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## **Identification of protein-ribulosamine-5-phosphatase as human low-molecular-weight protein-tyrosine-phosphatase-A**

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Abbreviations used: DTT, dithiothreitol; FN3K, fructosamine-3-kinase; FN3K-RP, FN3K-related protein; FN6Pase, fructosamine-6-phosphatase; FN6P, fructosamine 6-phosphate; LMWPTP, low-molecular-weight protein-tyrosine-phosphatase; MDP-1, Magnesium-Dependent Phosphatase-1; pNPPase, *p*-nitrophenyl-phosphatase; RN5Pase, ribulosamine-5-phosphatase; RN5P, ribulosamine 5-phosphate.

Short title: Protein-ribulosamine-5-phosphatase

## SYNOPSIS

Ribulosamines, which are substrates for the deglycating enzyme fructosamine-3-kinase-related protein, are presumably formed intracellularly through glycation of proteins with ribose 5-phosphate followed by dephosphorylation of resulting ribulosamine 5-phosphates by a putative ribulosamine-5-phosphatase. Ribose 5-phosphate is known to be a potent glycation agent and we show in the present work that it reacts  $\approx 10$ - and 80-fold more rapidly with protein than ribose and glucose, respectively. We also show that tissue and most particularly erythrocyte extracts contain a protein-ribulosamine-5-phosphatase. We have purified this enzyme from human erythrocytes to near-homogeneity and shown it to correspond to 'low-molecular-weight protein-tyrosine-phosphatase-A'. Human recombinant low-molecular-weight protein-tyrosine-phosphatase-A displayed a ribulosamine-5-phosphatase activity that was higher than its tyrosine-phosphatase activity, indicating that this phosphatase may participate in protein deglycation, a new form of protein repair.

Key words: glycation, ribose 5-phosphate, ribulosamine 5-phosphate, protein-repair, deglycation, protein-phosphatase.

## INTRODUCTION

Sugars with a free carbonyl group spontaneously react with the amino groups of proteins or low-molecular-weight compounds to form Schiff bases, which slowly rearrange into Amadori products (ketoamines). This reaction, designated glycation, is known to take place *in vivo* with glucose, giving rise to fructosamines. It must also take place with other monosaccharides or sugar phosphates, in particular glucose 6-phosphate and ribose 5-phosphate, which, though usually present at lower concentrations than glucose, are intrinsically more reactive than this hexose [1-4]. It should be noted, however, that the higher reactivity of ribose 5-phosphate has only been demonstrated by measuring the formation of advanced glycation end-products, and that this difference does not necessarily reflect a difference in the rate of early glycation.

Protein glycation is apparently detrimental and it is therefore not surprising that cells have enzymes that repair proteins by removing the accessible glycation adducts. The first deglycating enzyme that has been described is fructosamine-3-kinase (FN3K), which, by phosphorylating fructosamines on their third carbon, causes their destabilisation and shedding

from proteins [5,6]. The physiological occurrence of enzymatic deglycation was demonstrated by showing that FN3K deficiency leads to an increase in intracellular protein glycation [7,8]. FN3K takes care also of the glycation adducts formed from glucose 6-phosphate, after these have been dephosphorylated by a 'fructosamine-6-phosphatase' (FN6Pase) recently identified as Magnesium-Dependent Phosphatase-1 (MDP-1) [9].

FN3K is only found in higher vertebrates, such as mammals and birds. However, many species including mammals [10], birds, fishes [11], plants [12] and bacteria (R. Gemayel, unpublished results), contain a fructosamine-3-kinase homologue (FN3K-RP) that does not act on fructosamines, but only on ketoamines with a hydroxyl group in the D-configuration on C3, most particularly ribulosamines. We previously postulated that ribulosamines are produced by enzymatic dephosphorylation of ribulosamine 5-phosphates (RN5Ps), which form spontaneously from ribose 5-phosphate. The phosphatase involved in this process is, however, unknown. In the present work we have quantified the rate of early glycation by ribose 5-phosphate and identified in human erythrocytes a phosphatase, distinct from FN6Pase/MDP-1, that uses protein-RN5Ps as its best substrate.

## EXPERIMENTAL

### Materials

Reagents, of analytical grade whenever possible, were from Sigma, Acros or Merck. DEAE-Sephacrose, SP-Sephacrose, Q-Sephacrose, Sephacryl S-200, Superdex-200 10/300 GL column, were purchased from Amersham Biosciences; Biogel P2 and AG 50W-X4 (100-200 mesh) were from Bio-Rad. Dowex 1-X8-200 was purchased from Acros and Vivaspin-2 centrifugal concentrators from Vivascience. Radiolabelled compounds were purchased from Amersham Biosciences or American Radiolabelled Chemicals, or synthesised as described in [9]. [1-<sup>14</sup>C]Ribose 5-phosphate was prepared by phosphorylation of [1-<sup>14</sup>C]ribose with 25 mU of human recombinant ribokinase; the product was purified as previously described for [14C]glucose 6-phosphate [9]. FN3K-RP and MDP-1 were prepared as previously described [13,9].

### **Preparation of ribulosamine 5-phosphates**

Lysozyme glycosylated with [ $^{32}\text{P}$ ]ribose 5-phosphate was prepared as previously described for lysozyme glycosylated with [ $^{32}\text{P}$ ]glucose 6-phosphate [9], but the incubation time was reduced to 4 h and Tris was used instead of Hepes. For the preparation of lysozyme glycosylated with unlabelled ribose 5-phosphate, hen egg lysozyme (60 mg/ml) was incubated for 16 h at 50°C with 20 mM ribose 5-phosphate in a medium containing 25 mM Tris, pH 7.1, 1 mM EGTA and 0.5 mM  $\text{AlF}_3$ . The samples were purified on Dowex 1-X8 and Biogel P2 [9] and the concentration of lysozyme was determined [20].

Ribuloselysine 5-phosphate was prepared by incubating a solution (3 ml) containing 200 mM N- $\alpha$ -t-Boc-lysine and 100 mM ribose 5-phosphate for 4 h at 50°C. The t-Boc group was removed by overnight incubation in 1 N HCl at room temperature. The sample was diluted in 12 ml water and loaded on an AG 50W-X4 column ( $\text{H}^+$  form, 3  $\text{cm}^3$ ) equilibrated with water. The column was washed with 12 ml water and 36 ml 0.5 M NaCl. Fractions of 3 ml were collected, and those containing ribuloselysine 5-phosphate (assayed by the inorganic phosphate released by alkaline phosphatase, in the elution fractions) were concentrated under vacuum and desalted on Biogel P2 equilibrated with water.

### **Preparation of tissue extracts and purification of ribulosamine-5-phosphatase**

Tissues were collected from anaesthetized male Wistar rats. They were quick-frozen between Wollenberger clamps cooled in liquid nitrogen and kept at -70°C until used. The frozen tissues were homogenized in a Potter-Elvehjem device with 2 ml/g tissue of a buffer containing 25 mM Tris, pH 7.1, 1 mM dithiothreitol (DTT), 1  $\mu\text{g/ml}$  leupeptin, 1  $\mu\text{g/ml}$  antipain and 10 mM KCl (buffer A). Rat packed erythrocytes were washed twice with a solution containing 150 mM NaCl and 0.5 mM EDTA and once with 150 mM NaCl. 1 volume of washed erythrocytes was diluted in 10 volumes of buffer A. The homogenates and haemolysates were centrifuged for 20 min at 11 000 g and the resulting supernatants were used for enzyme assay. Protein concentration in tissue extracts was determined as described in [14] using bovine  $\gamma$ -globulin as a standard; haemoglobin concentration in erythrocyte extracts was determined by the method of Drabkin and Austie [15].

For the purification of human ribulosamine-5-phosphatase (RN5Pase), 80 ml of packed erythrocytes were washed three times with 150 mM NaCl and diluted in 400 ml of a buffer containing 10 mM Tris, pH 8, 1 mM DTT, 1  $\mu\text{g/ml}$  leupeptin and 1  $\mu\text{g/ml}$  antipain.

The hemolysate was centrifuged for 20 min at 11 000 g. The supernatant (300 ml) was diluted twice in buffer B (20 mM Tris, pH 8, 2 mM DTT, 1  $\mu$ g/ml leupeptin and 1  $\mu$ g/ml antipain) and applied to a DEAE-Sepharose column (200 cm<sup>3</sup>) equilibrated with the same buffer. The column was washed with 400 ml of buffer B and protein was eluted with a 0-0.25 M NaCl gradient in 1000 ml of buffer B. The most active fractions were pooled (55 ml), brought to 200 ml with buffer C (20 mM MES, pH 6, 2 mM DTT, 1  $\mu$ g/ml leupeptin and 1  $\mu$ g/ml antipain), and applied to an SP-Sepharose column (20 cm<sup>3</sup>) equilibrated with buffer C. The column was washed with 50 ml of equilibration buffer and protein was eluted with a 0-0.75 M NaCl gradient in 200 ml of buffer C. Fractions of 3 ml were collected. Three peaks of RN5Pase were found, each co-eluting with *p*-nitrophenyl-phosphatase (pNPPase) activity, one in the flow-through and washing fractions ("peak I"), and two eluted with the salt gradient (peaks II and III). Peak I was concentrated to 35 ml by ultrafiltration in a 200-ml Amicon cell equipped with a PM-10 membrane. This sample was brought to 150 ml with buffer D (20 mM glycine, pH 9, 2 mM DTT, 1  $\mu$ g/ml leupeptin and 1  $\mu$ g/ml antipain) and applied to a Q-Sepharose column (20 cm<sup>3</sup>) equilibrated with the same buffer. The column was washed with 50 ml of buffer D and developed with 200 ml of a 0-0.5 M NaCl gradient. Fractions of 2.5 ml were collected. The most active ones (10 ml) were concentrated to 0.5 ml (with Vivaspins-2) and gel-filtered on a Superdex-200 10/300 GL column equilibrated with buffer E (25 mM Tris, pH 7.1, 1 mM DTT, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml antipain and 100 mM NaCl). Peaks II (18 ml) and III (21 ml) of the SP-Sepharose column were also concentrated and gel-filtered on Superdex-200 equilibrated with buffer E. Fractions of 0.5 ml were collected. The bands co-eluting with the RN5Pase activity in each Superdex-200 chromatography were cut out from a 14% (w/v) polyacrylamide-SDS gel and digested with trypsin or chymotrypsin. Peptides were analyzed by nanoelectrospray-ionisation tandem mass spectrometry as previously described [16].

### **Expression and purification of human low-molecular-weight protein-tyrosine-phosphatases**

The open reading frame of human low-molecular-weight protein-tyrosine-phosphatases (LMWPTPs) A and B (Accession Numbers AAP35800.1 and AAC52065.1), two isoforms generated by alternative splicing [17], were PCR-amplified using Pwo polymerase, human liver cDNA as template, a 5' primer containing the initiator codon (**GCATATGGCGGAACAGGCTACCAAGT**) in an NdeI site (in bold) and a 3' primer

containing the putative stop codon (**CGGATCCTCAGTGGGCCTTCTCCAAGAAC**) flanked by a BamHI site (in bold). The 480-bp PCR-product was subcloned into pBlueScript. Restrictions with BstUI, HaeIII and AvaII were performed to discriminate between the two isoforms, which were checked by sequencing. NdeI-BamHI fragments of each form were removed from pBlueScript and inserted into pET-3a. The resulting plasmids were used to transform *E. coli* BL21(DE3)pLys [18]. The expression and preparation of bacterial extracts were performed as described previously [12]. The extract (12.5 ml) was diluted 3-fold with buffer F (25 mM Tris, pH 7.1, 5 mM DTT, 1  $\mu$ g/ml leupeptin and 1  $\mu$ g/ml antipain) and applied to a DEAE-Sepharose column (12 cm<sup>3</sup>) equilibrated with the same buffer. This column was washed with 25 ml of buffer F and the flow-through and washing fractions of this column were pooled, concentrated and gel-filtered on Sephacryl S-200 (70 cm<sup>3</sup>) equilibrated with buffer F. The fractions containing LMWPTP, as determined by SDS-PAGE analysis, were supplemented with 10% (w/v) glycerol and stored at -70°C. Protein concentration was estimated by measuring  $A_{280}$  assuming extinction coefficients of 18 260 and 15 130 M<sup>-1</sup>cm<sup>-1</sup> for LMWPTP-A and LMWPTP-B, respectively. Starting from 250 ml of culture, we obtained for both isoforms  $\approx$  10 mg of pure protein.

### Assay of phosphatase activities

FN6Pase and *p*-nitrophenyl-phosphatase activities were assayed as previously described [9]. All other enzymatic assays were performed at 30°C in a medium containing 25 mM Tris, 1 mM DTT and the indicated concentration of substrate. Ribuloselysine-5-phosphatase activity was measured through the formation of inorganic phosphate [19]. Activities on <sup>32</sup>P-compounds were determined in 50  $\mu$ l of a solution containing 1  $\mu$ M of phospho-groups (i.e.,  $\approx$  7000 cpm); this corresponded to a protein concentration of 0.5 mM (lysozyme), 15  $\mu$ M (myelin basic protein) and 50  $\mu$ M (haemoglobin). The reactions were stopped by the addition of 1 mg bovine serum albumin (as a carrier) and 100  $\mu$ l of ice-cold 10% (w/v) trichloroacetic acid. The samples were centrifuged for 10 min at 10 000 g and the radioactivity in the supernatant was counted. Unless otherwise stated, RN5Pase activity was measured using lysozyme glycosylated with [<sup>32</sup>P]ribose 5-phosphate as a substrate (tested at 1  $\mu$ M of phospho-groups).

## Phosphorylation by FN3K-RP of the dephosphorylation products of LMWPTP

Lysozyme glycosylated with ribose 5-phosphate (5 mg/ml) was incubated for 10 min at 37°C in 20  $\mu$ l of a solution containing 25 mM Tris, pH 7.1, 1 mM DTT and 20  $\mu$ g/ml LMWPTP-A. The samples were then incubated in 50  $\mu$ l of the same medium containing 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, 100  $\mu$ M ATP·Mg,  $10^6$  cpm [ $\gamma$ - $^{32}\text{P}$ ]ATP and 60  $\mu$ g/ml spinach leaf ribulose-3-kinase [12] or 280  $\mu$ g/ml human erythrocyte FN3K-RP [13]. After the indicated times, aliquots (12  $\mu$ l) were spotted on P81 papers, washed and counted for radioactivity as described previously [5].

## RESULTS

### Glycation of proteins with ribose 5-phosphate

To get a better appraisal of the relative reactivity of ribose 5-phosphate and other sugars in early glycation, we measured the incorporation of  $^{14}\text{C}$ -labelled ribose 5-phosphate, ribose and glucose 6-phosphate into lysozyme and albumin. As shown in Figure 1, ribose 5-phosphate reacted with lysozyme and albumin at rates that were 10- and 6-fold higher than ribose, and 15- and 24-fold higher than glucose 6-phosphate, respectively. As glucose 6-phosphate is itself 3- to 8-fold [9] more powerful than glucose as a glycating agent, we calculated that ribose 5-phosphate is about 80-fold more reactive than glucose towards proteins, being therefore a very significant glycating agent (see Discussion).

### Tissue distribution and purification of ribulose-5-phosphatase

As RN5Ps are not substrates for FN3K-RP (see below), we searched for an enzyme able to dephosphorylate RN5Ps in tissues and erythrocyte extracts. For this purpose, we used a radiolabelled substrate obtained by incubating lysozyme with [ $^{32}\text{P}$ ]ribose 5-phosphate. As shown in Figure 2, this substrate (tested at a concentration of phospho-group of 1  $\mu$ M) was dephosphorylated in all rat tissue extracts that were tested. Interestingly, the highest activity was observed with extracts derived from erythrocytes, a cell type in which both FN3K-RP and FN3K are particularly active [11]. In all cases, the enzymatic activity was >90% inhibited by 50 mM Hepes (a property of LMWPTP, see below).

The purification of ribulose-5-phosphatase (RN5Pase) was started by fractionation of a human erythrocyte lysate on DEAE-Sepharose at pH 8. RN5Pase was eluted



from this column as a single peak, which was separated from the peak of FN6Pase/MDP-1 but coincided with the peak of *p*-nitrophenyl-phosphatase (pNPPase) activity (Figure 3 A). Unlike FN6Pase/MDP-1, RN5Pase was not dependent on the presence of  $Mg^{2+}$  (not shown) but was inhibited by Hepes ( $IC_{50} = 1.2$  mM). Upon further purification on SP-Sepharose at pH 6 (Figure 3 B), about 60% of the RN5Pase activity was found in the flow-through and washing fractions ("peak I"), and the rest was eluted as two distinct peaks with  $\approx 200$  (peak II) and  $\approx 300$  mM NaCl (peak III). Peaks II and III were further purified by gel-filtration on Superdex-200. In both cases, the enzyme co-eluted with an 18-kDa polypeptide, as determined by SDS-PAGE analysis (Figure 4 B, C, E and F). This 18-kDa polypeptide was the only band present in some of the active fractions of the gel-filtration column on which peak III had been loaded. The 18-kDa bands derived from peaks II and III were submitted to chymotrypsin digestion and analysed by electrospray ionisation-tandem mass spectrometry. This led in both cases to the identification of two peptides matching residues 40-55 and 117-132 of low-molecular-weight protein-tyrosine-phosphatase-A (LMWPTP-A) (Figure 4 G). RN5Pase from peak I of the SP-Sepharose column was purified by chromatography on Q-Sepharose at pH 9 and Superdex-200. SDS-PAGE analysis of the fractions obtained at this stage revealed the presence of several bands co-eluting with the enzymatic activity (Figure 4 A and D), including an 18-kDa band that was also identified as LMWPTP-A (Figure 4 G). The other bands found in these fractions were digested with trypsin and mass spectrometry analysis led to their identification as (1) phosphohistidine phosphatase (NP\_054891), (2) thioredoxin (NP\_003320) and (3) SH3-domain binding glutamic acid-rich protein (NP\_112576).

### Expression and substrate specificity of LMWPTPs

Human LMWPTP-A and LMWPTP-B, which differs from LMWPTP-A by an internal sequence due to an alternative splicing event [21,17], were overexpressed in *E. coli* and purified to near-homogeneity by chromatography on DEAE-Sepharose and gel-filtration on Sephacryl S-200 (not shown). The activities of both recombinant proteins and of FN6Pase/MDP1 were tested on  $^{32}P$ -labelled RN5Ps bound to hen egg lysozyme or bovine haemoglobin, on fructosamine 6-phosphates (FN6Ps) bound to hen egg lysozyme and on phosphotyrosine bound to myelin basic protein. All substrates were used at the same, very low concentration of phospho-groups ( $1 \mu M$ ). As illustrated in Table 1 and Figure 5, RN5Ps (whether bound to lysozyme or to haemoglobin) were the best substrates for both forms of LMWPTP, followed by protein-phosphotyrosine and FN6Ps, whereas a different substrate

preference (FN6P >> RN5P > P-Tyr) was observed with FN6Pase/MDP1. Despite its high activity on protein-bound RN5Ps, LMWPTP-A was unable to dephosphorylate completely protein-RN5Ps : RN5Ps bound to lysozyme and haemoglobin were dephosphorylated to extents of 75% (Figure 5) and 55% (not shown), respectively. This suggests that some of the RN5P residues are inaccessible to the phosphatase. None of the two forms of LMWPTP enhanced the decomposition rate of ribulosamine 3-phosphates bound to lysozyme, indicating that they did not act as ribulosamine-3-phosphatases (not shown).

Interestingly, LMWPTP-A acted much better than LMWPTP-B on all four protein substrates (Table 1) as well as on free ribuloselysine 5-phosphate (activities at 1 mM :  $1.6 \pm 0.14$  *versus*  $0.08 \pm 0.01$   $\mu\text{mol/min/mg}$ ,  $n = 3$ ), whereas a similar activity was found when *p*-nitrophenylphosphate was used as a substrate (activities at 1 mM :  $4.7 \pm 0.35$  *versus*  $4.9 \pm 0.49$   $\mu\text{mol/min/mg}$ ,  $n = 3$ ).

### Conversion of ribulosamine 5-phosphates to substrates for FN3K-RP

Protein-bound RN5Ps were neither phosphorylated by human FN3K-RP, nor by its spinach leaf homologue, as indicated by the lack of incorporation of radioactivity from [ $^{32}\text{P}$ ]ATP into protein (Figure 6). As ribulosamine 3,5-bisphosphates are most likely more labile than ribulosamine 3-phosphates, we also measured the formation of [ $^{32}\text{P}$ ] inorganic phosphate under the same conditions and found it to be undetectable. This confirmed the absence of phosphorylation of RN5Ps by both ketoamine-3-kinases. As expected, preincubation of lysozyme-bound RN5Ps with LMWPTP-A led to the formation of a substrate for both plant and human ribulosamine-3-kinases (Figure 6).

## DISCUSSION

Previous work has shown that ribose 5-phosphate is a potent glycating agent [4]. However, the technique used to measure the glycation rate—the formation of UV absorbing material—does not allow one to make a precise comparison of the reactivity of different sugars, because the formation of UV-absorbing material involves reactions downstream of the formation of Amadori products and therefore largely depends on the reactivity of the Amadori products themselves. The present work shows that ribose 5-phosphate reacts with proteins about 10-fold more rapidly than ribose and 80-fold more rapidly than glucose. This higher reactivity was obtained with freshly prepared, purified radiolabelled compounds, and identical results

were obtained with [ $^{14}\text{C}$ ] and [ $^{32}\text{P}$ ]ribose 5-phosphate (not shown). This indicates that there was no interference of contaminating, highly reactive radioactive impurities, which could potentially lead to an overestimation of the glycation rate [22].

Since the intracellular ribose concentration appears to be  $< 10 \mu\text{M}$  (unpublished results) as compared to  $6\text{--}45 \mu\text{M}$  for ribose 5-phosphate [23,24], the much higher reactivity of ribose 5-phosphate compared to ribose indicates that ribulosamines most likely mainly arise from ribose 5-phosphate. At a (physiological) concentration of  $25 \mu\text{M}$ , ribose 5-phosphate has a glycating power amounting to about 40% of that of 5 mM glucose, which indicates that it is indeed a significant glycating agent. It may therefore make sense to have a system allowing the deglycation of RN5Ps.

FN3K-RP and its plant homologue are devoid of RN5P-3-kinase activity. A phosphatase is therefore needed to convert RN5Ps to ribulosamines. We show in the present work that such a phosphatase is present in mammalian tissues and erythrocytes. The human erythrocyte enzyme was purified and identified as LMWPTP-A. This identification was based on mass spectrometry analysis of purified RN5Pase, on the finding that like LMWPTP it is not dependent on  $\text{Mg}^{2+}$  but is inhibited by Hepes [25], and on the demonstration that recombinant LMWPTP-A acts as a RN5Pase. Erythrocyte LMWPTP-A separated into three peaks upon chromatography on SP-Sepharose. This is possibly the result of genetic heterogeneity (a common polymorphism replaces Gln105 by Arg, [26]), post-translational modification, oligomerization or association with other proteins.

LMWPTP was initially identified as a phosphatase acting on low-molecular-weight substrates, most particularly FMN and *p*-nitrophenylphosphate, but very poorly on sugar phosphates [27]. Later work showed it to act also as a protein-tyrosine-phosphatase, but not as a protein-serine/threonine-phosphatase. This, together with the presence of a sequence motif reminiscent of tyrosine phosphatases, led to this enzyme being renamed 'low-molecular-weight protein-tyrosine-phosphatase'. We now show that this enzyme acts particularly well as a protein-RN5Pase. Interestingly, this substrate is structurally similar to one of the best low-molecular-weight substrates, FMN, which contains a ribitol 5-phosphate portion. The crystal structure of LMWPTP shows it to have a deep and narrow catalytic pocket [28,29,25]. This is presumably why this enzyme is unable to remove phosphate groups bound to the first atom of an amino acid side-chain (serine and threonine phosphates), but that it is well active on tyrosine-phosphates and RN5Ps, where the phosphate is bound to the 5<sup>th</sup> and 10<sup>th</sup> atom of the amino acid side-chain, respectively. The low activity of LMWPTP on fructosamine 6-phosphates is most likely due to the fact that the latter are essentially under their furanose

forms [30], which are probably too bulky to enter the catalytic site. By contrast, RN5Ps are unable to cyclize. The lack of action of LMWPTP on ribulosamine 3-phosphates is presumably also related to the narrowness of the catalytic site, which is probably unable to accommodate a secondary phosphate group when the neighbouring carbons bear a hydroxyl group. The lack of ribulosamine-3-phosphatase activity is functionally important : its presence would prevent deglycation to take place since the latter involves phosphate elimination.

LMWPTP is unable to dephosphorylate all the RN5Ps that form on a protein that has reacted with ribose 5-phosphate. Similar observations have been made with other proteins that are *bona fide* protein-repair enzymes such as FN3K [31] and protein-isoaspartyl-methyltransferase [32]. This is most likely due to a problem of accessibility of some of the modified residues to these enzymes, as was previously discussed for fructosamine-6-phosphatase/MDP-1 [9].

In mammals and birds, two different forms of LMWPTP exist, which differ from each other by an internal sequence comprising residues 40-73 that border the catalytic cleft [21,26]. We found that the A form is intrinsically much better than the B form in its action as a RN5Pase both on low-molecular-weight and protein-bound substrates, but that this is not the case for *p*-nitrophenylphosphate. This difference may possibly be due to the presence in LMWPTP-A of a glutamate (Glu50, replaced by Asn in the B-form), which could potentially form an ionic pair with the amino group of RN5Ps.

FN3K-RP is widely distributed in mammalian tissues and is particularly active in erythrocytes, a cell type with no protein renewal and in which therefore protein-repair may be particularly critical. The tissue distribution of RN5Pase indicates that it is also widely distributed in tissues, consistently with other studies [33,34], and particularly active in erythrocytes. Although we do not know at this stage if this high activity reflects or not a high relative content of LMWPTP-A, it is consistent with the idea that LMWPTP has a role in protein maintenance besides its role in cell signalling.

Further work is needed to appreciate the physiological importance of the dephosphorylation of RN5Ps as a protein repair mechanism. The identification of an enzyme that dephosphorylates RN5Ps is an important step in this endeavour.

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## LEGENDS TO THE FIGURES

### Figure 1 Glycation of proteins with ribose 5-phosphate, ribose or glucose 6-phosphate

Hen egg lysozyme (A) or bovine serum albumin (B) were incubated with [ $^{14}\text{C}$ ]ribose 5-phosphate (R5P; *triangles* and *squares*), ribose (*circles* and *diamonds*) or glucose 6-phosphate (G6P; *cross*) and the indicated concentrations of unlabelled compound. After different times, the radioactivity bound to proteins was measured, as described in [9]. Means  $\pm$  S.E.M. ( $n = 3$ ).

### Figure 2 Tissue distribution of ribulosamine-5-phosphatase

The enzymatic activity was determined on crude extracts (1-3 mg protein/ml in the assay), using lysozyme glycosylated with [ $^{32}\text{P}$ ]ribose 5-phosphate as a substrate (tested at 1  $\mu\text{M}$  of phospho-group). Conditions used for these assays were such that the activity was proportional with time and with extract concentration and that less than 20% of the protein-RN5Ps were dephosphorylated. The results are the means  $\pm$  S.E.M. for independent determinations performed on samples from 3 different animals. They are expressed as the activity per mg of protein or haemoglobin (in the case of erythrocytes).

### Figure 3 Purification of ribulosamine-5-phosphatase from human erythrocytes

(A) A human erythrocyte extract was applied to a DEAE-Sepharose column and a linear NaCl gradient was applied. A $_{415}$  (*circles*), RN5Pase (*squares*), pNPPase (*diamonds*) and FN6Pase (*triangles*) were assayed. The flow-through and washing fractions (1000 ml; not shown) contained 17 mg/ml hemoglobin and less than 10% of the total RN5Pase activity. (B) RN5Pase was further purified on SP-Sepharose at pH 6. Three peaks of phosphatase were found, one in the flow-through and washing fractions (I) and two eluted with the NaCl gradient (II and III).

### Figure 4 Further purification of the three peaks of RN5Pase on Superdex-200 and co-purification with an 18-kDa polypeptide

The three peaks resulting from the SP-Sepharose chromatography were further purified on Q-Sepharose (peak I only; not shown) and Superdex-200. Protein concentration (*circles*) and RN5Pase activity (*squares*) were measured (A, B and C), and the fractions were analysed by SDS-PAGE (D, E and F). The arrows (and numbers) show the bands that were cut out from



the gel, digested with chymotrypsin (or trypsin) and analysed by tandem mass spectrometry. The sequence of human LMWPTP-A and the variable region corresponding to LMWPTP-B are shown; the identified peptides are *underlined* (G).

### Figure 5 Dephosphorylation of protein substrates by human LMWPTP-A

<sup>32</sup>P-labelled RN5Ps (*triangles* and *diamonds*), fructosamine 6-phosphates (*circles*) and tyrosine-phosphates (*squares*) were tested as substrate (at 1  $\mu$ M of phospho-groups) with the indicated concentrations of human recombinant LMWPTP-A.

### Figure 6 Formation of a substrate for FN3K-RP upon incubation of lysozyme with ribose 5-phosphate and dephosphorylation by LMWPTP-A

Lysozyme glycosylated with ribose 5-phosphate was incubated for 10 min with (*closed symbols*) or without (*open circles and squares*) LMWPTP-A (20  $\mu$ g/ml). The samples were then incubated with [ $\gamma$ -<sup>32</sup>P]ATP and human (*circles*) or spinach leaves (*squares*) FN3K-RP or without kinase (*triangles*). Unglycosylated lysozyme was also incubated with LMWPTP-A and with human (*open triangles*) or spinach leaves (*open diamonds*) FN3K-RP. The phosphorylation levels were measured after different times. Means  $\pm$  S.E.M. ( $n = 3$ ).

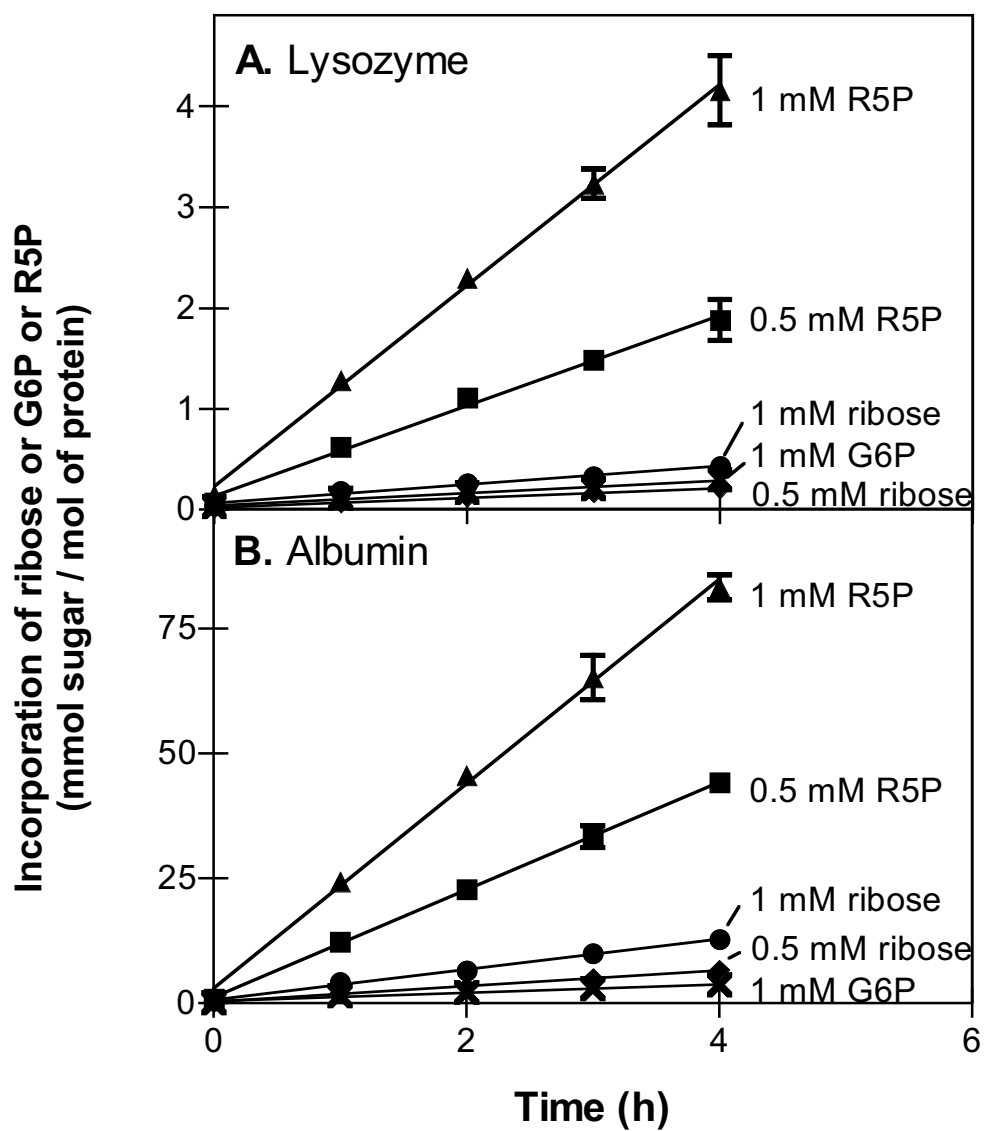
**Table 1 Substrate specificity of human LMWPTPs and MDP-1**

The activities were determined with pure recombinant enzymes. Means  $\pm$  S.E.M. ( $n = 3$ ) or individual values.

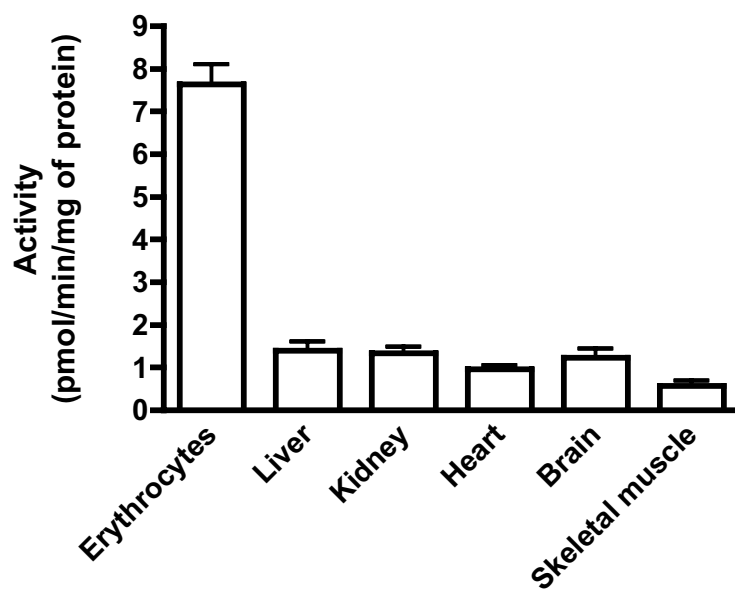
Substrate	Specific activity <sup>a</sup> (nmol/min/mg)		
	LMWPTP-A	LMWPTP-B	MDP-1/FN6Pase
Lysozyme glycated with [ <sup>32</sup> P]ribose-5P	5.95 $\pm$ 0.005	0.28 $\pm$ 0.007	1.46 $\pm$ 0.156
Lysozyme glycated with [ <sup>32</sup> P]glucose-6P	0.26 $\pm$ 0.121	0.04 $\pm$ 0.001	10.8 $\pm$ 0.298
Myelin basic protein-[ <sup>32</sup> P]P-Tyr	0.93 $\pm$ 0.038	0.14 $\pm$ 0.014	0.31 $\pm$ 0.049
Hemoglobin glycated with [ <sup>32</sup> P]ribose-5P	2.63 ; 3.05	0.66 ; 0.86	1.16 ; 1.29

<sup>a</sup>At 1  $\mu$ M of phospho-groups.

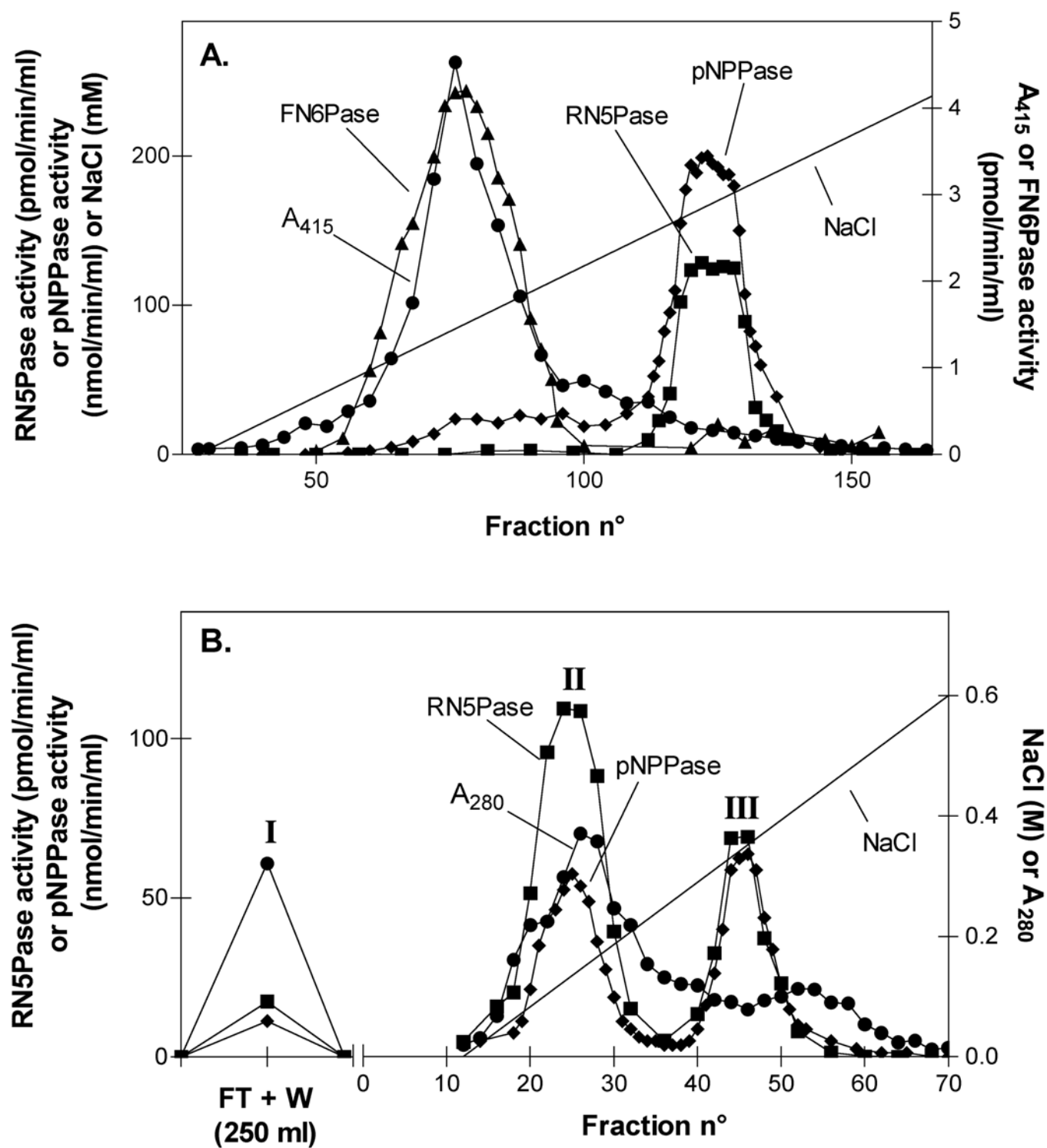
**Fig. 1**



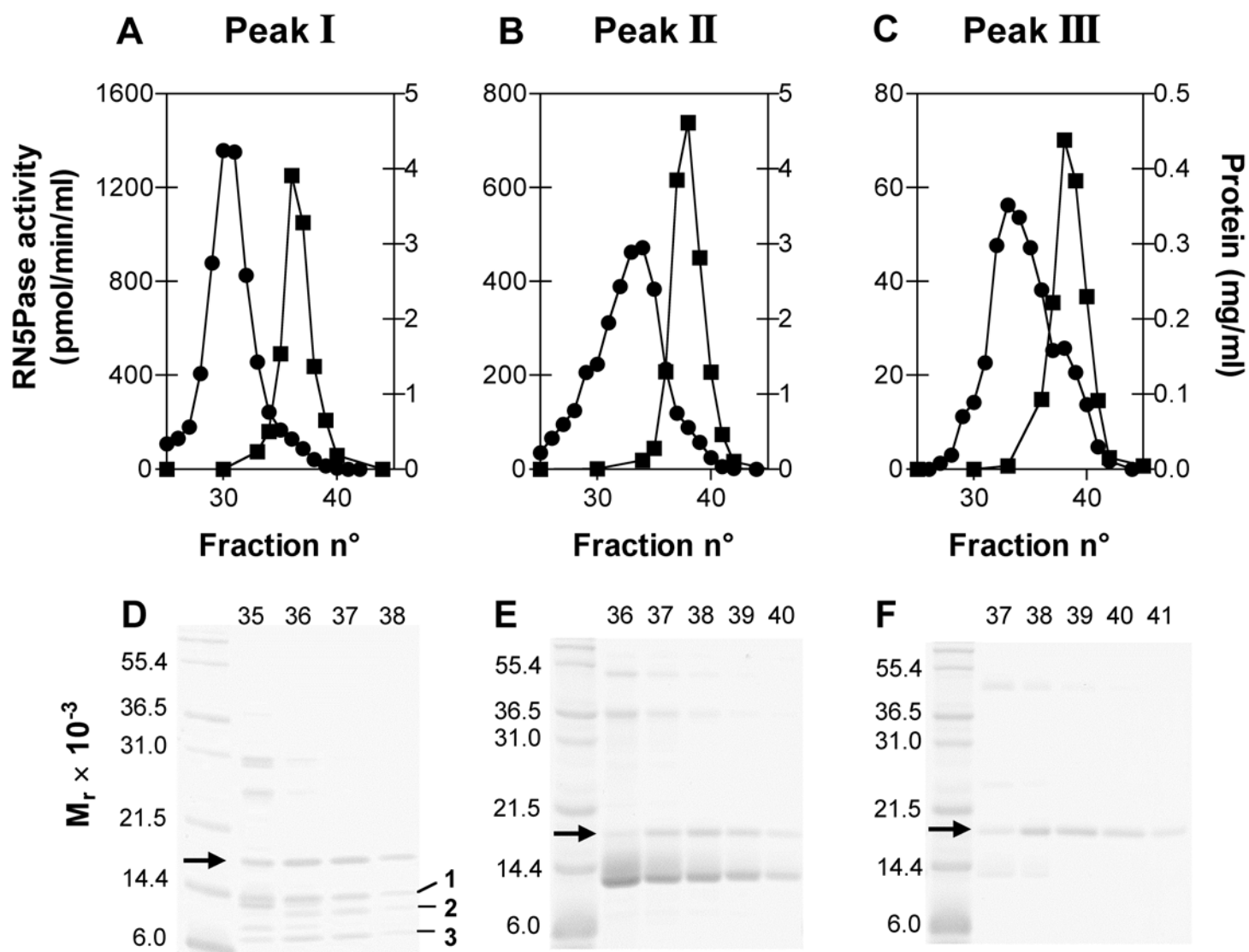
**Fig. 2**



**Fig. 3**



**Fig. 4**



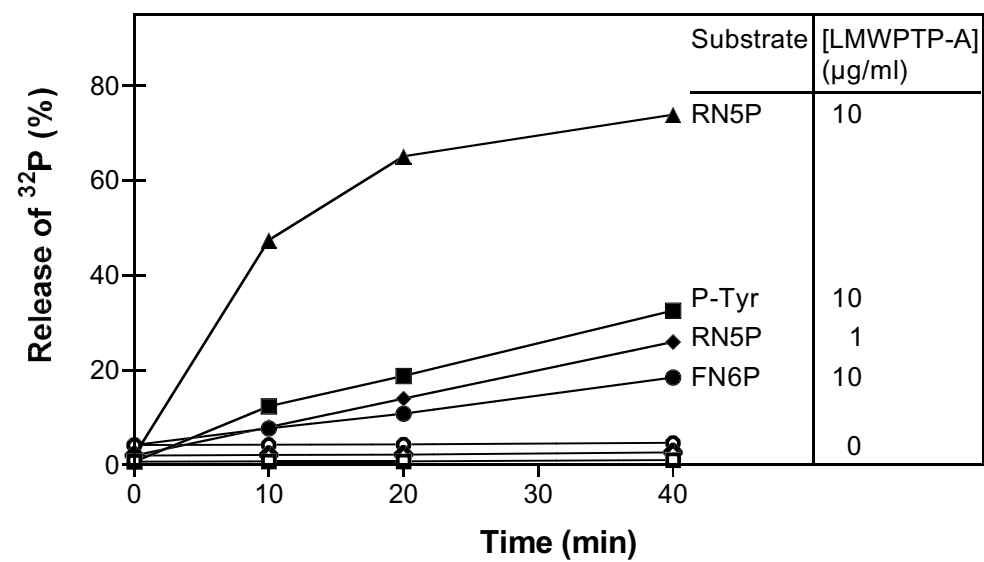
**G**

LMWPTP-A AEQATKSVLFVCLGNICRSPIAEAVFRKLVTDQNISENWRVDSAATSGYEIGNPP 55  
LMWPTP-B VIDSGAVSDWNVGRSP

LMWPTP-A DYRGQSCMKRHGIPMSHVARQITKEDFATFDYILCMDESNLRDLNRKSNQVKTK 110  
LMWPTP-B DPRAVSCLRNHGIHTAHK

LMWPTP-A AKIELLGSYDPQKQLIIEDPYYGNDSDFETVYQQCVRCRAFLEKAH 157

Fig. 5



**Fig. 6**

