Gel-Immobilized Protein Phosphatase 2A from *Yarrowia lipolytica* Dephosphorylates Phosvitin and Modifies Its Functional Properties

Claudine Queiroz-Claret,* Pascale Jolivet, Caroline Delérable, Edith Bergeron, and Jean-Claude Meunier

Laboratoire de Chimie Biologique, INRA, INA-PG, Centre de Biotechnologie Agro-Industrielle, F-78850 Thiverval-Grignon, France

The catalytic subunit of a protein phosphatase 2A (PP2Ac) purified from *Yarrowia lipolytica* was immobilized by covalent coupling on CNBr-Sepharose 4B with a fixation yield of about 70−85%. The specific activity of free PP2Ac for substrate phosvitin was almost totally preserved by the immobilization process. The immobilized enzyme exhibits strongly improved thermostability. Phosvitin dephosphorylation by immobilized PP2Ac attained 14% and 35% yield after 3 and 17 h incubation, respectively, and resulted in modified phosvitin properties, *e.g.*, improved solubility and alteration of ultraviolet absorption spectrum. Electrophoretic data indicated that β-phosvitin was preferentially dephosphorylated by the immobilized enzyme. Presence of a reductant such as DTT in the reaction medium improved dephosphorylation efficiency by inducing formation of a phosphate complex which would prevent enzyme inhibition by the released phosphate. This work indicates that immobilization of protein phosphatases is a performant tool to achieve modifications of highly phosphorylated proteins through dephosphorylation, with easy retrieval of the modified protein from the reaction medium.

Keywords: *Protein phosphatase 2A; immobilized enzyme; phosvitin dephosphorylation; yeast Yarrowia lipolytica*

INTRODUCTION

Functional properties of food proteins are influenced by the phosphorylated state of the protein. Thus, changes in the phosphorylation/dephosphorylation balance of food proteins not only mediate metabolic processes but also play an important role in the industrial utilization of these proteins for the development of new products. Modification of the phosphorylated state by chemical methods might restrict food applications in particular because of the difficulty of eliminating unreacted reagents from the product. These drawbacks are avoided by the utilization of enzymatic methods. Attention has focused mainly on modifications of functional properties upon phosphorylation by protein kinases (Hunter, 1987). In contrast, information is still scarce on intentional or unintentional changes of protein functionality by the action of protein phosphatases and on their utilization in processing and/or storage of food materials. Casein, a protein carrying relatively low phosphate levels, was particularly studied in this concern. Its dephosphorylation by acid or alkaline phosphatases was shown to improve acid clotting properties and digestibility (Li-Chan and Nakai, 1989) and creaming stabilization (Lorenzen and Rimerdes, 1992) and to increase solubility and reduce emulsion and foaming ability (Van Hekken and Strange, 1993), opening valuable possibilities of industrial applications. The aim of the present work is to extend insights into the conditions of food protein dephosphorylation by other types of protein phosphatases and the resulting effects on the functional properties of highly phosphorylated food proteins such as phosvitin.

Protein phosphatases type 2A (PP2A) have oligomeric structures associating one catalytic subunit (PP2Ac) with one or several regulatory subunits which may inhibit the catalytic activity (Cohen, 1989). Activity can be revealed by alcohol treatment of the extracted enzyme (Usui *et al.*, 1983). Among the diverse protein phosphatases 2A present in the yeast *Yarrowia lipolytica* utilized in the food industry, one form exhibiting high specific activity and present in large amounts was selected for this study.

Phosvitin from egg yolk, M_r 40 kDa, was utilized as substrate because of its abundance in serine (about 50%) with most of the seryl groups phosphorylated (Grizzuti and Perlmann, 1970; Shainkin and Perlmann, 1971). Phosphorus content ranges from 9% to 12% (w/w) (125−136 mol of P/mol of phosvitin) accounting for at least 60% of total protein phosphorus in yolk. The resulting negative charge and potential cation-binding ability of phosphoseryl groups are related to phosvitin hydrophobicity, viscosity, solubility, and emulsifying ability (Grizzuti and Perlmann, 1970; Causeret *et al.*, 1991). It was also known from early work by Mecham and Olcott (1949) that dephosphorylation by grapefruit phosphatases shifted the soluble phosvitin form into a gelified state and finally insoluble forms.

Immobilized PP2Ac was utilized in this study for several reasons. Generally, active subunits derived from oligomeric proteins are more easily stabilized when attached to an insoluble support than in solution. The techniques described in this work afford, thus, an efficient tool for studying the action of protein phosphatase subunits. Moreover, in the particular case of PP2Ac from *Y. lipolytica* utilizing phosvitin as substrate, the immobilization method avoids analytical difficulties arising from the small difference between their M_r (33 and 40 kDa, respectively) and facilitates elimination of the enzyme by centrifugation after the reaction. The method also provides an approach to compare the behavior of the enzyme in free solution and when bound to a matrix, a situation probably more consistent with

* Author to whom correspondence should be addressed (fax 33-1-30-81-53-73).

S0021-8561(97)00079-4 CCC: $14.00

that occurring in cellular location. From an industrial point of view, embedding the enzymes in an artificial matrix may allow the possibility of their reutilization. The present work investigated the kinetics of free and immobilized PP2Ac activity with phosvitin and casein as substrates and the resulting modifications of phosvitin properties.

EXPERIMENTAL PROCEDURES

Materials. Chemically dephosphorylated casein (#4765), rabbit muscle 3′,5′-cAMP-dependent protein kinase, Brij 35, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2-mercaptoethanol, bovine serum albumin (BSA), 2-(*N*-morpholino)ethanesulfonic acid (MES), tetrabutylammonium phosphate, phosvitin, and sodium pyrophosphate were purchased from Sigma. Tris(ethanolamine) (TEA)-HCl, Tris-HCl, dithiothreitol (DTT), cold ATP, and phenylmethanesulfonyl fluoride (PMSF) were from Boehringer. Malachite green, H_2SO_4 suprapur, and TSK (diethylamino)ethane (DEAE) Toyopearl 650S were obtained from Merck. [^{32}P]Sodium phosphate was from Isotopchim; [α-^{32}P]ATP, [γ-^{32}P]ATP, and scintillation liquid (Ecolite TM) were from ICN. Acetonitrile was from BDH. Sephadex gel 50, CNBr-activated Sepharose 4B, Superose 12HR 10/30, Mono Q HR 5/5, and fast desalting HR 10/10 fast protein liquid chromatography (FPLC) columns were purchased from Pharmacia; Ultrasphere ODS 5 μm column was from Beckman. Diaflo membranes were from Amicon. All other chemicals were of analytical grade.

Methods. *Cell Culture.* The yeast *Y. lipolytica* (wild strain W-29) was grown on an inorganic phosphate (P_i)-deficient medium (Galabova *et al*., 1993). The cells were sampled at the beginning of exponential growth (13 h), centrifuged at 4400*g* for 15 min, and washed with distilled water to eliminate culture medium.

Purification of the Enzyme PP2Ac. Fresh cells were ground in a MSK Braun ball homogenizer (ball diameter 0.11−0.12 mm; Braun Sciencetec, France) and cooled under a stream of CO_2 for 1.5 min in 20 mM TEA buffer, pH 7.0, with 1 mM EGTA, 0.1 mM DTT, 5% (v/v) glycerol, 0.01% (v/v) Brij 35 (hereafter designated as buffer A), and 0.4 mM PMSF. Proportions for grinding were 1/1/5 (w/w/v) cells/balls/buffer. In the experiments described here PP2Ac was purified from about 20 g of fresh cells. After centrifugation (48000*g* for 45 min at 4 °C) the supernatant was batch-processed on a TSK gel DEAE Toyopearl ion exchange chromatography column (2.6 cm × 30 cm), and the enzyme was eluted with buffer A containing 0.1 mM PMSF in a 0.05−0.6 M NaCl gradient.The fractions corresponding to 0.3−0.4 M gradient were pooled and subjected to 80% ethanol treatment in order to unmask the catalytic unit of PP2A (Usui *et al*., 1983). After concentration on a Diaflo membrane, the sample PP2Ac was eluted through a Superose 12HR FPLC column with buffer A containing 0.2 M NaCl (purification activity yield 350−650). Active fractions were pooled, loaded on a Mono Q FPLC column, and eluted with a 50 mM−0.5 M NaCl gradient. The enzyme PP2Ac was purified to homogeneity controlled by electrophoresis (purification activity yield 950). The molecular mass of the catalytic unit was 33 kDa, optimum pH 7−7.3. The experiments described in this paper utilized especially fractions obtained after the Superose step; the Mono Q step was utilized for kinetic studies with casein (Table 1). It was ascertained with effectors, *e.g*., heparin, that the enzyme was a PP2Ac and not a PP1 (Jolivet *et al*., 1997).

Enzyme Immobilization. The purified enzyme PP2A without ethanol treatment and PP2Ac with ethanol treatment was immobilized on CNBr-activated Sepharose 4B as described by Pharmacia. The gel was resuspended either in buffer A or in 50 mM Tris-HCl with 0.1 mM EGTA, pH 7.0. Immobilized enzyme (3−8 pmol of enzyme/mg of dry support) was utilized immediately. It was checked that immobilized enzyme could be stored in small batches in 25% ethanol for up to 1 week with only slight activity loss.

Protein Determination. Free protein concentration was measured with the Bradford method (Bradford, 1976). The bicinchoninic acid (BCA) method was used specially for direct determination of protein covalently bound to CNBr-Sepharose 4B (Smith *et al*., 1985; Stich, 1990) because the Bradford method was inadequate in this case. In both methods bovine serum albumin was utilized as standard. We checked that the gel did not interfere with BCA protein determination.

Phosvitin Preparation. Commercial phosvitin was diluted in water and purified on a fast desalting HR 10/10 FPLC column to remove free phosphate. Purification was controlled by electrophoresis. Protein concentration was measured by the micro-Kjeldahl method (30−45 mg sample; Bradstreet, 1965) because its structure was composed of many seryl groups and protein concentration could not be determined by the dying BCA or Bradford method. Aliquots of purified phosvitin were sampled and stored at −20 °C up to 9 months.

[^{32}P]Casein. Labeled casein was obtained from [γ-^{32}P]ATP according to McGowan and Cohen (1988) by the reaction of rabbit cAMP-dependent protein kinase on commercial chemically dephosphorylated casein, M_r 25 kDa (determined by electrophoresis). The amount of phosphate remaining in the utilized commercial casein was shown with the Eckman−Jäger method to be 4.2 mol of phosphate/mol of casein. As native casein contains 0.8% (w/w) phosphorus/mol, *i.e.*, 6.4 mol of phosphate/mol of native casein, it can be considered that the dephosphorylation rate of the utilized casein was about 30%. The labeled casein (2.4 μmol of P/g of casein or 0.1 μmol of P/μmol of casein) was stored for up to 3 weeks at 4 °C. Prior to use, nondegradation of casein was checked by electrophoresis.

Enzyme Assays. Casein dephosphorylation was studied by incubating aliquots of free or immobilized enzyme for 20 min at 30 °C, pH 7.0, in 50 mM Tris-HCl containing labeled casein, 0.1 mM EGTA, and 40 mg/L BSA to facilitate protein precipitation. Protein was precipitated with cold 20% trichloracetic acid containing 100 mM sodium pyrophosphate by 6 min centrifugation in a labstop (12000*g*). Radioactivity was counted in the supernatant by liquid scintillation (Tricarb 1500, Packard). The activities are expressed as ^{32}P removed/enzyme/time.

Phosvitin dephosphorylation was carried out by incubating (65−120 nM) free or immobilized enzyme with 12.5 μM substrate phosvitin in 15 mM TEA buffer with 0.5 mM EGTA, 3% (v/v) glycerol, 0.05 mM DTT, and 0.005% (v/v) Brij 35. Free enzyme aliquots were directly mixed with color reagent which stopped the enzymatic reaction. Sampled immobilized enzyme aliquots were spun for 5 min at 5000*g* to remove the gel. Free phosphate released from phosvitin during the reaction was measured in the supernatant by the technique of Baykov *et al*. (1988). After 15 min incubation with the color reagent (malachite green), absorbance was measured at 630 nm in a double-beam spectrophotometer (Uvikon 941, Kontron).

Trypsin effect on enzyme activity was evaluated by incubating free or immobilized PP2Ac with trypsin (enzyme/trypsin w/w ratio = 100) for 15 min at 30 °C. The catalytic activity in the presence of 11.5 μM phosvitin was measured by phosphate production as determined by the Baykov *et al*. (1988) method after 30 min reaction at 30 °C.

Response to pH of free and immobilized PP2Ac was established in 50 mM TEA for pH 4.5−5.5, 50 mM MES for pH 5.5−6.5, or 50 mM TEA for pH 6.5−8.5.

Thermostability was studied by incubating free or immobilized PP2Ac 30 min at temperatures ranging from 20 to 60 °C and then measuring activity with 11.5 μM phosvitin as substrate at 30 °C for 30 min.

Study of Dephosphorylated Phosvitin by Immobilized PP2Ac. After 3 and 17 h incubation in the reaction medium, the gel carrying the enzyme was removed by centrifugation for 5 min at 5000*g*. The supernatant was utilized to measure phosvitin phosphate content, UV absorption spectra, and solubility and to determine phosvitin subunits. Controls were achieved by incubating phosvitin with gel without enzyme.

A. Determination of Phosphate Content in Phosvitin. After 15 min hydrolysis at 100 °C with 2 N NaOH, phosphate was determined with malachite green reagent (0.8 mL total volume; Eckman and Jäger, 1993). Two standard curves were

required utilizing either a KH_2PO_4 solution (0−2 nmol) or a phosvitin solution (0−10 pmol of phosvitin).

B. Phosvitin UV Absorption Spectra. Ultraviolet absorption of phosvitin samples before and after dephosphorylation by PP2Ac was measured between 190 and 320 nm.

C. Phosvitin Solubility. Phosvitin solutions were subjected to centrifugation (10000*g* for 20 min at 5 °C), and the soluble protein remaining in the supernatant was determined by the BCA method (Huang and Kinsella, 1986).

D. SDS−Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed in 4−20% gradient polyacrylamide gel (Novex, San-Diego, CA), and molecular weight determination utilized Novex see-blue prestained protein standards for SDS−PAGE. Electrophoresis was carried out as described by Laemmli (1970). After staining with Coomassie blue, densitometric analysis was performed with a Hoefer densitometer (Hoefer Instruments) coupled with a Beckman computer (System Gold chromatography software). Autoradiograms were obtained by exposing the film (Kodak X-Omat) to the gel in an intensifying Chromex cassette for 6 h at 70 °C.

Formation of a Phosphate Complex. Experiments were performed utilizing 0.15 mM phosphoric acid with either 15 mM TEA (control buffer) or 15 mM TEA containing 0.5 mM EGTA, 50 μM DTT, 0.005% (v/v) Brij, and 2.5% (v/v) glycerol (experiment buffer). The Baykov method was utilized to measure the free phosphate remaining in the experiment buffer. Total phosphate was determined after alkaline hydrolysis by the Eckman−Jäger method.

Enzyme Phosphorylation with [^{32}P]Phosphate. The enzyme was incubated with 13−50 μM [^{32}P]sodium phosphate (sa = 3700 Bq/nmol) for 15 min at 30 °C in 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, and 5% (v/v) glycerol (buffer B).

Enzyme Phosphorylation with [γ-^{32}P]ATP or [α-^{32}P]ATP. Enzyme incubation (30 min at 30 °C) was performed as described by Abdel-Ghany *et al.* (1993). The reaction medium contained 50 mM Tris-HCl buffer (pH 7.0), 0.1% (v/v) 2-mercaptoethanol, 0.5 mM $AlCl_3$, 50 μM ATP, and 17.6 μM [γ-^{32}P]-ATP (sa = 167 TBq/mmol) or 26.4 nM [α-^{32}P]ATP (sa = 111 TBq/mmol). Phosphate content incorporation in protein was determined utilizing [^{32}P]ATP specific activity.

Isolation of [^{32}P]Phosphoenzyme. After incubation with labeled sodium phosphate or ATP, the [^{32}P]phosphoenzyme was isolated by elution through a Sephadex G50 gel filtration column (1 cm × 30 cm). Alternatively, the phosphorylated enzyme was separated by HPLC utilizing a reverse-phase C_{18} column (Ultrasphere ODS 5 μm). This method was modified from Casnellie *et al.* (1982) and Ferry *et al.* (1990). The column was washed with aqueous buffer (40 mM sodium phosphate, pH 7.4, 5 mM tetrabutylammonium phosphate) to elute the radioactive P_i and ATP. The enzyme was then eluted with a linear gradient of 0−75% acetonitrile containing 5 mM tetrabutylammonium phosphate over 30 min at 1 mL/min. Detection of eluted ^{32}P-labeled compounds was carried out with a Berthold LB-506 C on-line radioactivity monitor. The analysis cell was an empty 500 μL cell (Z 500) in which β-radiation of high energy (1.7 MeV) led to Cerenkov radiation when passing through the transparent material (light emitted at a velocity greater than that of natural light). Detection yield was about 40% according to Ferry *et al.* (1990). Under these experimental conditions, P_i and ATP were detected during the first 5 min elution, while PP2Ac was eluted with 44% acetonitrile.

RESULTS AND DISCUSSION

Immobilization of PP2A. The CNBr-activated Sepharose 4B is an immobilization matrix allowing spontaneous coupling of enzyme by a primary amino group. Coupling yields for the catalytic subunit PP2Ac and for enzyme including both the catalytic and regulatory subunits (PP2A) proved to be very favorable as checked with the BCA method which showed that about 70−85% of the proteins were bound. Measurement of phosphate produced during 30 min reaction (initial rate was linear between 0 and 30 min) utilizing up to 30 μM phosvitin concentrations established that the catalytic specific activity of the free PP2Ac was almost totally preserved by immobilization (Figure 1A).

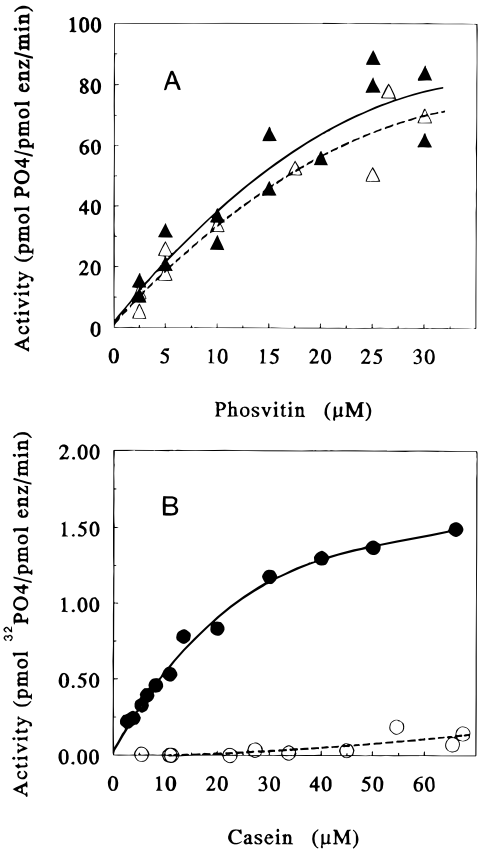


Figure 1. Activity of free and immobilized PP2Ac (10 μM) with (A) substrate phosvitin, (▲) free enzyme and (△) immobilized enzyme, released phosphate measured after 30 min reaction; (B) substrate casein, (●) free enzyme and (○) immobilized enzyme, released ^{32}P measured after 10 min reaction.

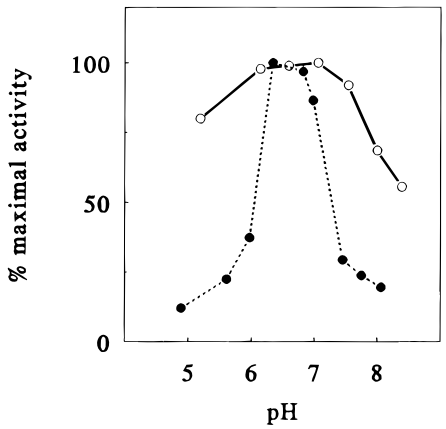


Figure 2. pH dependence of the activity of free (○) and immobilized (●) PP2Ac. Activity expressed as percent of maximum. Substrate: 11.5 μM phosvitin.

Immobilization increased PP2Ac sensitivity to trypsin. After 15 min incubation with trypsin, the activity of free enzyme was unaffected while the immobilized enzyme lost 55% activity. Optimum pH (between 6.3 and 7.0) was pratically not modified by immobilization, as expected with a noncharged support, but the response to lower or higher pH was amplified (Figure 2). Noteworthy is the difference in response to temperature resulting from immobilization. As it appears from data shown

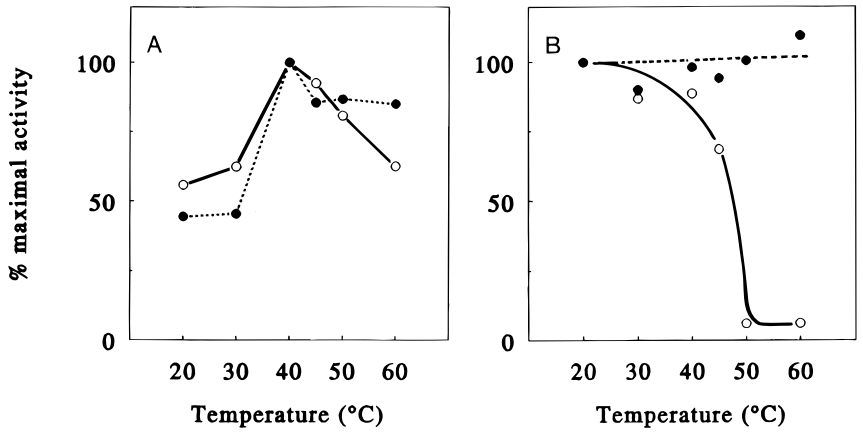


Figure 3. (A) Effect of temperature on free (○) and immobilized (●) PP2Ac activity. (B) Thermostability of free (○) and immobilized (●) PP2Ac after 30 min incubation at the indicated temperatures. Activity measured by the phosphate produced after 30 min reaction at 30 °C with 11.5 μM phosvitin as substrate. Results in percent of the activity observed after incubation at 20 °C.

in Figure 3A, optimum temperature is not modified but immobilized PP2Ac performs with higher activity at high temperatures. Moreover, immobilization prevents thermodegradation of the enzyme (Figure 3B).

K_m(phosvitin) values of both enzymes were respectively 22 μM (free PP2Ac) and 16 μM (immobilized PP2Ac). In contrast, data in Figure 1B show that the ability to dephosphorylate casein was apparently lost upon immobilization, at least for the range of casein concentrations utilized. K_m(casein) of free enzyme is 36 μM. However, data in Figure 1A indicate that this apparent loss was not the result of enzyme alteration by the immobilization process. As it is known that casein presents a spontaneous denatured conformation (Holt, 1992), it might be hypothesized that its access to the catalytic site was hindered by fixation of the enzyme on the gel, and thus the method is not convenient for casein.

From the results in Figure 1A it appears that activity of immobilized PP2Ac is particularly remarkable in comparison to other enzymes. For instance, lignin peroxidase was reported to bind to the same matrix with a yield of 80%, but the immobilized enzyme retained only about 40% of the initial specific activity under the experimental conditions (Asther and Meunier, 1993).

Free and Immobilized PP2Ac Activity with Substrate Phosvitin. When phosvitin is used as substrate for free PP2Ac, the time course curve of activity obtained by measuring phosphate concentration (expressed as pmol of P/pmol of enzyme) in the reaction medium exhibits an anomalous shape (Figure 4). After about 1 h reaction during which the product accumulated in the reaction medium, its concentration suddenly decreased during about 1 h and then net accumulation resumed. Similar kinetics occurred when phosvitin dephosphorylation was carried with immobilized PP2Ac and with the same level of specific activity except that apparent resumption of activity after 2−3 h was stronger in the case of the free enzyme. Experiments carried out with enzyme containing the regulatory subunit (PP2A; Figure 4, insert) showed specific activities about 40 times weaker than those of PP2Ac for both free and immobilized enzyme. The curves of net phosphate concentration in the reaction medium also presented the succession of increase and decrease periods but shifted in time: with free PP2A two peaks were observed at 1 and 2.5 h, but in the case of immobilized PP2A the two peaks occurred earlier.

Efficiency of Phosvitin Dephosphorylation by Immobilized PP2Ac. In control medium containing

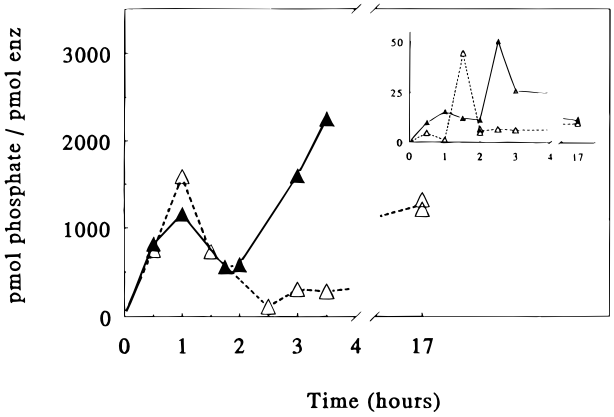


Figure 4. Time course of apparent phosphate release from phosvitin (12 μM) by (▲) free or (△) immobilized PP2Ac (90 nM). Insert: Experiment with PP2A.

phosvitin and gel without enzyme, measurements of phosphate content in phosvitin by the Eckman−Jäger method indicated 134 mol of phosphate/mol of phosvitin, which is in agreement with data in the literature reporting 125−136 mol of phosphate/mol of phosvitin (Grizzuti and Perlman, 1970). In a typical experiment, after 17 h dephosphorylation by immobilized PP2Ac, the amount of phosphate retained in phosvitin was 87 mol of phosphate/mol of phosvitin, *i.e.*, the dephosphorylation yield attained was 35% (14−17% dephosphorylation was attained after 3 h), which agrees with data reported by Kato *et al.* (1987) who obtained 40% dephosphorylation of phosvitin in 10 h by alkaline phosphatase.

Modification of Phosvitin Properties by Action of Immobilized PP2Ac. To investigate modifications of phosvitin properties upon dephosphorylation by immobilized PP2Ac, the reaction medium was subjected to 5 min centrifugation at 5000*g* to discard the gel containing the enzyme, and the supernatant was used for the analyses.

(a) Alteration of the Ultraviolet Absorption Spectrum of Phosvitin. In our experiments absorption spectra between 190 and 320 nm were determined for phosvitin and enzyme-dephosphorylated phosvitin (Figure 5). Dephosphorylation shifted the 223 nm absorption peak to 230 nm. Difference between the two spectra indicates a strong absorbance modification at 235 nm attaining 0.2−0.7 according to different experiments. For example 3 h dephosphorylation led to 0.26 absorbance difference at 235 nm, and after 17 h dephosphorylation this value attained 0.7. This result proved that phos-

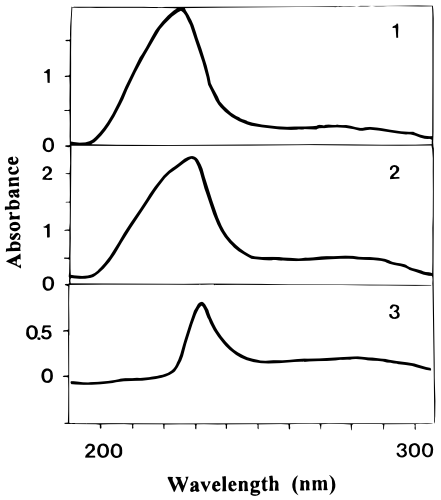


Figure 5. Ultraviolet absorption spectra of (1) phosvitin (λ_{max} = 223 nm) and (2) dephosphorylated phosvitin (λ_{max} = 230 nm); (3) difference between spectra 1 and 2 (λ_{max} = 235 nm, Δ_{abs} = 0.7).

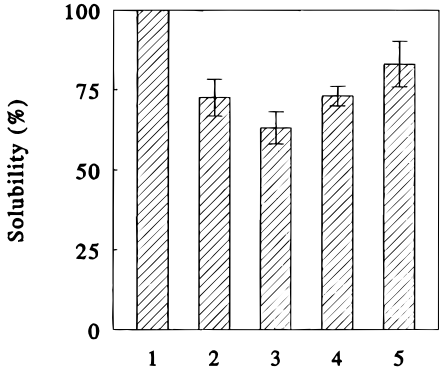


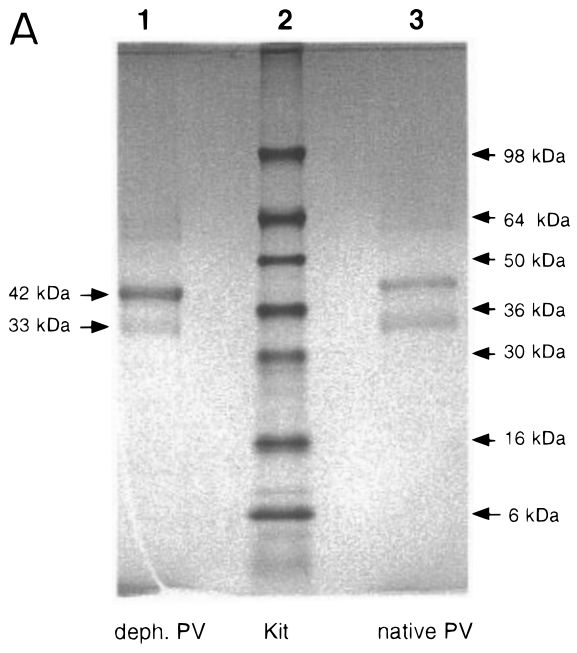
Figure 6. Variations of phosvitin solubility, expressed in percent of control (±SD), in the presence or absence of immobilized enzyme: (1) native phosvitin, (2) incubated phosvitin in control medium at 30 °C during 3 or 17 h; (3) incubated phosvitin in the presence of gel support at 30 °C during 3 or 17 h; (4) phosvitin after 3 h dephosphorylation by immobilized PP2Ac, (5) phosvitin after 17 h dephosphorylation by immobilized PP2Ac.

vitin degradation continued after 3 h reaction. In their study on the characteristics of phosvitin, Mecham and Olcott (1949) showed that phosvitin dephosphorylation with 0.25 M KOH at 35 °C during 4 h resulted in 36% phosphorus release with a 10-fold increase in optical density of 250 nm. The authors assumed that under the action of dilute alkali peptide serines form dehydroalanyl (α-aminoacrylic acid) residues which absorb very strongly in the UV range (Carter and Greestein, 1946). After 24 h alkali treatment 95% dephosphorylation was obtained but with only a small increase in density possibly because of degradation of dehydroalanyl groups. Similar feature would occur in our experiments.

(b) Solubility. After 20 min centrifugation at 10000*g*, phosvitin solubility was evaluated in the supernatant by the BCA method. In their study with soy protein isolate Campbell *et al.* (1992) reported that protein phosphorylation improved protein solubility over the pH 3−6 range but not at pH 7. Huang and Kinsella (1966) showed by electrophoresis that phosphorylation of yeast nucleoprotein favored cross-linking and that during centrifugation a gellike network structure was formed and only 45% of protein remained in true solution. In our experiments phosvitin was incubated for 3 and 17 h at 30 °C in the presence or absence of enzyme. In control medium (without enzyme) a spontaneous 27−28% decrease in phosvitin solubility was observed after 3 and 17 h incubation (Figure 6). This effect was augmented to about 37% when phosvitin was incubated in the presence of gel support. In contrast dephosphorylation by immobilized PP2Ac resulted in efficient preservation of phosvitin solubility (27% loss after 3 h and 17% after 17 h dephosphorylation). Absence of interference by bacterian development during the incubation experiments was checked by adding 1 mM NaN_3 in the control medium containing phosvitin. It may be hypothesized that dephosphorylation impaired or prevented the cross-linking network and thus hindered trapping phosvitin into a gellike structure. Our results seem to be in contradiction with those of Melcham and Olcott (1949) who reported progressive gelification of phosvitin upon dephosphorylation with grapefruit phosphatase. However, it is difficult to compare these results because the authors utilized a noncharacterized, unfractionated enzyme preparation from grapefruit which appeared to cause some proteolysis and did not proceed to solubility controls as a function of time.

(c) Electrophoretic Analysis of Native and Dephosphorylated Phosvitin. Electrophoresis on 4−20% gradient SDS−polyacrylamide gels showed similar migration patterns for native (Figure 7A, lane 3) and dephosphorylated (lane 1) phosvitin. Two main bands were obtained corresponding to M_r 33 and 45 kDa for native phosvitin and 33 and 42 kDa for dephosphorylated phosvitin after 17 h reaction. The 3 kDa difference was confirmed and may be ascribed to the loss of about 30 PO_4^{2-}, fitting rather well with the measurements by the Eckman−Jäger method reported above. Our results are in good accordance with those (35−45 kDa) found by Mok *et al.* (1961) and correspond respectively to the main components of phosvitin subunits, first isolated by Conelly and Taborsky (1961) and designated α-phosvitin and *β*-phosvitin (Abe *et al.*, 1982; Itoh *et al.*, 1983; Wallace and Morgan, 1986; Causeret *et al.*, 1991). These two components differ from each other in phosphorus content. The *β*-phosvitin contained 9.28% phosphorus, while α-phosvitin contained 2.97% (Abe *et al.*, 1982). Densitometric scans of SDS gels (Figure 7B) showed that upon 17 h dephosphorylation the surface ratio between peak 2, which corresponds to the slow-moving *β*-component, and peak 3, corresponding to the α-subunit, increased from 1.5 to 4.3. As peak 3 was unchanged, the difference resulted from the apparent increase in peak 2. After 3 h dephosphorylation the ratio between peaks 2 and 3 was 1.05 in the control and 2.4 after dephosphorylation (results not shown). Studies by Grant and Taborsky (1966) showed that dephosphorylation changes the phosphoseryl residues into α-aminoacrylic acid residues by *β*-elimination reaction, and therefore, the decreases in negative charge of the dephosphorylated *β*-subunit resulted in stronger fixation of Coomassie blue dye. On this assumption it can be concluded from the data presented in Figure 7 that *β*-phosvitin was more sensitive to dephosphorylation by immobilized PP2Ac than α-phosvitin.

Mechanisms Involved in the Dephosphorylation Process. Tentative explanations for the observed successive increases and decreases in the net content of phosphate released into the reaction medium during the course of phosvitin dephosphorylation by both free and immobilized PP2Ac or PP2A (Figure 4) might consider different hypotheses, *viz.*, (a) phosphate released from



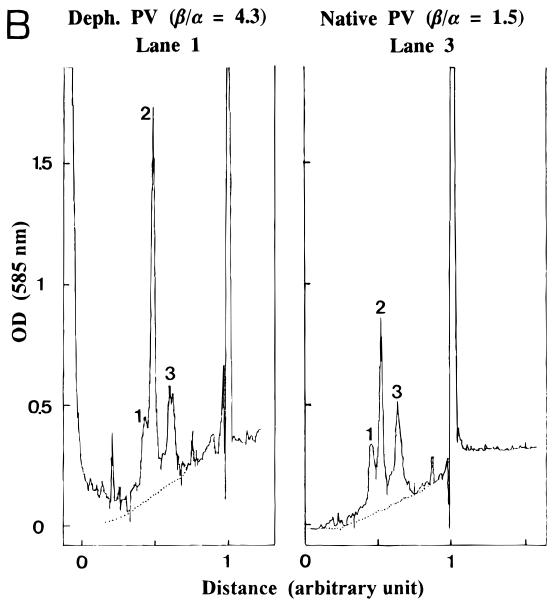


Figure 7. (A) SDS−polyacrylamide gel electrophoresis of dephosphorylated (lane 1) and native (lane 3) phosvitin. (B) Densitometric scans of lanes 1 and 3 of the SDS gels shown in panel A. See text for details.

phosvitin would fix nonenzymatically on the PP2Ac molecule or/and (b) phosphate would precipitate as a complex not detectable by the Baykov method. In both cases the dephosphorylation reaction was not prevented or was even facilitated as suggested by the following results.

(a) Phosphate Fixation on PP2Ac. Nonenzymatic fixation of phosphate was shown for enzymes able to bind P_i (Penefsky, 1977) or the triphosphate moiety of ATP (Abdel-Ghany *et al.*, 1993). In order to investigate the ability of PP2Ac to fix phosphate, we carried out comparative experiments in which the enzyme was incubated with labeled phosphate or labeled ATP.

Free PP2Ac was incubated with labeled phosphate for 15 min at 30 °C and then eluted through a Sephadex G50 column. Results shown in Figure 8A,B support the hypothesis that PP2Ac is able to fix P_i, the incorporation

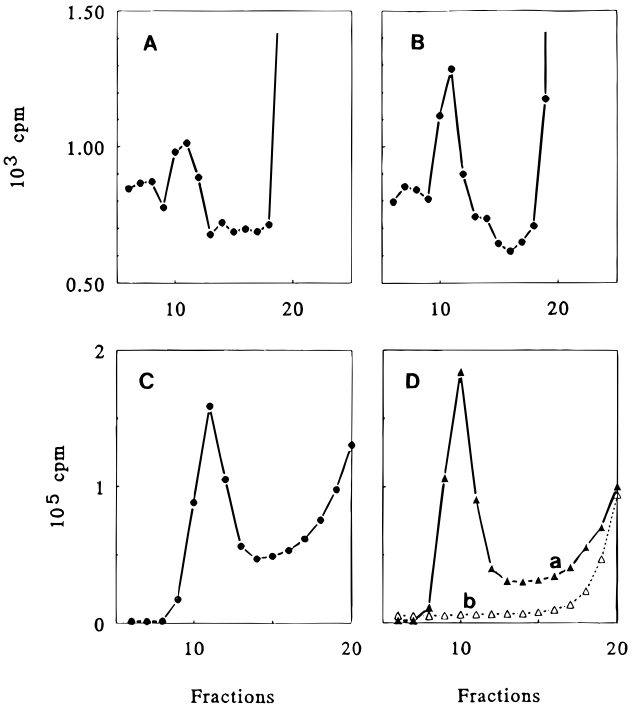


Figure 8. (A, B) Gel filtration chromatography on Sephadex G50 of phosphorylated PP2Ac after 30 min incubation at 30 °C with $^{32}P_i$ in buffer B. PP2Ac (130 pmol) was incubated with 25 (A) or 50 (B) nmol of ^{32}Pi, incubation volume 0.45 mL; $[P_{fixed}]/[enzyme] = 0.040$ and 0.064, respectively. **(**C, D) Gel filtration chromatography on Sephadex G50 of phosphorylated PP2Ac after incubation with [γ-^{32}P]ATP: 0.45 μM PP2Ac, 50 μM ATP, 17.6 μM [γ-^{32}P]ATP, incubation volume 0.25 mL; (C) $[P_{fixed}]/[enzyme] = 1.13$, **(**Da) elution with 0.5 M NaCl, $[P_{fixed}]/[enzyme] = 1.16$, (Db) elution with 2 M NaCl.

increasing with P_i concentration in the medium. The enzyme−^{32}P bond was disrupted by electrophoresis (results not shown). Reverse-phase HPLC with 0−75% acetonitrile gradient resulted in almost total loss of ^{32}P from the enzyme eluted with the 44% acetonitrile fractions (results not shown). Label also disappeared from the enzyme after elution through a Sephadex G50 column with buffer B containing 0.5 M NaCl. In the whole, these results suggest that phosphate was attached to the enzyme by an ionic bond. The $[P_{fixed}]/[E]$ ratio attained was 0.22.

Incubating PP2Ac with [α- or γ-^{32}P]ATP in the presence of $AlCl_3$ induced ^{32}P incorporation into the enzyme (Figure 8C). The incorporated label persisted after reverse-phase HPLC or after elution through Sephadex G50 with buffer B containing 0.5 M NaCl (Figure 8Da) in contrast with labeling with [^{32}P]phosphate. However the linkage was broken by elution through Sephadex G50 with buffer B with 2 M NaCl (Figure 8Db). Stoichiometry of labeling from [γ-^{32}P]ATP was about $[P_{fixed}]/[E] = 1.13$ and 1.16 with 0.2 and 0.5 M NaCl, significantly higher than labeling from [^{32}P]phosphate. The amounts of ^{32}P associated to PP2Ac from α- or γ-ATP were of the same order (1.06 and 1.13). Linkage lability in the presence of 2 M NaCl indicates involvement of a coordination bond.

To obtain information on the action of phosphate on the enzyme, free PP2Ac was incubated with phosphate concentrations close to those released into the medium during the course of enzyme reaction (see Figure 4), and kinetic properties were evaluated utilizing [^{32}P]casein as substrate. The kinetic parameters K_m(casein) and k_c calculated from the saturation curves are shown in

Table 1. Effect of Incubation with P_i on the Kinetic Properties of PP2Ac[a]

[P]/[E]	K_m(casein) (μM)	k_c (s^{-1})	k_c/K_m (s^{-1} M^{-1})
0	36	0.060	1667
118	25	0.043	1720
264	22	0.033	1500
550	18	0.040	2220

[a] K_m and k_c calculated from activity curves of 72 μM PP2Ac (from the Mono Q purification step) on casein (2.2−30 μM), buffer A.

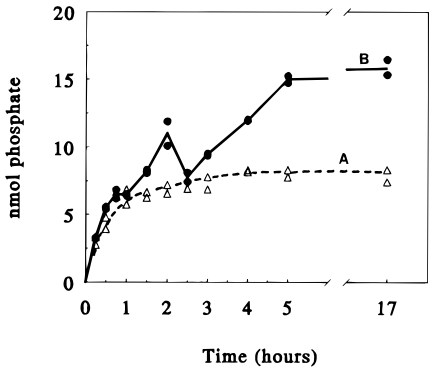


Figure 9. Time course of 6 μM PP2Ac activity on 12.5 μM phosvitin in the (A) absence and (B) presence of 50 μM DTT.

Table 1 for different values of the [phosphate]/[enzyme] concentration ratio in the incubation mixture. It appears that contact of the enzyme with phosphate modifies its catalytic properties: K_m and k_c decreased as phosphate concentration increased.

(b) Formation of a Phosphate Complex. Phosphoric acid was incubated for 17 h in control and experiment buffers (see Methods), and the content in free PO_4^{2-} was measured by the Baykov method and total PO_4^{2-} by the Eckman−Jäger method. The Baykov method showed a 20% decrease in free phosphate remaining in the experiment buffer, while by alkaline hydrolysis (Eckman−Jäger method) the total PO_4^{2-} was recovered. Similar results were obtained with 4 mM ascorbic acid in place of DTT. It is suggested that during the dephosphorylation reaction the presence of a reductant might induce formation of a complex for critical levels of phosphate released into the medium, which would account for the disappearance of free phosphate in solution. Time course curves of phosvitin dephosphorylation in the control buffer without DTT and in the experiment buffer containing DTT (Figure 9) support this assumption: the kinetic abnormality was not visible in the absence of reductant, and the reaction was practically stopped after about 1−2 h. It is also clear that the presence of a reducing agent improved dephosphorylation efficiency. Incidentally, withdrawal of phosphate from the medium by complex formation would prevent enzyme inhibition (Lorient and Linden, 1976). Computation from data in Figures 4 and 9B compared to the stoichiometry of P association by PP2Ac suggests that the transient decrease in free P content in the medium should be mostly ascribed to formation of a P complex.

CONCLUSIONS

The catalytic subunit of PP2A was attached by covalent bond to the insoluble support CNBr-Sepharose 4B with 70−85% fixation yield. Activity of the immobilized enzyme was checked on phosvitin and casein. Immobilization impaired the utilization of casein as substrate but did not modify the ability of the enzyme to dephosphorylate phosvitin. Immobilization increased enzyme sensibility to trypsin but in contrast allowed strong resistance to temperatures up to 60 °C.

Analysis of the processes involved in phosphate release from phosvitin by free PP2Ac indicated that besides significant noncovalent phosphate binding into PP2A, inclusion of DTT in the reaction medium induced removal of phosphate from the solution by formation of a complex. It appears from the results that the presence of a reductant during the dephosphorylation reaction would prevent enzyme inhibition by produced phosphate and thus favors catalytic efficiency.

It was established that dephosphorylation by the immobilized PP2Ac improved phosvitin solubility. Polyacrylamide gel electrophoresis provided evidence that the catalytic activity affected the phosphorus content of the slow-moving component β-phosvitin and not that of α-phosvitin. Dephosphorylation involved transformation of the phosphoseryl residues of phosvitin into α-aminoacrylic acid as shown by changes in the UV absorption spectrum and increased fixation of Coomassie blue.

In conclusion, the present results indicate that immobilization of protein phosphatases might afford a performant technique for modifying the properties of highly phosphorylated proteins through dephosphorylation and facilitates separation of the enzyme from the modified protein.

LITERATURE CITED

Abdel-Ghany, M.; El-Sebae, A. K.; Shalloway, D. Aluminium-induced nonenzymatic phosphoincorporation into human tau and other proteins. *J. Biol. Chem.* **1993**, *268*, 11976−11981.

Abe, Y.; Itoh, T.; Adachi, S. Fractionation and characterization of hen's egg yolk phosvitin. *J. Food Sci.* **1982**, *47*, 1903−1907.

Asther, M.; Meunier, J.-C. Immobilization as a tool for the stabilization of lignin peroxidase produced by *Phanerochaete chrysosporium* INA-12. *Appl. Biochem. Biotechnol.* **1993**, *38*, 57−67.

Baykov, A. A.; Evtushenko, O. A.; Avaeva, S. M. A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase based enzyme immunoassays. *Anal. Biochem.* **1988**, *171*, 266−270.

Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Bradstreet, R. B. *The Kjeldahl method for organic nitrogen*; Academic Press: New York, 1965.

Campbell, N. F.; Shih, F. F.; Marshall, W. E. Enzymatic phosphorylation of soy protein isolate for improved functional properties. *J. Agric. Food Chem.* **1992**, *40*, 403−406.

Carter, C. E.; Greenstein, J. P. A spectrometric method for the determination of dehydropeptidase activity. *J. Biol. Chem.* **1946**, *165*, 725−726.

Casnellie, J. E.; Harrison, M. L.; Pike, L. J.; Hellstrom, K. E.; Krebs, E. G. Phosphorylation of synthetic peptides by a tyrosine protein kinase from the particulate fraction of a lymphoma cell line. *Proc. Natl. Acad. Sci. U.S.A.* **1982,** *79*, 282−286.

Causeret, D.; Matringe, E.; Lorient, D. Ionic strength and pH effects on composition and microstructure of yolk granules. *J. Food Sci.* **1991**, *56*, 1532−1536.

Cohen, P. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **1989**, *58*, 453−508.

Connely, C.; Taborsky, G. Chromatographic fractionation of phosvitin. *J. Biol. Chem.* **1961**, *236*, 1364−1368.

Eckman, P.; Jäger, O. Quantification of subnanomolar amounts of phosphate bound to seryl and threonyl residues of

phosphoproteins using alkaline hydrolysis and malachite green. *Anal. Biochem*. **1993**, *214*, 138−141.
Ferry, G.; Ernould, A.-P.; Genton, A.; Boutin, J. A. Assay of tyrosine protein kinase activity from HL-60 by high performance liquid chromatography for specificity studies. *Anal. Biochem*. **1990**, *190*, 32−38.
Galabova, D.; Tuleva, B.; Balasheva, M. Phosphatase activity during growth of *Yarrowia lipolytica*. *FEMS Microbiol. Lett.* **1993**, *109*, 45−48.
Grant, C. T.; Taborsky, G. The generation of labile, protein-bound phosphate by phosphoprotein oxidation linked to the autoxidation of ferrous ions. *Biochemistry* **1966**, *5*, 544−555.
Grizzuti, K.; Perlman, G. E. Conformation of phosphoprotein phosvitin. *J. Biol. Chem*. **1970**, *245*, 2573−2578.
Holt, C. Structure and stability of bovine casein micelles. *Adv. Protein Chem*. **1992**, *43*, 63−143.
Huang, Y. T.; Kinsella, J. E. Functional properties of phosphorylated yeast protein: solubility, water-holding capacity, and viscosity. *J. Agric. Food Chem*. **1986**, *34*, 670−674.
Hunter, T. A thousand and one protein kinases. *Cell* **1987**, *50*, 823−829.
Jolivet, P.; Queiroz-Claret, C.; Bergeron, E.; Meunier, J.-C. Purification and characterization of a type 2A protein phosphatase from *Yarrowia lipolytica* grown on a phosphate-deficient medium. *C. R. Acad. Sci. Paris* **1997**, in press.
Itoh, T.; Abe, Y.; Adachi, S. Comparative studies on the α- and *β*-phosvitin from hen's egg yolk. *J. Food Sci*. **1983**, *48*, 1755−1757.
Kato, A.; Miyazaki, S.; Kawamoto, A.; Kobayashi, K. Effects of phosphate residues on the excellent emulsifying properties of phosphoglycoprotein phosvitin. *Agric. Biol. Chem*. **1987**, *51*, 2989−2994.
Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
Li-Chen, E.; Nakai, S. Enzymatic dephosphorylation of bovine casein to improve acid clotting and digestibility for infant formula. *J. Dairy Res*. **1989**, *56*, 381−390.
Lorensen, P. C.; Reimerdes, E. H. Enzymatic dephosphorylation of caseins and creaming behaviour of o/w emulsions stabilized with dephosphorylated casein fractions. *Nahrung* **1992**, *6*, 595−599.
Lorient, D.; Linden, G. Dephosphorylation of bovine casein by milk alkaline phosphatase. *J. Dairy Res*. **1976**, *43*, 19−26.
McGowan, C. H.; Cohen, P. Protein phosphatase-2C from rabbit skeletal muscle and liver: an Mg^{2+}-dependent enzyme. *Methods Enzymol*. **1988**, *159*, 416−426.
Mecham, D. K.; Olcott, H. S. Phosvitin, the principal phosphoprotein of egg yolk. *J. Am. Chem. Soc*. **1949**, *71*, 3670−3678.
Mok, C. C.; Martin, W. G.; Common, R. H. A comparison of phosvitin prepared from hen's serum and from hen's egg yolk. *Can. J. Biochem. Physiol*. **1961**, *39*, 109−117.
Penefsky, H. S. Reversible binding of Pi by beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem*. **1977**, *252*, 2891−2899.
Shainkin, R.; Perlman, G. E. Phosvitin, a phosphoglycoprotein. I. Isolation and characterization of a glycopeptide from phosvitin. *J. Biol. Chem*. **1971**, *246*, 2278−2284.
Smith, P. K.; Krokn, R. L.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Frijimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klent, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem*. **1985**, *150*, 76−85.
Stich, T. E. Determination of protein covalently bound to agarose supports using bicinchoninic acid. *Anal. Biochem*. **1990**, *191*, 343−346.
Usui, H.; Kinohara, N.; Yoshikawa, K.; Imazu, M.; Amaoka, T.; Takeda, M. Phosphoprotein phosphatases in human erythrocyte cytosol. *J. Biol. Chem*. **1983**, *258*, 10455−10463.
Van Hekken, D. L.; Strange, E. D. Functional properties of dephosphorylated bovine whole casein. *J. Dairy Sci*. **1993**, *76*, 3384−3391.
Wallace, R. A.; Morgan, J. P. Isolation of phosvitin: retention of small molecular weight species and staining characteristics on electrophoretic gels. *Anal. Biochem*. **1986**, *157*, 256−261.

Received for review January 3, 1997. Revised manuscript received April 25, 1997. Accepted April 28, 1997.[⊗]

JF9700790

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1997.