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Influence of physicochemical conditions and technological treatments on the iron binding capacity of egg yolk phosvitin

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Abstract

The iron binding capacity of egg yolk phosvitin was evaluated at different conditions of pH (3.0–7.0) and ionic strength (0.10–0.60). The best value was obtained at pH 6.5 and 0.15 M of ionic strength (115 μg of iron/mg of phosvitin). For pH values under 3.5, iron fixation was slow and weak. When increasing ionic strength at pH values higher than 5.2, iron binding capacity was weakened. On the contrary, between 5.2 and 3.5 of pH values, the higher the ionic strength, the better the iron fixation. Once iron was fixed in favourable conditions, further acidification does not modify iron binding capacity of phosvitin. Only the presence of a strong chelating agent could remove iron from phosvitin. Phosvitin treated by thermal or high pressure methods did not result in aggregation and kept its high iron binding capacity, even at the strongest treatment conditions (90 °C or 600 MPa).

Keywords: Phosvitin; Iron binding; Technological treatments; pH; Ionic strength

1. Introduction

Egg yolk phosvitin has a very specific amino acid composition, presenting about 50% of serine (Byrne, van het Schip, van de Klundert, Arnberg, Gruber, & Geert, 1984; Losso & Nakai, 1994). All of them are phosphorylated (Clark, 1985), so phosvitin is a heavily phosphorylated protein. Moreover, the phosphoserines are arranged in a singular way, forming blocks that can carry up to 15 consecutive residues (Byrne et al., 1984; Mabuchi, Yamamura, Adachi, Aoki, Nakamura, & Nakamura, 1996). This unique primary structure makes of this protein one of the strongest metal chelating agent (Grizzuti & Perlmann, 1973, 1975; Hegenauer, Saltman, & Nace, 1979). One phosvitin molecule can anchor 113 ions of Mn^{+2} or 120 of Co^{+2}, and the binding is not affected by temperature up to 60 °C (Grizzuti & Perlmann, 1975). These authors have also described the interaction with Mg^{+2} and Ca^{+2}: at pH 6.5 phosvitin binds up to 103 and 127 ions/molecule, respectively. Even though, at pH 4.5 it can only bind 40 and 32 ions of Mg^{+2} and Ca^{+2}, respectively.

Iron is an important metal in food and health sciences (Gaucheron, 2000). By equilibrium dialysis, Hegenauer et al. (1979) described a great affinity between ferric ions and phosvitin ($K_f = 10^{18}$). Even more, this protein can chelate ferric ions better than molecules like citrate or nitrilotriacetate. Iron/phosvitin interaction depends on the method of ferric ion addition. If phosvitin is directly mixed with a ferric solution, the iron/phosvitin interaction is mainly intermolecular, and consequently the complexes are insoluble (McCollum, Gregory, Williams, & Taborsky, 1986; Taborsky, 1991). However, when mixing phosvitin and ferrous iron solutions, iron will be oxidised into ferric iron by autoxidation (Grogan & Taborsky, 1986), and in this case, they form soluble complexes based on intramolecular interactions. The high iron binding capacity brings about the bactericidal properties of phosvitin (Sattar Khan, Nakamura, Ogawa, Akita, Azakami, & Kato, 2000) or its antioxidant activity (Lee, Han, & Decker, 2002; Lu & Baker, 1986, 1987; Nakamura, Ogawa, Nakai, Kato, & Kitts, 1998). It was shown that both pH and temperature affect the antioxidant activity. In general, decreasing pH values

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will decrease the antioxidant activity of phosvitin (Lu & Baker, 1987), and this potential is also affected by drastic thermal treatments like autoclaving, but not by mild conditions like those employed in pasteurisation or similar treatments. In the same way, Albright, Gordon, and Cotterill (1984) have studied the influence of heat on the release of iron from phosvitin, and they have determined that heating phosvitin solution at 110 °C for 40 min did not release iron.

Phosvitin structure is affected by pH, because of modifications in electrostatic repulsion (Taborsky, 1968; Yasui et al., 1990). Iron binding has not the same effect on the structure, even if the interaction between iron and protein reduces electrostatic repulsions (Taborsky, 1980). Phosvitin is poorly modified by thermal treatments owing to its unordered structure. Nonetheless, an irreversible thermal transition of protein structure at 79 °C was described (Chung & Ferrier, 1995).

Even though there are some studies about the iron binding capacity of phosvitin, the influence of physicochemical and technological treatments on iron fixation by phosvitin has not been deeply studied. Our objectives in this research were: to improve the knowledge of the iron binding capacity of phosvitin at different conditions of pH and ionic strength, to study this capacity when phosvitin has been previously processed by technological treatments, and to evaluate the stability of the iron/phosvitin complexes when external conditions have been modified.

2. Materials and methods

2.1. Materials

Isabrown eggs were obtained from local wholesale distributor, they were 3 or 4 days old. All chemicals (analytical grade) were purchased from Sigma (Saint Quentin-Fallavier, France). Purified phosvitin (Pvt) was obtained by isolation and further purification method developed in our laboratory (Castellani, Martinet, David-Briand, Guérin-Dubiard, & Anton, 2003). At first, phosvitin was isolated from egg yolk granules by solubilization in water. Then, isolated phosvitin was purified by anion exchange chromatography using a SOURCE 15Q column from Amersham Pharmacia Biotech AB (Upsala Sweden). Purified phosvitin was virtually free of iron (0.03 atoms of iron per phosvitin molecule).

2.2. Methods

2.2.1. Iron determination

Free iron content was determined according to Sandell (1950), as reported by Grogan and Taborsky (1986). The method is based on the colorimetric determination of ferrous iron/o-phenanthroline complex, which absorbs at 508 nm. Because of the reducing action of hydroquinone, all free iron (in ferrous and ferric forms) present in solution were measured. Final concentrations of hydroquinone and o-phenanthroline in the colorimetric tube were 9.15 and 12.5 mM, respectively, and the final pH value of colorimetric reaction was around 5.0.

2.2.2. Iron binding capacity of phosvitin

Iron binding capacity was studied in solution following the method described by Guérin-Dubiard, Anton, Dhene-García, Martinet, and Brulé (2002) with some modifications. Increasing quantities of iron from freshly prepared solutions of $Fe(NH_4)_2(SO_4)_2.6H_2O$ in 10 mM HCl were added to phosvitin solutions of 0.77 mg/ml (AU_{280} 0.21). These solutions were named interaction solutions. Sixteen different concentrations, from 0 to 2.7 mM of ferrous ions were tested. These concentrations represented 0–180% of saturation of available sites. The last estimation was based on the three following parameters: 100% of iron saturation was defined by an Fe/P ratio of 0.5 (Grogan & Taborsky, 1986), average molecular mass of phosvitin is 32,000 Daltons, and protein has 123 serine residues per phosvitin molecule (Byrne et al., 1984), which are all phosphorylated (Clark, 1985). We have performed controls which were corresponding with the phosphorous/protein ratio derived from last parameters. After one hour of interaction, except for some specific cases where the interaction time was modified (indicated in the text), interaction solutions were diluted ten times in 10 mM HCl. To this diluted solution, an equal volume of reaction mixture (18.3 mM hydroquinone and 25 mM o-phenanthroline in 50% ethanol solution) was added. Immediately after that, the solution was stirred (Top-mix vortex, Bioblock Scientific, Germany) and the absorbance at 508 nm was measured using a spectrometer Lambda 12 (Perkin-Elmer, Germany). The optical density was related to free iron quantities by applying the same colorimetric method to ten standard solutions. The calculation of bound iron was made by plotting $y = f(x)$ (where y was free determined iron, and x was total added iron), and making an extrapolation to zero value of the ordinate.

2.2.3. Study of influence of pH and ionic strength on iron binding capacity of phosvitin

Different 50 mM buffers were employed to control the pH of the interaction solutions: glycine (pH between 2.9 and 3.5), acetic acid/sodium acetate (3.5–5.0), MES (6.5–7.1) and Tris–HCl (7.1). The tested pair values of pH and ionic strength (I) were: pH = 2.9, I = 0.35 M; pH = 3.5, I = 0.15 M and 0.55 M; pH = 5.0, I = 0.07 M, 0.35 M and 0.63 M; pH = 6.5, I = 0.15 M and 0.55 M; pH = 7.1, I = 0.35 M. When necessary, desired ionic strength values were obtained by addition of NaCl. We have

used a central composite model design. It was constituted of 10 tries distributed on a circle. The central try (pH = 5.0 and I = 0.35 M) was replicated four times to estimate the repeatability. A surface response was fitted to the experimental points following a quadratic equation by using STATGRAPHICS software (Statistical Graphics Corporation, Rockville, USA). The pH values were also checked after addition of phosvitin and ferrous iron solution, and we did not detect any modification of the pH.

2.2.4. Effect of high pressure and thermal treatments on the iron binding capacity of phosvitin

High pressure processing was carried out in a 1.5 l unit (ACB Pressure System, Nantes, France) equipped with both temperature and pressure regulator devices. Prior to pressure processing (pre-treatment process), phosvitin solutions were vacuum packaged in a polyethylene bag (La Bovida, France). Samples were subjected to high pressure treatment at 300 and 600 MPa (±7 MPa) for 10 min. The level of pressure was reached at 6.5 MPa/s, then released at 20 MPa/s. The temperature of transmitting medium in the vessel was settled at 10 °C (±2 °C) during pressure treatment. Phosvitin solutions (0.77 mg/ml) at pH 6.5 and ionic strength 0.15 M were employed. After treatments the iron binding capacity of phosvitin was determined like already mentioned, and two controls were employed, in one of them iron binding capacity of phosvitin without any treatment was measured, and in the other one, pretreated phosvitin solutions (vacuum packaging) without high pressure treatment were analysed.

For thermal treatments, phosvitin solutions at the same concentration (0.77 mg/ml) were kept in a closed tube at 50 or 90 °C by 60 min (Ter2, IKA®, Germany). Volume correction (<5%) was performed with the corresponding buffer, due to evaporation process. Then the determination of iron binding by phosvitin was carried out.

2.2.5. Stability of iron/phosvitin complex

After the iron fixation by phosvitin (interaction solution) at pH 6.5 and ionic strength 0.05 M, the stability of iron/phosvitin complexes was studied as follow:

- by acidification: the pH was abruptly lowered to 3 using HCl solution (1.2 M). The iron binding capacity was determined after 0, 1.5 and 3 h.
- by using a chelating agent (o-phenanthroline): iron binding capacity determination was employed, but the colorimetric measures were carried out after 0, 1.5, 3 and 24 hours after the addition of the hydroquinone/o-phenanthroline mixture. To inhibit light interference during the colouring process (Grogan & Taborsky, 1986), the tubes were kept in the darkness.

2.2.6. Native polyacrylamide gel electrophoresis

Stacking gel was constituted by 3.5% of acrylamide in 0.124 M Tris–HCl buffer at pH 6.8, and the running gel by 6.5% of acrylamide in 0.05 M Tris–HCl/0.36 M glycine at pH 8.8. Electrophoresis buffer was constituted by 0.05 M Tris–HCl and 0.42 M glycine at pH 8.8. Corresponding phosvitin solutions were diluted (4:5) with sample buffer (0.25 M Tris–HCl pH 6.8, 0.04% bromophenol blue and 30% glycerol). About 30 μg of total protein were loaded onto the gels. Electrophoretic migration was performed at 30 mA per gel for 1.5 h in an Amersham Biosciences system (Hoefer, Mighty Small II SE250/SE260, San Francisco, USA). Staining was performed with Stains-all (Wallace & Begovac, 1985). The gel images were taken with a GS-710 Imaging Densitometer and analysed using Quantity One (Quantitation software, version 4.1.0), all from BioRad Laboratories (Marnes la Coquette, France).

2.2.7. Circular dichroism

Phosvitin solutions (0.77 mg/ml) were freshly prepared at pH 6.5 and 0.15 M of ionic strength, using MES (0.05 M) buffer system, as already described. Iron was added from a 100 mM $Fe(NH_4)_2(SO_4)_2$ solution, and the interaction was allowed for 1 h (like for the iron binding capacity determination). Then, circular dichroism measures were performed at 25 °C on a Jobin Yvon CD6 recording spectrometer (Longjumeau, France), in 0.1 mm path-length cells. Recorded dichroism data, in the wavelength range of 250–190 nm, were converted to values of mean residue ellipticity, $[\theta]$ ($degree \times cm^2 \times decimole^{-1}$) taking the average molecular mass of phosvitin 32,000 Da, and 217 amino acids by molecule. Samples were analysed by triplicate and three spectra of each sample were used.

2.3. Statistical analysis

Except for the experimental design (see influence of pH and ionic strength on iron binding capacity of phosvitin), three replicates were made for all measurements. Stability of iron/phosvitin complex and technological treatment studies were subjected to a 1-way analysis of variance using STATGRAPHICS software (Statistical Graphics Corporation, Rockville, USA). Confidence intervals were set at 95% ($P < 0.05$).

3. Results

3.1. Effect of pH and ionic strength on iron binding capacity of phosvitin

Fig. 1 shows the estimated response surface of iron fixation by phosvitin versus pH and ionic strength. The analytical results indicate a significant relationship at a

confidence level of 99%. The equation which describe the estimated response surface is:

$$FI = -343.7 + 293.7 \times IS + 123.0 \times pH - 56.4 \times IS \times pH - 7.8 \times pH^2 \ (r2 = 0.97)$$

where FI is the fixed iron in μg/mg of phosvitin, pH is the pH value and IS represents the ionic strength value. We did not observe any correlation between FI and IS^2 even at 95% confidence level.

These results mean that there is a relationship between the iron binding capacity of phosvitin and both the pH and ionic strength conditions. The best capacity was obtained in the zone of high pH and low ionic strength values (nearby 6.5 and 0.15, respectively): 115 μg of iron/mg of phosvitin, or almost 100% of saturation of disposable sites.

The influence of ionic strength on the iron binding capacity of phosvitin depends on the pH value. For pH values upper than 5.2, the higher the ionic strength, the lower the iron binding capacity. On the contrary, for pH values lower than 5.2, iron binding capacity increases when increasing the ionic strength.

At acid pH values (lower than 3.5), and specially at low ionic strength, the iron binding capacity of the protein is strongly diminished (nearby zero). In the analysis conditions, the interaction time between protein and iron is 1 h, but if we increase the interaction time up to 5.5 h it is possible to have some iron fixation (44.6 μg/mg of phosvitin).

We have carried out iron binding studies with two different types of buffer molecules at the same conditions of pH and ionic strength (see Section 2). The objective was to verify that the influence of pH and ionic strength values on iron binding capacity of phosvitin was not correlated to the nature of different buffer molecules. We have concluded that this capacity is not affected by the type of ion used for buffer systems in this study, because the obtained results for pH 3.5 or 7.1 were comparable using two different buffer systems.

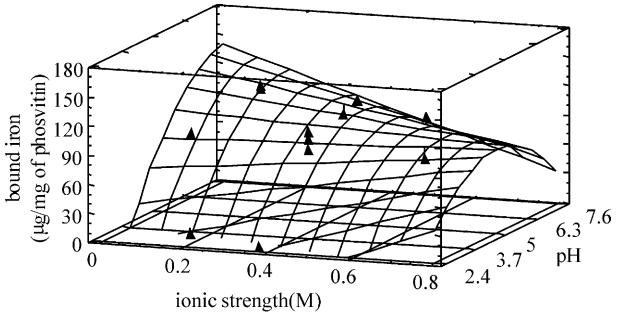

Fig. 1. Iron fixation by phosvitin versus pH and ionic strength (M) values. The estimated response surface is represented in μg of bound iron per mg of phosvitin. Triangles: experimental points.

3.2. Characterization of iron/phosvitin complex

Possible phosvitin changes in secondary structure at different levels of iron saturation were investigated by circular dichroism (Fig. 2). The profile obtained for a free iron phosvitin solution at pH 6.5 and ionic strength 0.15 (0% of iron in the figure) indicates an unordered secondary structure of phosvitin. In these conditions of pH and ionic strength, iron/phosvitin complexes formed at 33 and 93% of iron saturation have similar CD profiles. These results are in accordance with those of Taborsky (1980). For 178% of iron saturation and after 1 h of interaction, a precipitate was detected. These insoluble particles impair the circular dichroism determination under this conditions.

Native electrophoresis of the same samples is shown in Fig. 3. Free iron phosvitin (0% in the figure) showed the three phosvitin polypeptides (β, α_1, α_2), and additional phosphorylated polypeptides with high electrophoretic mobility named phosvettes (Castellani et al., 2003; Wallace & Morgan, 1986). When increasing the iron concentration until 93% of saturation, these four principal proteins have a higher mobility, and from 93% of iron saturation, a smear appeared. When iron saturation was 178%, the electrophoresis profile showed proteinaceous material having lesser mobility than free iron phosvitin, which indicates a possible aggregation in the system. This later result is in agreement with those of circular dichroism.

3.3. Effect of technological treatments on the iron binding capacity of phosvitin

We also investigated the iron binding capacity of phosvitin, which was previously treated by two different technological process. We have studied the influence of thermal and high pressure treatments. We have treated phosvitin at 50 and 90 °C for 60 min, and we have observed that temperature does not modify the capacity

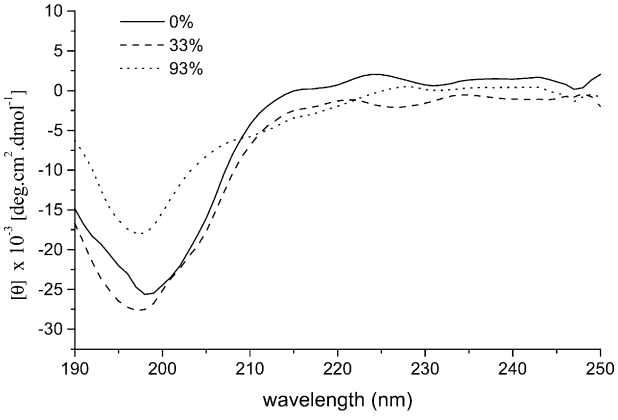

Fig. 2. Circular dichroism of free iron phosvitin (0%) and iron/phosvitin complexes at 33 and 93% degree of iron saturation. Determinations were made at pH 6.5 and 0.15 M of ionic strength.

of phosvitin to interact with iron atoms, even after relatively hard treatments like heating the protein at 90 °C for 60 min (data not shown).

We have studied the secondary structure of phosvitin after thermal treatments by circular dichroism (Fig. 4). The profile at 50 °C is similar to that of the protein without thermal treatment (control) indicating that the protein keeps its unordered structure. However, the profile changes for the treatment at 90 °C: the peak minimum shifts from 198 to 209 nm and a small positive peak at 200 nm appears. Remarkably, this change in secondary structure has no influence on phosvitin iron

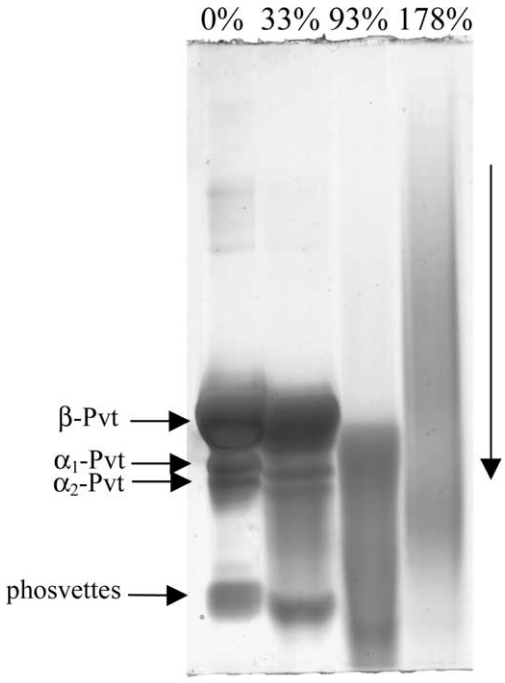

Fig. 3. Native polyacrylamide gel electrophoresis (6.5% of acrylamide) of phosvitin solutions at different percentages of iron saturation. The degree of iron saturation of phosvitin (0–178%) is indicated on the top of the gel. Vertical arrow indicates the direction of negatively charged protein migration. Dye: stains all.

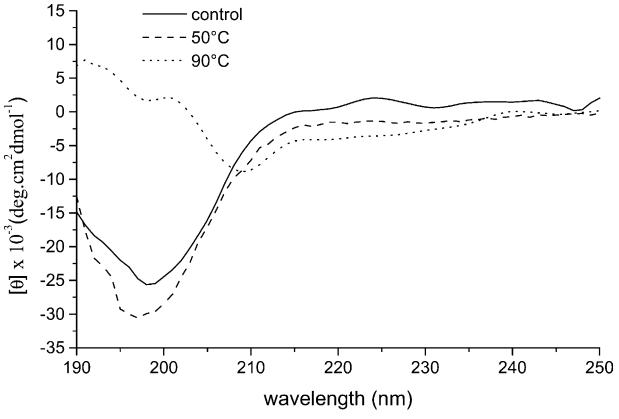

Fig. 4. Circular Dichroism profiles of phosvitin solutions before (control) and after (50 and 90 °C) heat treatments. Determinations were made at pH 6.5 and 0.15 M of ionic strength.

binding capacity. Chung and Ferrier (1995) have detected, by differential scanning calorimetry, a phosvitin structure modification at 77 °C and some changes in its emulsifying activity when heating the protein over 60 °C. This structure modification could be related to the changes that we have observed by circular dichroism when heating phosvitin at 90 °C.

Concerning the high pressure treatment only a little decrease to 95% during the pre-treatment process (vacuum packaged in polyethylene bag) was observed. After that, pressure treatments did not shown any significant difference. With respect to the secondary structure, no change in circular dichroism profile was detected, and the profiles were all typical of unordered structure (results not shown). Consequently, this protein appears to be very resistant to hydrostatic pressure.

3.4. Stability of iron/phosvitin complex

We have studied the stability of iron/phosvitin complexes in non-favourable conditions. Before the study, these complexes were formed in favourable conditions (pH 6.5 and ionic strength 0.15 M). We have employed two different method: decrease of pH or presence of a strong chelating agent. In Table 1, we can observe that acidification of solution did not show a relevant modification on iron binding capacity. So, at pH 3 the protein have a low capacity to bind iron atoms, but at the same pH iron/phosvitin complex previously formed at pH 6.5 are stable. The iron fixation is strong and is not weakened by the high proton activity present at this pH value. On the contrary, in the presence of a chelating molecule like o-phenanthroline and of a reducing agent (hydroquinone), iron/phosvitin complex is unstable (Table 1) and, after 24 h of contact, all initially bound iron formed coloured complexes with o-phenanthroline.

4. Discussion

4.1. pH and ionic strength influence iron binding capacity of phosvitin

The study of the influence of pH and ionic strength on the iron binding capacity of purified phosvitin indicates that the best conditions for iron fixation are almost neutral pH (6.5) and low ionic strength (0.15 M). This is probably because, at this pH, phosphates are completely ionized, and there are not enough salt ions to prevent the iron binding by phosvitin. The maximum iron binding capacity was 115 μg of iron/mg of phosvitin, which corresponds to one iron per two phosphate groups. This saturation value was also described by Taborsky (1963), and indicates that, in spite of the structure of iron/phosvitin complex, which could be tetrahedral or octahedral (Webb, Multani, Saltman,

Table 1
Percentage of retained iron by phosvitin after different treatments. The initial conditions of interaction were: pH 6.5, ionic strength 0.15 M, for 60 min

Time of treatment (min)	Acidic treatment (pH 3)	Chelating treatment (o-phenanthroline)
0	100±5	100±8
90	92±3	75±4
180	93±3	70±4
1440	nd	0

nd, not detected.

Beach, & Gray, 1973), iron has four coordinating positions occupied by oxygen atoms which belong to phosphoserines.

For the other conditions, lower iron quantity was bound, and in more acidic conditions (pH values lower than 3.5), there was no significant fixation. These results are in agreement with the ultrafiltration studies performed by Taborsky (1980) at pH 2, where he also mentioned an acidic precipitation. In this study, however, we did not detect any acidic precipitation of iron/phosvitin complexes or of other species up to one hour of interaction. It is likely that precipitation occurs at pH values lower than 2.9 (our lowest pH value studied), where the secondary structure of phosvitin changes. The weak fixation obtained at acidic pH is not related to an inhibition of iron autoxidation because, at this pH value and for a time of 1 h, this process is largely allowed (Taborsky, 1980). So, even if little proportion of iron could remain in ferrous form, ferric ions must predominate and we did not detect any iron binding. We could infer from these results that the presence of partially protonated phosphates could inhibit the formation of iron/phosvitin complex. A lower capacity of interaction with Ca^{+2} and Mg^{+2} ions when lowering the pH value has also been observed for phosvitin (Grizzuti & Perlmann, 1973). These authors have described that metal binding capacity decreases to 40% and 25% for magnesium and calcium, respectively, when pH value is modified from 6.5 to 4.5. Another phosphoprotein, the β-casein of milk, whose metal binding properties have been well studied, behaves similarly, even though it has a notably lower degree of phosphorylation (only between 4 and 5 phosphoserines per molecule). Calcium binding capacity of this milk protein decreases with 70% when pH goes down from 7 to 5.5 values (Baumy & Brulé, 1988).

It was observed that under acidic conditions phosvitin increases its iron binding capacity when it has more time to interact with iron. One explanation for this phenomenon is that the interaction between ferric ions and single charged phosphate esters is weak, so the iron is detected as free iron during the iron binding determination. When iron and phosvitin have more time to interact, the presence of ferric ions nearby the electronic system of partially protonated phosphate esters may induce the dissociation of the residual proton and, as a consequence, they could form a more stabilised complex. This hypothesis is illustrated in Fig. 5. At this pH value, this process would be kinetically more relevant than in other conditions where the proton activity is lower.

The influence of the ionic strength on the iron binding capacity of phosvitin shows, in the range of studied values, a linear relationship. This indicates that, for this variable, a maximum is not achieved under the tested conditions. More interesting is the fact that the slopes at different pH values have opposed signs, the critical pH value is 5.2. Like mentioned before, for higher pH values the increase in salt concentration leads to a decrease in iron binding capacity, and for lower pH values the system describes the opposed tendency.

The pH value of 5.2 is suggestive because it is just below the pH of the second pK_a of phosphoserines (5.8), and so, the proportion of partially protonated phosphates begins to be important. Consequently, when phosvitin has a large proportion of completely dissociated phosphates, the presence of salt ions might have a screening effect between iron atoms and phosvitin when increasing NaCl concentration. On the contrary, when increasing the proportion of partially protonated phosphates, the addition of NaCl induces the proton ionisation by the reduction of it pK_a value, as described by Baumy, Guenot, Sinbandhit, and Brulé (1989) for the β-casein system. This effect could improve the iron binding capacity of phosvitin. In spite of the fact that interactions at pH values under 5.2 are improved in the presence of NaCl, the iron binding capacity of phosvitin is lower than the optimal value, as it presents, at pH 3.6, 40% of its maximal capacity.

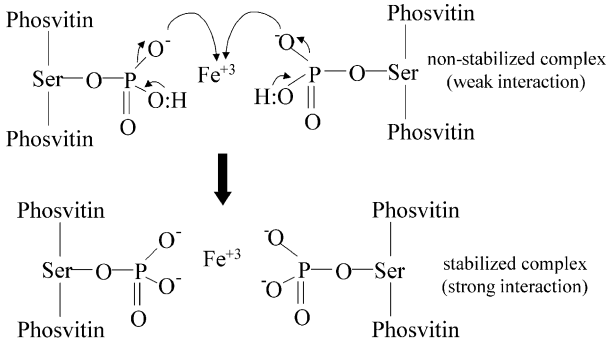

Fig. 5. Proposed mechanism for the transition from a weak to strong interaction between ferric ion and phosvitin at pH 3.5. The thin arrows show the direction of the electronic density induction. The phosvitin chain is always the same molecule, in view of the fact that we are working with intramolecular complexes. Only four sites of coordinate valences are represented, even though it does not mean if the real coordination is tetrahedral or octahedral.

4.2. During iron binding, phosvitin structure is unordered and compact

The study of different iron/phosvitin complexes at pH 6.5 and ionic strength 0.15 (for 0, 33, 93 and 178% of iron saturation) has indicated that until 93% of iron saturation the protein does not change its unordered structure. Even if iron could partially neutralize negative charges of phosphoserines, the iron/protein complex keeps the same secondary structure as the free iron protein. This is in agreement with Taborsky (1980), and it is possible that the remaining charges of phosvitin are negative enough to maintain the unordered structure. For the highest saturation value (178%) the measure of secondary structure was not possible because of protein aggregation, which was also observed by native electrophoresis. Actually, we had already observed this phenomenon at a 150% of saturation (data not shown). If we keep in mind that the atomic Fe/P ratio for phosvitin saturation is 0.5, when saturation is 150%, the atomic ratio between Fe/P is 0.75. In this case, considering only ferric ions (+3) and phosphoserines (−2), the charges in the system have a ratio of 1.1. It is very close to the electrostatic neutrality, and the interactions between different protein molecules become facilitated because of reduction of electrostatic repulsion. Moreover, a ratio of 1.1 indicates a scarcely excess in positives charges, that could be explained by the fact that we have not considered the presence of other charged amino acids.

From native electrophoresis results it was also possible to obtain information about the spatial organisation of iron/phosvitin complexes. When phosvitin forms complexes with iron atoms, these complexes have higher molecular mass and also lower negative charge than free iron phosvitin, so one expects that complexes have less electrophoretic mobility. Our results at 33 and 93% of iron saturation degree showed the opposite tendency. We can hypothesize that when phosvitin binds iron the complex has a more compact spatial shape. These more compact molecules may show a higher native electrophoretic mobility even if they are molecules with a lower charge/mass ratio, because of a reduction in the resistance to migrate through the network gel. These results are in accordance with those of Taborsky (1980), where the more saturated iron/phosvitin complexes resulted more retained by gel filtration chromatography, and consequently more compact in shape. In the presence of iron, more compact molecules could elute at higher elution volumes, even if they have a higher molecular mass than free iron phosvitin. This conclusion does not disagree with circular dichroism results, because it is possible to have spatial changes that not affect the main distribution of secondary protein structure. For 33% of iron saturation, there are the same electrophoretic bands as for iron free polypeptides but having higher electrophoretic mobility, that could indicate that the phosvitin polypeptides have bound iron atoms in similar proportions. At 93% iron saturation, there is a higher number of proteinaceous species (smear), and the β-phosvitin band is not present. This could indicate that, when iron saturation is about 100%, the different phosvitin molecules have bound different quantities of iron, generating many different iron/phosvitin complexes.

4.3. Iron binding capacity of phosvitin is not modified by technological process

Iron binding capacity of phosvitin, at pH 6.5 and ionic strength 0.15, have not been changed by thermal treatments, even with a harsh treatment like 90 °C for 1 h. These results correlate well with the fact that the protein has an unordered structure, so the influence of heat treatment, that usually unfold proteins, is not observed. Our results complement those of Albright et al. (1984), who detected that heating iron/phosvitin complexes did not release bound iron. Grizzuti and Perlmann (1975) described that the interaction between phosvitin and metals (Mg^{+2}, Ca^{+2}, Mn^{+2}, Co^{+2}) are not modified until 60 °C. Nonetheless, the stability of free metal protein and of a metal/protein complex could be not the same. Complexes should be more stable than free proteins. Our results, concerning phosvitin solutions, are coincident with those of the already mentioned authors, so we could assert that the heat resistance, particularly with respect to its iron binding capacity, is not only a property of the iron/phosvitin complex, and that the protein by itself has a high thermal resistance. In the same way, for pressure treatments no modification was detected, being also possible that its unordered structure and high negative charge prevents modifications on the iron binding capacity of phosvitin. From these results, we could confirm that, at least with respect to the iron binding capacity, the protein is not modified during these kind of processes.

4.4. Good stability of iron/phosvitin complex

We have also studied the influence of acidification and chelation treatments on the iron/phosvitin complex. Complexes formed under favourable conditions are very stable. In this way, the decrease of pH to values where the formation of iron/phosvitin complex is normally prevented, did not release the iron bound by phosvitin. These results imply that there must be some modifications when the complex is formed. Between them, ion distances or the addition of species to complete the coordination sites of the complex are highly possible. This type of modifications should stabilize the complex. With respect to this later hypothesis, one could think that in an initial step the iron ions interact with only one phosphate group, and once their interaction is formed, another phosphate completes the process (tetrahedral

stoechiometry), or in the case where the complex has an octahedral stoechiometry two other molecules (i.e. water molecules) could complete the system. Once this condition is reached, the activation energy to dissociate the complex could become high. Consequently, once the complex is formed, its dissociation by protons is not observed, even in the presence of a high proton activity and in conditions where the complex formation is prevented. Only the presence of a chelating agent (o-phenanthroline) with stronger iron binding capacity than phosvitin could release iron from the complex. This result is in agreement with the one reported by Albright et al. (1984), where EDTA was capable of releasing iron from phosvitin. These results could have interesting repercussions in the industry application, because it is possible to find conditions where iron could rest sequestered by phosvitin or could be released from it.

5. Conclusion

Phosvitin presents a natural high iron binding capacity, and the best physicochemical conditions are pH 6.5 and ionic strength 0.15 M. In these conditions, phosvitin binds one iron every two phosphoserines, which is considered to be its saturation capacity. The ionic strength has different influences on the iron binding capacity of the protein depending on the pH value. Aggregates between iron and phosvitin arise when the neutrality of the system is reached (150% of saturation). We have also observed that iron/phosvitin complex are stable at conditions that do not allow their formation (acidic conditions). Iron phosvitin is poorly affected by food processing treatments, and only the presence of a strong chelating agent could release the iron fixed by phosvitin. Finally, it is possible to improve or to weaken the iron binding capacity of phosvitin by manipulating external parameters (i.e. pH, ionic strength, etc.). More studies are needed to better understand the molecular mechanism implicated in this behaviour.

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