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Effect of pH and ionic strength on the binding of bivalent cations to β -casein

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Summary

The binding capacity of β -casein for Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+} was studied at various pHs (from 8.00 to 5.00) and different ionic strengths (about 0, 0.05, 0.10).

β -casein can bind 7 bivalent cations atoms, at pH higher than 7.00 and low ionic strength, before precipitation occurs. For Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} the binding ability decreases with pH or when ionic strength increases. For Cu^{2+} and Fe^{2+} , no effect of pH and ionic strength were detected. Affinity constants of β -casein towards Ca^{2+} , Mg^{2+} , Zn^{2+} and Mn^{2+} were investigated using SCATCHARD's plot at pH 6.60 and ionic strength=0.10 ; it shows different kinds of binding sites (phosphoserilyl, carboxylic) and different affinities for the cations corresponding to different kinds of links.

Key words : β -casein - Bivalent cations - Binding - pH - Ionic strength.

Résumé

Effet du pH et de la force ionique sur la fixation des cations bivalents par la caséine β

Une étude sur les capacités de fixation de la caséine β pour Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+} a été réalisée à différents pH (de 8,00 à 5,00) et à différentes forces ioniques (0, 0,05, 0,10).

A pH supérieur à 7,00 et à force ionique faible, la caséine β peut fixer jusqu'à 7 atomes de cations bivalents sans précipiter. Pour Ca^{2+} , Mg^{2+} , Mn^{2+} et Zn^{2+} , ces capacités de fixation diminuent quand le pH chute ou quand la force ionique s'accroît. Ces deux caractéristiques du milieu n'ont pas d'influence sur la fixation du Cu^{2+} et Fe^{2+} . Les constantes d'affinité déterminées par la représentation de SCATCHARD de cette protéine vis-à-vis de Ca^{2+} , Mg^{2+} , Zn^{2+} et Mn^{2+} ont été déterminées à pH 6,60 et pour une force ionique de 0,10. Différents types de sites (phosphosériques et carboxyliques) ont été mis en évidence ; ils ont des affinités variables pour les cations et correspondent à différents types de liaison.

Mots clés : Caséine β - Cations bivalents - Complexation - pH - Force ionique.

Introduction

β -casein represents 33 % of cow's milk caseins. This protein, which is composed of 209 amino acids, has a molecular weight of 24000 ; it shows 7 variants containing 5 phosphoserines in position 15, 17, 18, 19 and 35 except for C and D variants (4 phosphoserines) (EIGEL *et al.*, 1984).

The peptide composed of the 50 N-terminal amino acids of the protein is hydrophilic, while the C-terminal chain is hydrophobic. In solution, when physicochemical characteristics of the aqueous phase (pH, ions) reduce the net charge of the protein, the hydrophobic interactions become preponderant over charge repulsion ; it leads to aggregation and precipitation (WEST, 1986) at temperature higher than 4 °C (SCHMIDT, 1982) ; at 4 °C only soluble monomers are present.

Caseins bind various bivalent cations : Ca^{2+} , Mn^{2+} (LONNERDAL *et al.*, 1985), Zn^{2+} (HARZER and KAUER, 1982) Fe^{2+} , Mg^{2+} , Cu^{2+} (BRULE and FAUQUANT, 1982 ; FRANSON and LONNERDAL, 1983). Their binding ability depends on pH, ionic strength, temperature (PARKER and DALGLEISH, 1981) and the amount of phosphate in the solution (CREAMER and YAMASHITA, 1976). The binding of more than 6.75 calcium ions on a β -casein molecule causes its precipitation (PARKER and DALGLEISH, 1981) : this result shows that binding to phosphoseryl and carboxylic residues is involved as demonstrated by ONO *et al.* (1976) for α_{s1} -casein and by ONO *et al.* (1980) for K-casein.

HUMPHREY and JOLLEY (1982) have demonstrated, using ^{31}P NMR, that the phosphoserines of β -casein have different pK. The pK are modified by calcium binding and ionic strength ; the calcium binding does not occur simultaneously on all the phosphoserines (BAUMY *et al.*, 1988).

This work investigates the effect of pH and ionic strength on the binding capacities of β -casein for various cations (Ca^{2+} , Mg^{2+} , Zn^{2+} , Mn^{2+} , Cu^{2+} , Fe^{2+}), and the affinity constants of the protein towards different cations.

I. Materials and methods

A. Purification of β -casein

β -casein was isolated from sodium caseinate (ARMOR PROTEINES - 35460 Saint-Brice-en-Cogles, France) using a modification of the method of MANSON and ANNAN (1971). The caseinate was dissolved (2 g.l^{-1}) in 1100 l. of cold water (2 °C) ; the pH was adjusted to 4.37. After 16 hours, the supernatant containing 0.42 g.l^{-1} β -casein was filtered on SEITZ Zenith V 40 filter (Supra EK) then concentrated at pH 7.00 and 30 °C by ultrafiltration on a ROMICON equipment composed of 2 cartridges PM 50 (4.9 m^2) up to a volume concentration ratio of 50, then the retentate was further concentrated on a cartridge PM 10 (1.4 m^2) up to a concentration ratio of 250. A further purification was performed using ion exchange chromatography on FRACTOGEL TSK DEAE-650 M (PHARMACIA) (column : length 240 mm - diameter 65 mm - flow rate 200 ml.h^{-1} - Tris buffer :

0.02 M, pH 7.00, urea 4.5 M, mercaptoethanol 0.01 M - elution : gradient up to 0.35 M NaCl - sample 100 ml at 10 % (w/w) total solids.

The different steps of protein purification were followed by FPLC according to the procedure of ANDREWS *et al.* (1985). The preparation was then demineralized and the urea was removed using dialysis, and was finally freeze dried. Amino acid analysis permitted the calculation of a correlation coefficient equal to 0.994 between the determined and theoretical composition. The β -casein-content of the preparation calculated using N determination (KJELDHAL procedure on a KJELTEC SYSTEM 1003 - TECATOR) was equal to 86 % (w/w) and using phosphorus determination (norme AFNOR NFVB 04 - 284) was equal to 82 % (w/w).

An average of 84 % β -casein (w/w) was used for calculation ; the remainder corresponds to bound water.

B. Sample preparation

The β -casein preparation was dissolved in Milli Q (MILLIPORE) filtered water at 2 g.l^{-1} ; it gives 1.68 g β -casein per liter. Mineral chlorides ($\text{CaCl}_2, 2\text{H}_2\text{O}$; $\text{MgCl}_2, 6 \text{H}_2\text{O}$; ZnCl_2 ; $\text{FeCl}_2, 4 \text{H}_2\text{O}$; $\text{MnCl}_2, 2 \text{H}_2\text{O}$; $\text{CuCl}_2, 2 \text{H}_2\text{O}$ - MERCK - Purity > 99 %) were predissolved at 0.140 M for Ca^{2+} , Mg^{2+} , Mn^{2+} and at 0.049 M for Fe^{2+} , Zn^{2+} , Cu^{2+} , then separately added to the solutions at pH 8.00. The pH was then lowered with HCl (N - N/10) using 0.25 pH steps (20°C) and samples in which β -casein content was constant were isolated to be analyzed. Ionic strength (μ) was modified using NaCl (MERCK).

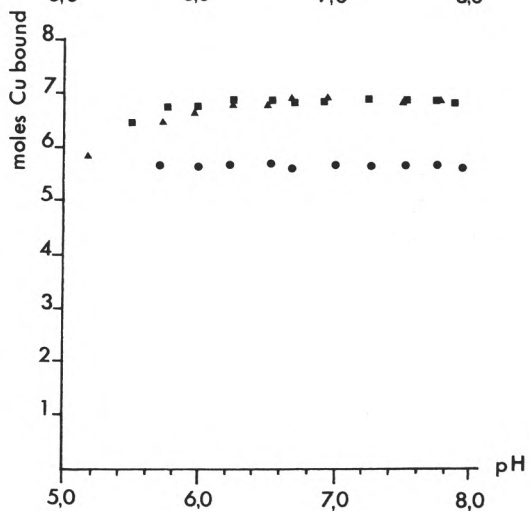
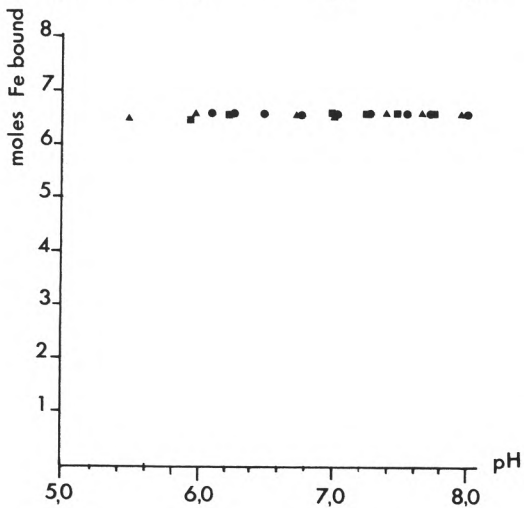
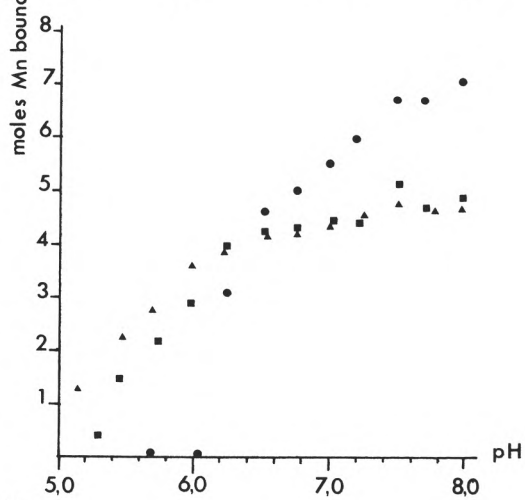
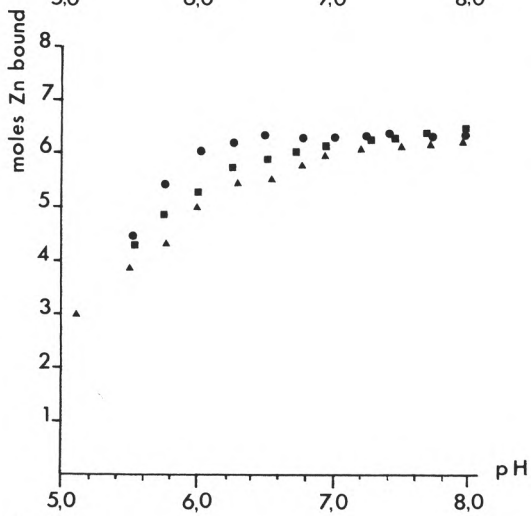
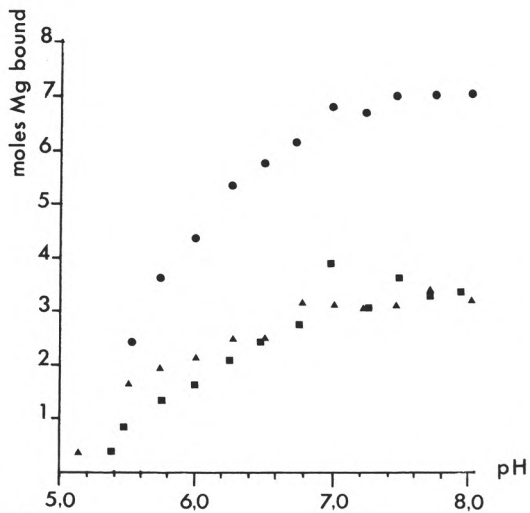
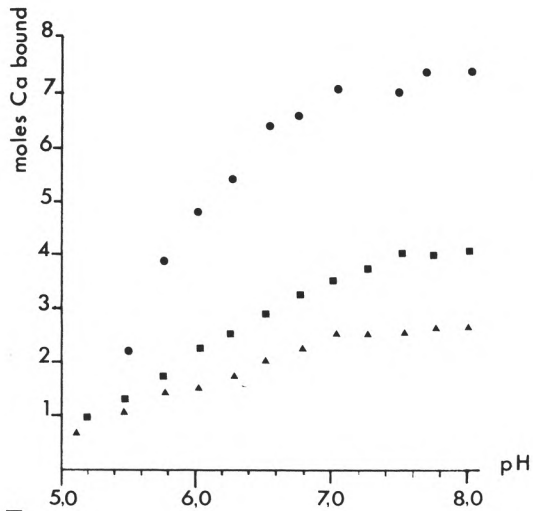
A preliminary investigation of the solubility of β -casein at different pHs and with different concentrations of mineral chlorides was done in order to obtain solutions without any precipitate. The solutions were at first cloudy then aggregates appeared. The maximum concentration of mineral which did not give precipitation was about 20 moles of Ca^{2+} , Mg^{2+} , Mn^{2+} ($1.40 \times 10^{-3} \text{ mol.l}^{-1}$) and 7 moles ($4.9 \times 10^{-4} \text{ mol.l}^{-1}$) of Fe^{2+} , Zn^{2+} , Cu^{2+} per β -casein mole. Precipitation occurred at pH below 5.00 ; the effect of pH was studied between 8.00 and 5.00.

C. Phase separation

Mineral separation between bound protein form and free form was performed using ultrafiltration on Centricron 10 (AMICON) (Cut off : 10 000) with a centrifuge JOUAN CR 4.11 (2 000 g - 90 min - 20°C). The amounts of solution and permeate were respectively equal to 2 and 1 ml. A preliminary ultrafiltration of the studied solution was done in order to saturate the membrane with the cation.

D. Mineral content

Mineral content was determined by Atomic Absorption Spectrophotometry on a VARIAN AA 1275 equipment. Ca and Mg were determined according to the procedure of BRULE *et al.* (1974) (Precision = 0.02 mg.l^{-1}). The determination of Zn, Cu and Mn was made using dilutions of the samples in order to obtain amounts of cation in the solution between 0.5 and $2.0 \times 10^{-3} \text{ g.l}^{-1}$.



The absorption of the iron-protein complex at 248 nm hindered direct determination of total iron content of the samples : accurate amounts of iron were added to the solutions and the determination was only performed for free iron.

II. Results

A. Effect of pH on cation binding to β -casein

The amount of Ca^{2+} , Mg^{2+} , Zn^{2+} bound to β -casein is constant between pH 7.00 and 8.00 then decreases with lowering pH to reach very low values at pH 5.00 (fig. 1). The binding of Mn^{2+} is only constant between pH 7.50 and 8.00. One molecule of protein binds 7 ions of Ca^{2+} at pH 8.00 but only 2 at pH 5.50. On the other hand, the amount of Fe^{2+} and Cu^{2+} bound to protein was not pH dependent. At pH 6.60 (ionic strength about 0) the number of bound cations per mole of protein were respectively equal to 7.4, 7.1, 7.1, 6.4, 6.6 and 5.7 and the binding ratios (amount of mineral bound to protein / total amount of mineral) equal to 36 %, 32 %, 36 %, 98 %, 100 % and 99 % for Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Fe^{2+} and Cu^{2+} . At pH 5.50 the ratios were equal to 11 %, 11 %, 0 %, 71 %, 100 % and 99 %.

B. Effect of ionic strength on cation binding to β -casein

When ionic strength was increased (fig. 1) the amount of bound Ca^{2+} , Mg^{2+} , Zn^{2+} and Mn^{2+} decreased but ionic strength had no effect on the binding of Fe^{2+} and Cu^{2+} . At $\mu=0.10$ and pH higher than 7.50 the maximal bound amounts were respectively equal to 2.7, 3.3, 4.7, 6.3, 6.6 and 6.9 ions of Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Fe^{2+} , Cu^{2+} per β -casein molecule ; the binding ratios are then respectively equal to : 13 %, 15 %, 21 %, 83 %, 99 % and 99 %.



Fig. 1

Amounts of bound mineral (bound mineral moles / β -casein mole) to β -casein (1.68 g.l^{-1}) versus pH for Ca^{2+} , Mg^{2+} , Zn^{2+} , Mn^{2+} , Fe^{2+} and Cu^{2+} for three ionic strengths : about 0 (●), 0.05 (■) and 0.10 (▲).

Total amount of mineral (total mineral moles per β -casein mole) is equal to :

Ca (●) 20.56 ; (■) 20.60 ; (▲) 20.43

Mg (●) 22.16 ; (■) 20.20 ; (▲) 22.19

Zn (●) 6.48 ; (■) 7.32 ; (▲) 7.57

Mn (●) 19.73 ; (■) 21.27 ; (▲) 21.94

Fe (●) 6.64 ; (■) 6.64 ; (▲) 6.64

Cu (●) 5.72 ; (■) 6.98 ; (▲) 7.00

Teneur minérale fixée (moles minéral fixé / mole caséine β) à la caséine β ($1,68 \text{ g.l}^{-1}$) en fonction du pH pour Ca^{2+} , Mg^{2+} , Zn^{2+} , Mn^{2+} , Fe^{2+} et Cu^{2+} à trois forces ioniques : voisine de 0 (●), 0,05 (■) et 0,10 (▲).

La teneur minérale totale est égale aux valeurs indiquées.

C. Affinity determination

The affinity constants and numbers of binding sites on β -casein for Ca^{2+} , Mg^{2+} , Zn^{2+} , Mn^{2+} at pH 6.60 and $\mu=0.10$ were determined using SCATCHARD's plot (1949) : $R/A=f(R)$ (R and A are respectively the concentration of ligand bound to protein and concentration of free ligand calculated for one β -casein mole). Figure 2 shows the presence of two kinds of sites for Zn^{2+} : the first 5 sites have a high affinity of $143\,490\text{ M}^{-1}$ and the two following of $31\,580\text{ M}^{-1}$. The affinities and number of binding sites are respectively equal to $3\,400\text{ M}^{-1}$ (4.4 sites), $1\,880\text{ M}^{-1}$ (2.4 sites), $1\,150\text{ M}^{-1}$ (2.7 sites) for Mn^{2+} , Ca^{2+} and Mg^{2+} .

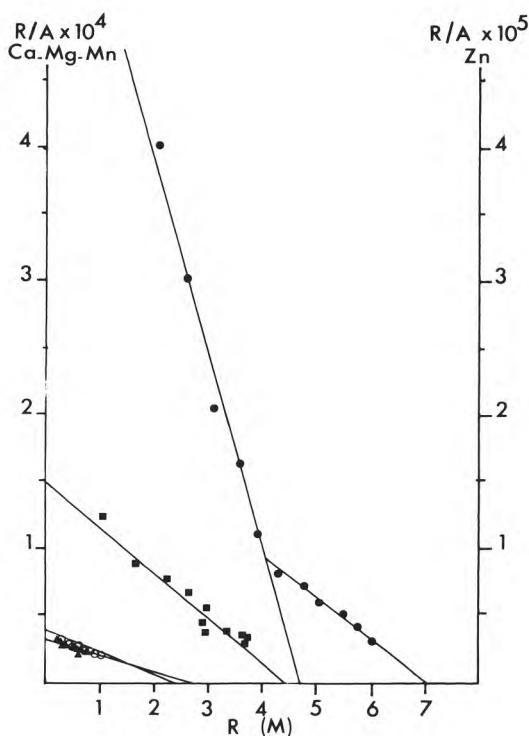


Fig. 2

SCATCHARD's plot (R/A versus R) for β -casein (1.68 g.l^{-1}) and Zn^{2+} (●), Mn^{2+} (■), Ca^{2+} (○) and Mg^{2+} (▲) at pH 6.60 and $\mu = 0.10$.

Représentation de SCATCHARD (R/A en fonction de R) : Caséine β (1.68 g.l^{-1}) en présence de Zn^{2+} (●), Mn^{2+} (■), Ca^{2+} (○) et Mg^{2+} (▲) à pH 6,60 et $\mu = 0,10$.

III. Discussion

The binding capacity of β -casein is at least 7 cations of Ca^{2+} , Mg^{2+} , Zn^{2+} , Fe^{2+} , Cu^{2+} , Mn^{2+} for pH values higher than 7.00 and low ionic strengths. All the Zn^{2+} , Fe^{2+} and Cu^{2+} ions of the solution were bound to β -casein so we can assume that the total concentration of cation, which was imposed to avoid precipitation, was a limiting factor of the binding. These sites are probably composed of 5 phosphoserines and of 2 carboxylic sites, as demonstrated by DICKSON and PERKINS (1971) with alkaline-earth metal ions. SCATCHARD's plot concerning the binding of Zn to β -casein at pH 6.60 and $\mu = 0.10$ shows that phosphoserines have a 4.5 times higher affinity than carboxylic sites. The affinity order which is $\text{Zn} > \text{Mn} > \text{Ca} > \text{Mg}$ depends on the characteristics of the cation. These constants equal to $1\ 880\ \text{M}^{-1}$ for Ca^{2+} and to $1\ 150\ \text{M}^{-1}$ for Mg^{2+} are similar to the ones deduced from the results of BRULE and FAUQUANT (1982) respectively equal to $1\ 180\ \text{M}^{-1}$ and $950\ \text{M}^{-1}$, but are higher than the $520\ \text{M}^{-1}$ value calculated for Ca^{2+} by PARKER and DALGLEISH (1981) (pH = 7.0 - 0.1 M NaCl). The binding constants are very dependent on pH and ionic strength of the aqueous phase (PARKER and DALGLEISH, 1981). The difference between the results of PARKER and DALGLEISH and ours may be due to the use of a higher concentration of β -casein ($5\ \text{g.l}^{-1}$) and of an imidazole buffer which increase ionic strength and so decrease the affinity constant. The maximal numbers of binding sites determined with bound mineral amount and with SCATCHARD's plot are equivalent within the precision of the determinations.

When all the sites are saturated, precipitation occurs. The protein sensitivity seems to be low with Ca^{2+} , Mg^{2+} , Mn^{2+} but more pronounced with Zn^{2+} , Fe^{2+} and Cu^{2+} because of the various affinities of protein towards these cations. At pH 6.60 and at low ionic strength precipitation occurs when all the phosphoserine sites are saturated as previously shown for α_{s1} -casein (AOKI *et al.*, 1985).

pH and ionic strength characteristics of the solutions of β -casein influence the binding capacities of the protein. The decrease in ionization of phosphoserine groups, which takes place at low pH, lowers the mineral binding ability except for Cu^{2+} and Fe^{2+} . Ca^{2+} , Mg^{2+} , Zn^{2+} , Mn^{2+} are bound through ionic links which are very sensitive to pH, and Cu^{2+} or Fe^{2+} also through ionic links with phosphoserine and coordination links with NH_2 , CO_2H , CONH , H_2O .

Ionic strength decreases the mineral binding ability of β -casein; its effect is strong between $\mu = 0$ and $\mu = 0.05$ and more reduced between $\mu = 0.05$ and $\mu = 0.10$: 7.4, 4.1 and 2.7 moles of Ca^{2+} are respectively bound to β -casein for $\mu = 0$, $\mu = 0.05$ and $\mu = 0.10$. The number of binding sites of β -casein for Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} at pH 7.00 and $\mu = 0.10$ are more reduced than the maximal number at pH 7.00 and $\mu = 0$. The phosphoserine groups of β -casein have different pK which depend on their position in the peptide chain (BAUMY *et al.*, 1988); the phosphoserine residues 17, 18 and 19 which have the highest pK may be the preferential binding sites. SCATCHARD's plot does not allow the determination of the affinity of each site because we have insufficient data.

Ionic strength reduces the pK of the second ionization of phosphoserine; they are more dissociated but have a lower affinity towards mineral (BAUMY *et al.*,

1988). The number of calcium binding sites determined at high ionic strength needs to be compared to the 4.6 and 3.4 sites respectively found by DICKSON and PERKINS (1971) (pH 7.4 – $\mu = 0.10$) and by IMADE *et al.* (1977) (pH 6.9 – $\mu = 0.07$); the differences in the results may be due to the pH and ionic strength effects. Cu^{2+} and Fe^{2+} binding ability is not altered by ionic strength because of the presence of coordination links.

The particularly high affinity of β -casein towards Cu^{2+} may explain the low mobility of this cation from inside to rind during ripening of cooked hard cheese (LE GRAET *et al.*, 1986).

This work demonstrates the binding ability of β -casein for Ca^{2+} , Mg^{2+} , Zn^{2+} , Fe^{2+} , Mn^{2+} , Cu^{2+} , the different affinities for Ca^{2+} , Mg^{2+} , Zn^{2+} and Mn^{2+} and the dependence of these links on pH and ionic strength. A further study needs to be done with a mixture of cations to investigate the competition and perhaps to detect specific binding sites.

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