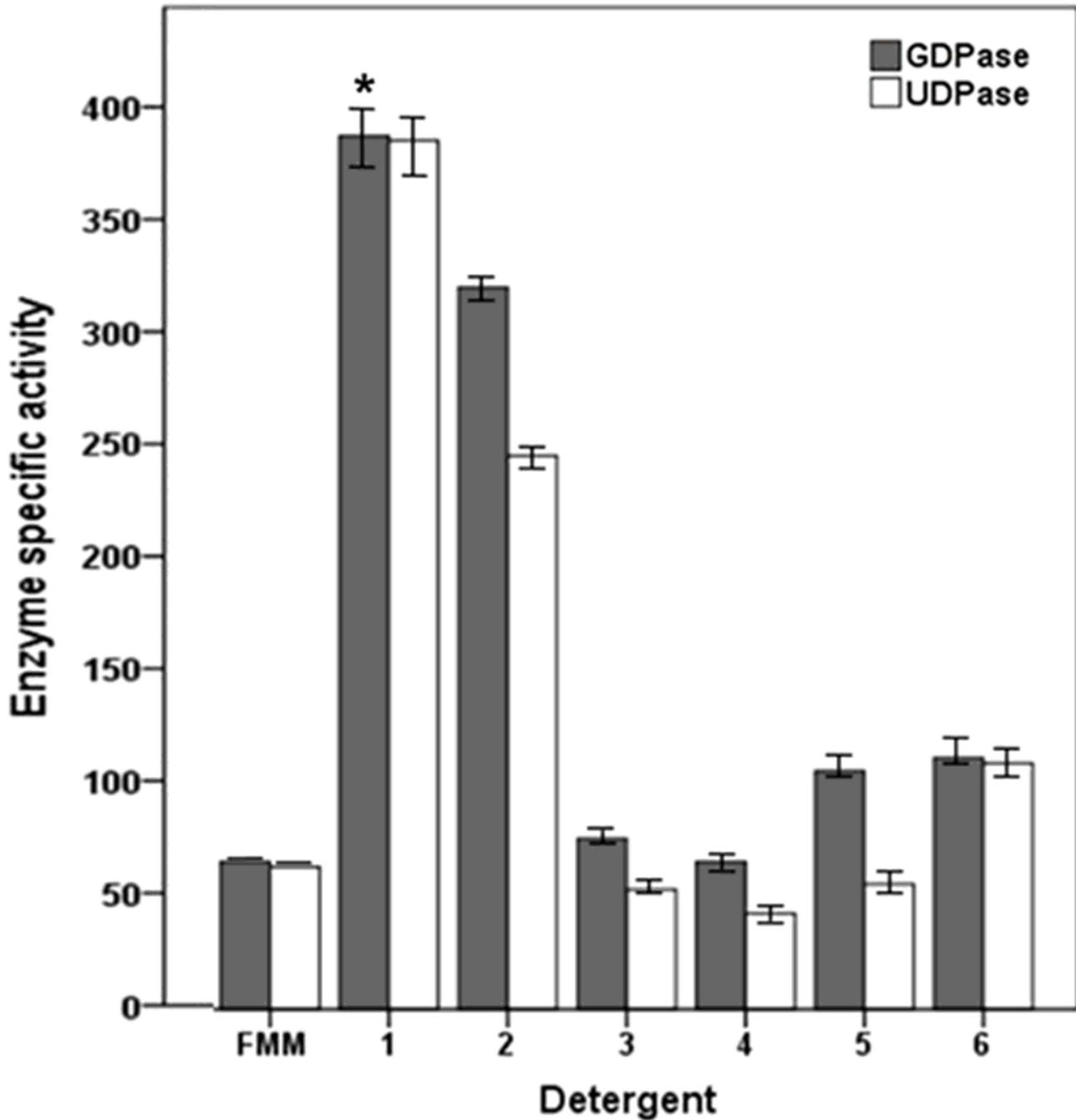


Figure 2

Solubilization of nucleotidase activity. The MMF (2-4 ml) obtained as described in Fig. 1b was mixed with the non-ionic detergents Lubrol WX (1, 2), Igepal CA-630 (3,4) or Triton X-100 (5,6) at concentrations of 0.5% (1,3,5) and 1% (2,4,6) (by vol) and gently shaken at 4 oC. A control MMF sample was mixed with

buffer. After 30 min, mixtures were centrifuged at 100,000 x g for 1 h. The supernatants, labeled as SF's, were collected and used to measure protein and GDPase and UDPase as described in the text.

Fig. 3 Bibián-García et al. 2021

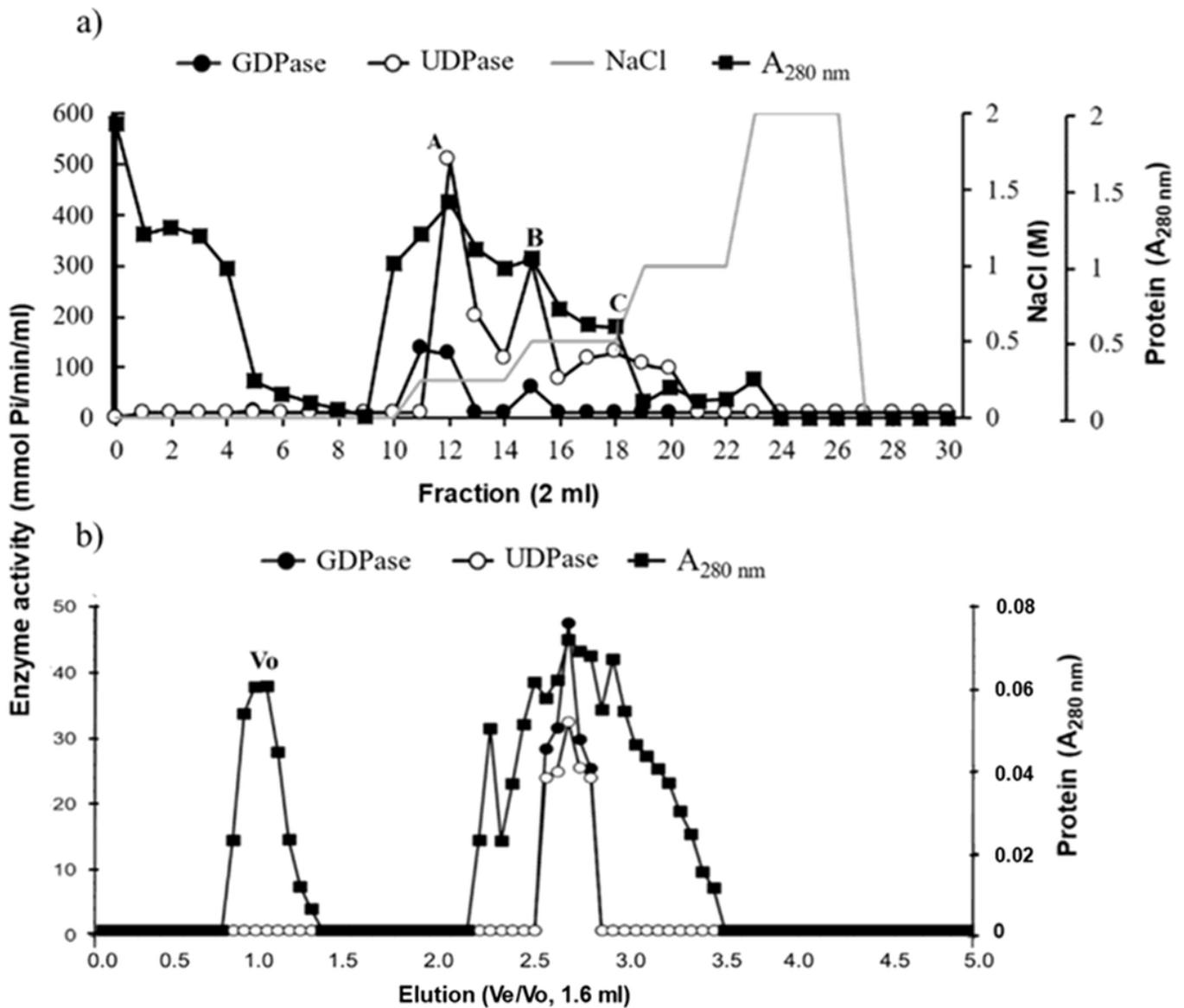


Figure 3

Purification of nucleotidase. A sample (routinely 4 ml) of the SF obtained after extraction of MMF with 0.5% Lubrol WX was layered on top of a column (1 x 10 cm) of DE-52 equilibrated with buffer B and the sample was eluted with the same buffer followed by a discontinuous 0-2 M NaCl gradient. Fractions (2 ml) were collected and used to measure protein and hydrolysis of GDP and UDP (a). Most active fractions of peaks A and B were pooled and the pool was subjected to size exclusion chromatography in a column (1 x 81 cm) of Bio-Gel P-100 equilibrated with buffer B. The sample was eluted with the same buffer and fractions (1.6 ml) were collected according to their V_e/V_o. Fractions were used to measure protein and

hydrolysis of GDP and UDP as described in the text (b). Only the most active fraction enriched with both nucleotidases was collected and saved for further analysis.

Fig. 4 Bibián-García et al. 2021

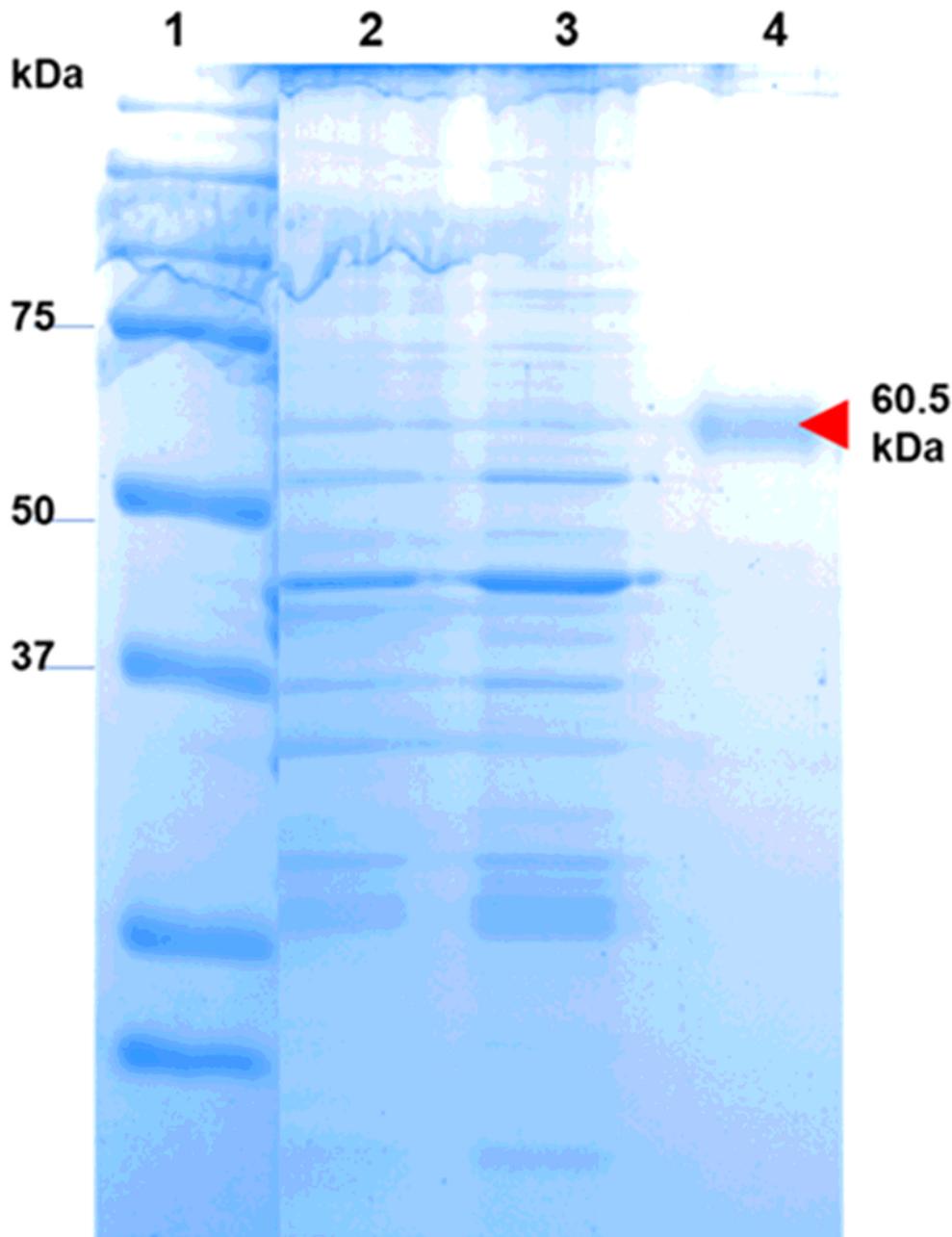


Figure 4

Analytical electrophoresis of nucleotidase. An aliquot of the enzyme separated in Bio-Gel P-100 was subjected to denaturing electrophoresis in 12% polyacrylamide gels of 1.5 mm according to Laemmli

(1970). Gel was processed and stained with Coomassie Brilliant Blue as described in Materials and methods. Bands were analyzed and images were captured in a CHEMIDDOCTMMP imaging system.

Fig. 5 Bibián-García et al. 2021

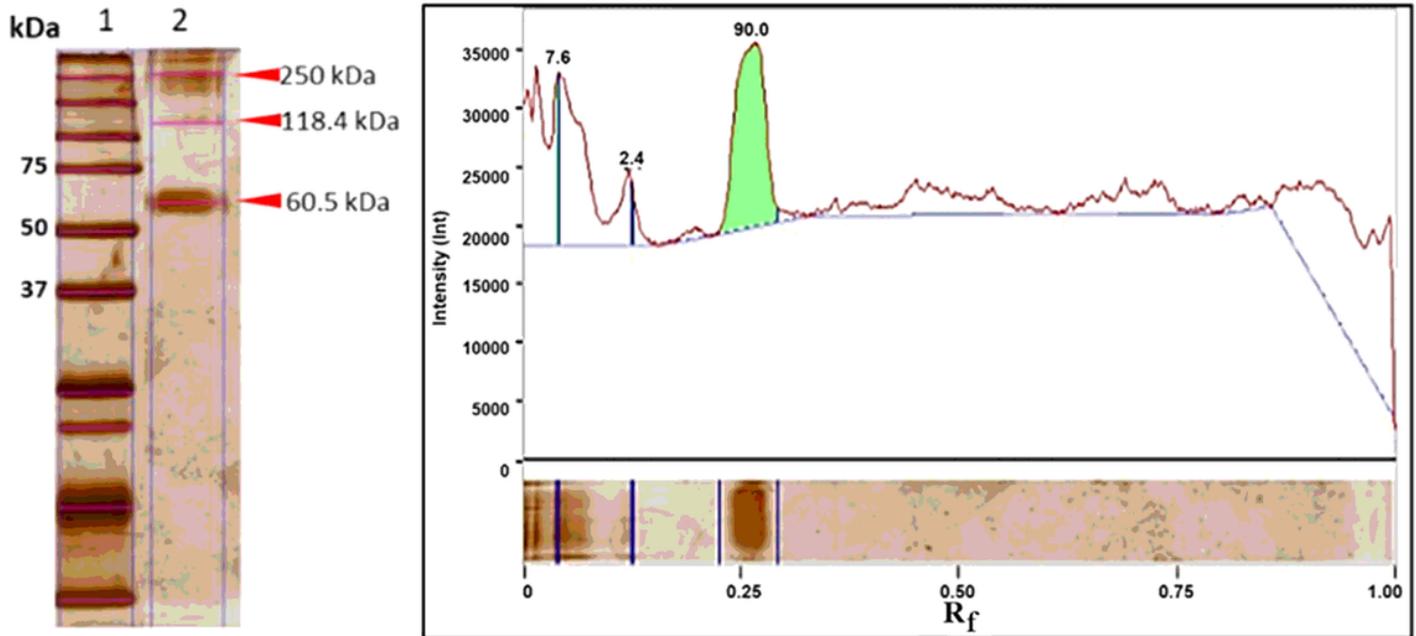


Figure 5

Same as legend of Fig. 4 except that the gel was stained with silver nitrate.

Fig. 6 Bibián-García et al. 2021

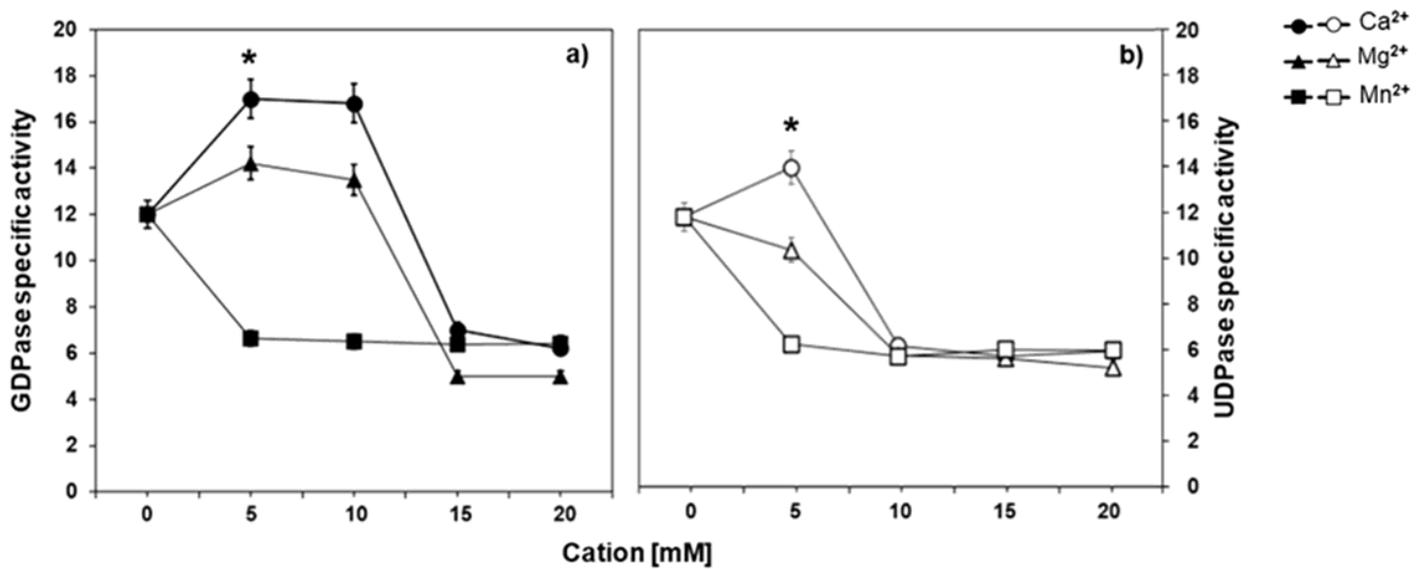


Figure 6

Effect of bivalent ions on the bifunctional nucleotidase enzyme. Hydrolysis of GDP (a) and UDP (b) by the purified nucleotidase was measured in the presence of the indicated concentrations of Ca²⁺, Mg²⁺ or Mn²⁺ as described in the text. All other ingredients of the reaction mixture remained unchanged. Results shown are the average of three independent experiments run in duplicate.

Fig. 7 Bibián-García et al. 2021

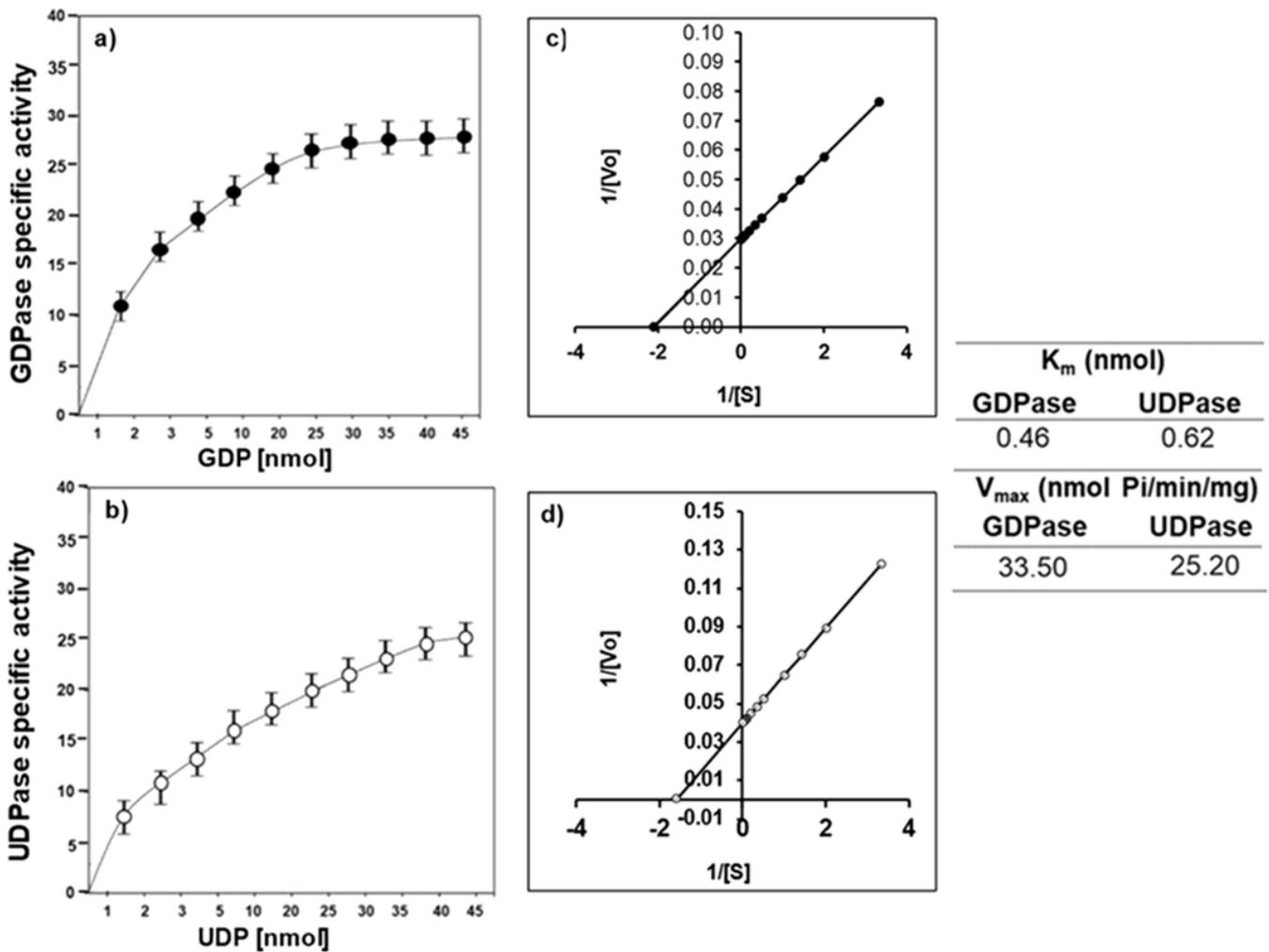


Figure 7

Measurement of nucleotidase activity as a function of increasing concentrations of substrate. Hydrolysis of GDP (a) and UDP (b) was measured at the indicated concentrations of diphosphates. All other ingredients of the reaction mixture remained unchanged. Activity data shown in (a) were used to determine K_m and V_{max} by the reciprocal plots of Lineweaver and Burk, as indicated in (c) and (d) for GDP and UDP, respectively. Values of enzyme constants are compiled in the table at right.

Fig. 8 Bibián-García et al. 2021

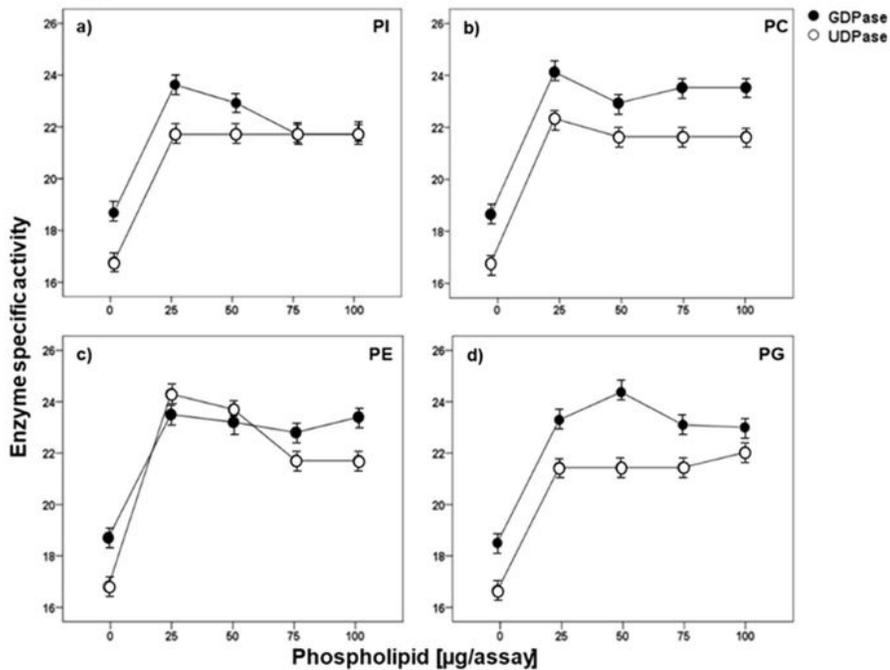


Fig. 8 Bibián-García et al. 2021 Continued...

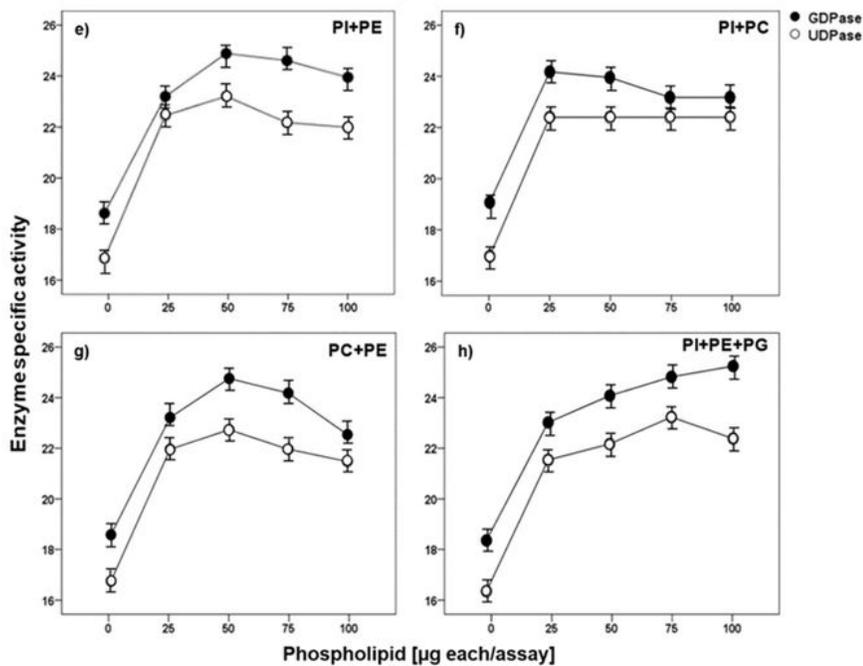


Figure 8

Influence of some common glycerophospholipids on hydrolysis of GDP and UDP by the purified nucleotidase. Hydrolysis of GDP (closed symbols) and UDP (open symbols) was measured in the presence of increasing concentrations of the indicated individual (a-d) and combined (e-h) phospholipids as described in the text. All other ingredients of the reaction mixture remained unchanged. Results shown are the average of two independent experiments run in duplicate.

Supplementary Files

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