Carbohydrate Polymers 84 (2011) 459–464

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Interaction behavior between chitosan and pepsin

Valeria Boeris[a,*], Yasmine Micheletto[b], Maria Lionzo[b], Nádya Pesce da Silveira[b], Guillemo Picó[a]

[a] Bioseparation Lab., Chemical Physics Department, Faculty of Biochemical and Pharmaceutical Sciences, CONICET, FonCyT and CIUNR, National University of Rosario, Suipacha 570 (S2002RLK) Rosario, Argentina
[b] Instituto de Química, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Agronomia 91501-970, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 19 July 2010
Received in revised form
17 November 2010
Accepted 2 December 2010
Available online 10 December 2010

Keywords:
Chitosan
Pepsin
Protein–polyelectrolyte interaction
Light scattering

ABSTRACT

The interaction between chitosan (a positively charged polysaccharide) and the acidic protein pepsin was studied by the use of dynamic light scattering, electronic spectroscopic approaches and measurements of thermodynamic functions. An interaction, mainly electrostatic, between the enzyme and the cationic polymer was found. The size of the soluble complexes formed between pepsin and chitosan was shown to depend on the pH value. Chitosan was seen to interact with the surface of the pepsin molecule, but no modification was observed in the pepsin secondary structure or in its chemical and thermal thermodynamic stability.

1. Introduction

Chitin (poly(β(1–4)-N-acetyl-D-glucosamine)), an abundant naturally occurring biopolymer, is the main structural component of the invertebrate's exoskeleton and the fungal cell wall. The waste produced in the processing of seafood, mainly crab, shellfish, lobster, and shrimp, is an abundant source of chitin. By alkaline deacetylation, it is transformed in chitosan (Chi). Chi is a weak base with a pKa of around 6.4 and forms water-soluble salts with hydrochloride, glutamic acid and acetic acid (Fan, Hu, & Shen, 2009; LeHoux & Dupuis, 2007; Rangel-Mendeza, Monroy-Zepedab, Leyva-Ramosb, Diaz-Floresa, & Shirai, 2009). The solubilization in aqueous media is caused by protonation of the $-NH_2$ function on the C-2 position of the D-glucosamine repeated unit, whereby the polysaccharide is converted into a polyelectrolyte in acidic media. Chi is the only pseudonatural cationic polymer and, for this reason, it has many applications. Due to its low toxicity, low immunogenicity, biocompatibility, and biodegradability, it is used as a flocculant for protein recovery, as a drug carrier and it is also used to eliminate contaminant substances (Chatterjee, Chatterjee, Chatterjee, & Guha, 2004; Rangel-Mendeza et al., 2009; Rungsardthong, Wongvuttanakul, Kongpien, & Chotiwaranon, 2006; Sinha et al., 2004; Wibowo, Velazquez, Savant, & Torres, 2005).

Chi forms complexes with acidic proteins by simple coulombic attraction (Li et al., 2010; Subramanian, Rau, & Kaligotla, 2006), since the positive charge of the amine polymer groups interacts with the negatively charged groups in the protein. Thus, these complexes are soluble at low pH value (below the pH of the soluble–non-soluble complex equilibrium) while at higher pH, they are non-soluble. This property has made Chi useful for the separation and purification of enzymes by precipitation (Boeris, Spelzini, Farruggia, & Picó, 2009b; Chen & Tianqing, 2008; Montilla et al., 2007; Singh & Gupta, 2008).

Polyelectrolyte precipitation uses a poly-charged macromolecule of opposite electrical charge to the target macromolecule, forming a soluble protein–polyelectrolyte complex under desired experimental conditions; these complexes interact with each other, producing insoluble macro aggregates. This is a suitable method for protein isolation because very low polyelectrolyte concentrations are used (up to 0.1%, w/v). This method sometimes offers a high selectivity and the insoluble complex can be re-dissolved by a pH change or by salt addition (Boeris et al., 2008; Boeris, Romanini, Farruggia, & Picó, 2009a; Montilla et al., 2007; Sperber, Schols, Cohen Stuart, Norde, & Voragen, 2009). This technique offers the possibility of concentrating and purifying the target macromolecule at a low cost.

Since PEP is an aspartic protease widely used in different biotechnological processes, much attention has been given to the development of techniques to isolate and purify this protein. In a previous paper we studied the purification of PEP using Chi as a precipitant (Boeris et al., 2009b). However, we are now interested in studying the Chi capacity to form soluble complexes with PEP. The

Abbreviations: PEP, pepsin; Chi, chitosan.

* Corresponding author. Tel.: +54 341 4804597x253; fax: +54 341 4804598.
E-mail address: valeriaboeris@conicet.gov.ar (V. Boeris).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.12.008

aim of this work is to study the molecular mechanism of the interaction between PEP and this cationic polyelectrolyte and to determine how the presence of Chi affects the structure and stability of this protein.

2. Materials and methods

2.1. Chemicals

Chitosan from crab shells—practical grade (medium degree of deacetylation 85%) and Bovine Pepsin (PEP), crystalline, 3.44 Units/mg Lot number 035k76701 were purchased from Sigma–Aldrich (St. Louis, MO) and were used without further purification. Chitosan (Chi) was dissolved in acetic acid 0.1 M at a concentration of 2% (w/v).

2.2. Dynamic light scattering measurements

The measurements were performed at 25 °C using a Brookhaven Instrument standard setup (BI200 M goniometer, BI9000AT digital correlator) with a vertically polarized Coherent He–Ne Laser (λ = 632.8 nm) as a light source. The scattering volume was minimized using a 0.2 mm aperture and an interference filter before the entrance of the photomultiplier. Polarized homodyne intensity autocorrelation functions $g_2(t)$ were obtained using a multi-τ mode correlator with 224 channels. The scattered light was analyzed after placing the sample cell containing the apparatus in decahydronaphthalene (Aldrich), which is an index-matching liquid.

2.2.1. Data analysis

Normalized electric field correlation functions $g_1(t)$, calculated from the intensity autocorrelation functions $g_2(t)$, were analyzed by using the regularized positive exponential sum (REPES), which employs the Laplace inversion. The resulting $A(\tau)$ is a distribution of relaxation times:

$$g_2(t) - 1 = \beta \left[\int A(\tau) \exp\left(\frac{-t}{\tau}\right) \int d\tau \right]^2 \quad (1)$$

In Eq. (1), t is the delay time of the correlation function and β is a coefficient accounting from ideal correlation. The relaxation time τ or the relaxation frequency $\Gamma(\tau^{-1})$ is associated with a diffusion coefficient D through the relation:

$$D = \frac{\Gamma}{q^2} \quad (2)$$

The scattering vector q takes into account the refractive index of the solvent, n, and the scattering angle, θ, as given in Eq. (3):

$$q = \frac{4\pi n}{\lambda} \sin\left(\frac{\theta}{2}\right) \quad (3)$$

The hydrodynamic radii (R_h) were then calculated from the diffusion coefficient using the well-known Stokes–Einstein equation:

$$R_h = \frac{kT}{6\pi\eta D} \quad (4)$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the solvent.

2.3. Chi effect on the PEP secondary structure

Circular dichroism spectrum scans of PEP in the absence and presence of different Chi concentrations were carried out using a Jasco spectropolarimeter, model J-8150. The ellipticity values [θ] were obtained in millidegree (mdeg) directly from the instrument. The cell pathlength of 0.1 cm was used for the spectral range 200–250 nm. The scanning rate was 5 nm/min. Bandwidth was 1 nm. Five scans of each sample were made.

2.4. Chi effect on the emission of the native PEP fluorescence

It was analyzed by obtaining the fluorescence emission spectrum of the protein in buffer acetate 50 mM medium, pH 3.80 at increasing Chi concentration. The scanning rate was 10 nm/min and the data acquisition was each 0.1 nm with a slit of 0.1 nm. The fluorescence spectra were obtained in a Amico Browman spectrofluorometer Serie 2000 using a thermostatized cuvette of 1 cm pathlength and were corrected using a software provided by the instrument manufacturer.

2.5. Chi effect on the quenching of the native PEP fluorescence by acrylamide

The quenching of the protein's tryptophan residues (TRP) fluorescence was carried out by titration with acrylamide in the absence and presence of different concentrations of Chi. The data were analyzed using the mathematical model for the sphere of action according to Lakowicz (1983):

$$\frac{F_0}{F_i} = 1 + K_D[Q] \exp\left(\frac{v[Q]N}{1000}\right) \quad (5)$$

where F_0 and F_i are the protein fluorescencies at 340 nm (exciting at 280 nm) in the absence and presence of quencher, respectively; K_D, the Stern Volmer constant related to the lifetime of the fluorophore and the bimolecular quenching constant; [Q], the quencher concentration; N, the Avogadro's number and v is the volume of the "sphere of action", i.e., the sphere within which the probability of immediate quenching is unity, and whose radius is only slightly larger than the sum of the radius of the fluorophore and quencher.

2.6. Measurements of the protein surface hydrophobicity (S_0)

The relative surface hydrophobicity of the protein was determined by applying a fluorescence method using 1-Anilino-8-Naphthalene Sulfonate (ANS) as a probe (Cardamone & Puri, 1992; Tubío, Nerli, & Picó, 2004). Stock solutions of 8 mM ANS and 3 mM PEP were prepared in 50 mM acetate buffer (pH 3.80). Aliquots of the protein were added to a sample containing 3 mL of buffer solution containing a final ANS concentration of 2 μM, while the PEP concentration varied from 0 to 10 μM. The fluorescence emission intensities at 470 nm (while exciting at 360 nm) for each protein concentration were measured at a Jasco FP 770 spectrofluoromether. Under the above mentioned experimental conditions (with ANS excess), the initial slope (S_0) of the fluorescence intensity versus protein concentration plot has been shown to be correlated to the relative surface hydrophobicity of the protein (Alizadeh-Pasdar & Li-Chan, 2000). Surface hydrophobicity values were determined using at least triplicate analyses. In all cases, r^2 values of 0.99 were obtained for the linear regression analyses used to calculate surface hydrophobicity (S_0) values.

2.7. Protein thermal stability

Thermally induced unfolding was monitored by absorbance at 280 nm, as it was previously reported (Boeris, Farruggia, Nerli, Romanini, & Picó, 2007). The analysis of the data was made assuming an approximation of a two-state model of denaturation where only the native and unfolded states were significantly populated and the absorptivity coefficients of both protein states were differ-

ent. The unfolded protein fraction was calculated from:

$$\alpha = \frac{A_i - A_D}{A_N - A_D} \quad (6)$$

where α is the unfolded protein fraction, A_N and A_D are the absorbancies of the native and unfolded states respectively, A_i is the absorbance at any temperature. We have used non-linear least squares to fit the unfolded protein fraction versus temperature data, and the temperature at the mid-point of denaturation (T_m) was determined. The equilibrium constant, K, for the unfolding process can be calculated:

$$K = \frac{\alpha}{1 - \alpha} \quad (7)$$

the free energy (ΔG^0) was calculated as:

$$\Delta G^0 = -RT \ln K \quad (8)$$

where R is the gas constant. From a plot of ΔG^0 versus T, the unfolded entropy ΔS^0 was calculated according to:

$$\Delta S^0 = -\frac{\partial \Delta G^0}{\partial T} \quad (9)$$

The enthalpic change was calculated from the equation (Pace, Laurents, & Thomson, 1990; Picó, 1997):

$$\Delta H^0 = \Delta G^0 + T\Delta S^0 \quad (10)$$

Absorbance measurements were recorded on a Jasco 550 spectrophotometer. Sample temperature was controlled by a peltier heating and measured with a thermocouple immersed inside the cuvette. The heating rate was 1 °C/min. The data of absorbance versus temperature were collected by the software given by the instrument manufacturer.

2.8. PEP chemical stability in the presence of Chi

Chemical stability of PEP was assayed by measuring native fluorescence emission, exciting at 280 nm in media of increasing urea and constant Chi and PEP concentrations. We assumed that urea – a denaturant agent – induces unfolding due to its interaction with the protein unfolded form, D (Greene & Pace, 1974; Pace & Vanderburg, 1979). Analysis of the data was performed assuming an approximation of a two-state model of denaturation where only the native and unfolded states were significantly populated and the wavelengths of the maximum of fluorescence of both states were different. A non-linear least squares method was used to fit the unfolded protein fraction versus urea concentration data and the urea concentration at the mid-point of denaturation (C_m) was determined. The unfolded protein fraction was calculated from:

$$\alpha = \frac{\lambda_i - \lambda_N}{\lambda_D - \lambda_N} \quad (11)$$

where λ_N, λ_D and λ_i are the maxima of fluorescence emission of PEP (exciting at 280 nm) in the absence of denaturant, of the unfolded state (at high denaturant concentration) of PEP and in the presence of different urea concentrations, respectively. From Eq. (7) the equilibrium constant can be calculated. The free energy (ΔG^0) was calculated from Eq. (8).

3. Results and discussion

3.1. Determination of the hydrodynamic radii of the soluble PEP–Chi complexes

Fig. 1 shows the size distribution of Chi in a pH 3.00 medium. The size was obtained by extrapolating the apparent radii values obtained at different scattering angles to scattering angle 0°. A typical autocorrelation function for Chi at pH 3.00 and scattering angle of 90° is shown in the inset of Fig. 1. Table 1 shows the mean hydrodynamic radii (R_h) of Chi in solution at three different assayed pHs obtained from dynamic light scattering measurements. The pH range selected was 3–4 as the complex is soluble in this range; a higher pH was not assayed because of the possibility of turbidity. A slight decrease in the R_h was observed with the increase in pH. However, this observation is not conclusive. When the same experiment was performed in the presence of a constant PEP concentration, a dramatic decrease in R_h value was observed when the pH varied from 3 to 4. Two effects take part in the Chi-PEP complex contraction: first, a loss of the positive electrical charge of Chi by increasing the pH of the medium, and second, the presence of PEP, which has a net negative electrical charge, thus inducing the neutralization of a significant number of positive electrical charges of Chi molecule. Pepsin has a very acid isoelectrical point (pI 1.0) due to the high amount of acid aminoacid residues in its composition (43 out of 327) (Chow & Kassell, 1963; Horn & Heuck, 1983). This means that PEP carries net negative charge from this pH value. Considering the aminoacidic composition of PEP, it is possible to calculate its charge, which increases from −3 to −21 in the pH range assayed. As the negative charge of PEP increases, the interaction with Chi becomes higher. The higher attraction between PEP and Chi results in a lower repulsion between the positively charged amino groups of Chi. This induces an approximation of the Chi chains and, as a result, a decrease in the molecule diameter.

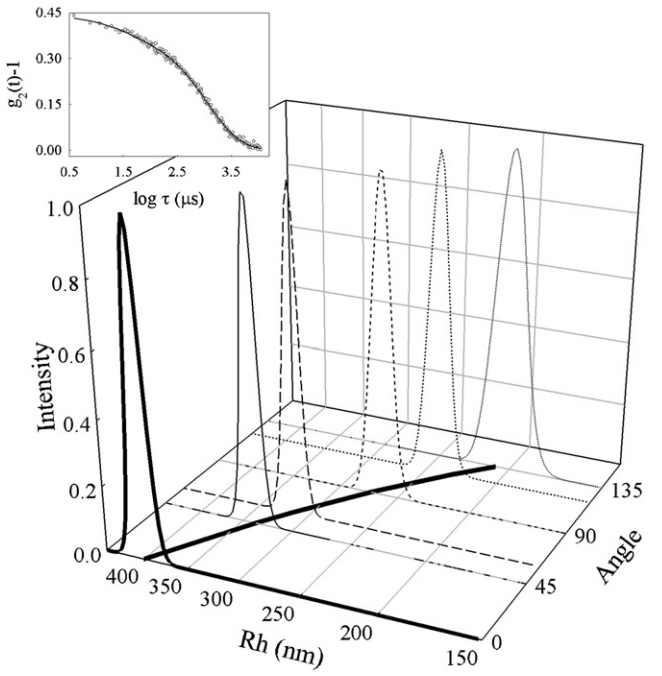

Fig. 1. Size distribution of Chi in a medium of pH 3.00 obtained at different scattering angles and extrapolated to scattering angle 0°. Inset: autocorrelation function for Chi at pH 3.00 and scattering angle of 90°. Temperature 25 °C. PEP concentration 0.25 mg/mL. Chi concentration 0.1% (w/v).

Table 1
Mean radii of the polymer and soluble complexes PEP–Chi at different pH of the medium obtained by dynamic light scattering measurement. PEP concentration 0.25 mg/mL. Chi concentration 0.1% (w/v).

Medium acetate 50 mM	R_h Chi (nm)	R_h Chi–PEP (nm)
pH 3.00	400 ± 10	410 ± 10
pH 3.50	410 ± 10	240 ± 10
pH 4.00	370 ± 10	180 ± 10

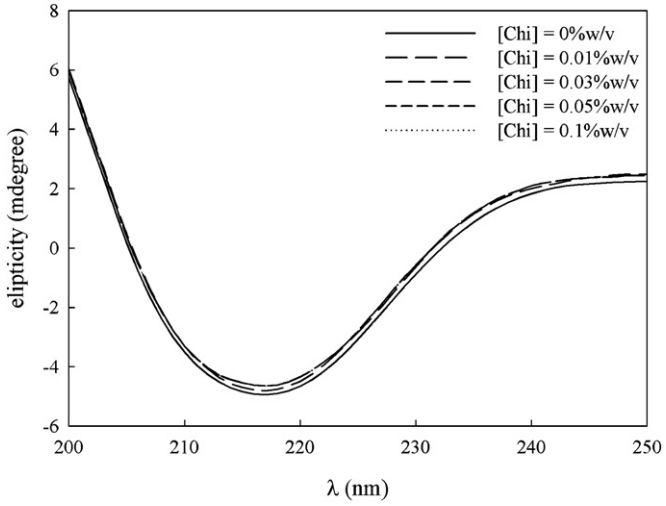

Fig. 2. CD spectrum of PEP in the absence and presence of different concentrations of Chi. Medium acetic 50 mM pH 3.7. Temperature 25 °C. PEP concentration 0.25 mg/mL.

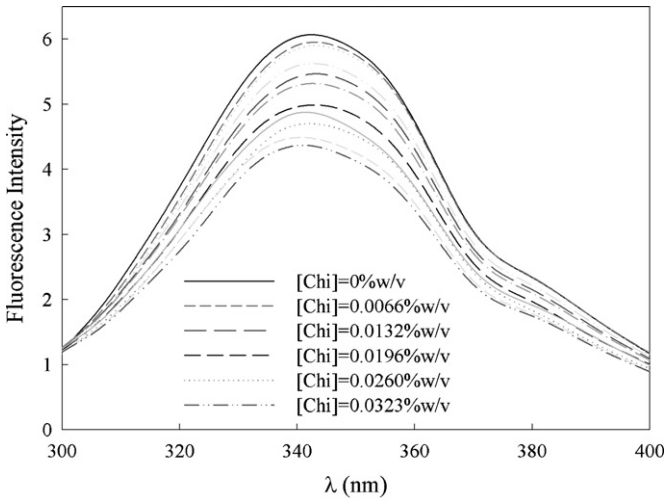

Fig. 3. Fluorescence emission spectrum of PEP in the presence of increasing concentration of Chi. Excitation 280 nm. Medium acetic 50 mM pH 3.7. Temperature 25 °C. PEP concentration 0.25 mg/mL.

3.2. Chi effect on the PEP secondary structure

Fig. 2 shows the circular dichroism spectra of PEP in the presence of different Chi concentrations. No modification in the PEP circular dichroism spectrum was observed by the presence of Chi, which is consistent with the fact that PEP keeps its secondary structure unaltered. This finding is important since the design of protein isolation methods using a polyelectrolyte as a precipitant has as a first step: the study of the interaction between the protein and the polymer, and in many cases this interaction induces dramatic changes in the macromolecule secondary structure with loss in its biological activity.

3.3. Chi effect on the native fluorescence emission peak of PEP

The fluorescence emission spectrum of a protein is sensitive to changes in the polarizability that occurs in the environment of the tryptophans; therefore, the fluorescence signal can be used as optical probe to analyze the effect of a cosolute on the tertiary structure of the protein domain that contains the tryptophan. PEP has 5 tryptophan residues; 2 of which are accessible to the solvent while the others are buried. The fluorescence band of tryptophan is centered at 340 nm. Fig. 3 shows the effect of increasing Chi concentration on the native fluorescence emission. Chi induced a quenching in the intensity but did not change the band position. This last finding suggests that Chi does not change the polarizability of the TRP environments but induces a decrease in the number of particles in the excited state. This is a proof of an interaction of Chi with the environment of the TRP of PEP during the formation of the soluble complex.

3.4. Quenching of the native PEP fluorescence by acrylamide in the presence of Chi

The fluorescence quenching of a fluorophore by a quencher is a powerful technique that gives information about the quencher accessibility to the fluorophore. Moreover, this technique gives information about any modification in the tryptophan microenvironment (Lakowicz, 1983) induced by the presence of a cosolute. The quenching of the native PEP fluorescence, measured at 340 nm while exciting at 280 nm, shows positive deviations from the Stern Volmer equation. This phenomenon is frequently interpreted as a mechanism of "sphere of action" within which the probability of quenching is unity. Table 2 shows the K_D and ν values calculated from Eq. (5). The presence of Chi did not change K_D; however, the radium of the sphere of action decreased in agreement with a minor number of molecules of quencher in the environment of the tryptophan residues. This last finding suggests that the presence of Chi has a protective effect as regards the fluorophores to the quencher.

Table 2
Stern Volmer parameters for the quenching of the native fluorescence of PEP by acrylamide. PEP concentration 0.25 mg/mL.

[Chi] (%, w/v)	K_D (M^{-1})	ν (nm^3/molecule)
0	15.09 ± 0.08	7.2 ± 0.1
0.05	14.01 ± 0.06	6.3 ± 0.1
0.1	15.0 ± 0.1	4.0 ± 0.2

3.5. Effect of Chi on the PEP relative surface hydrophobicity

Fig. 4 shows the S_0 values calculated as the slope of the straight line obtained by plotting the fluorescence emission of ANS (exciting at 360 nm, while measuring its fluorescence emission at 470 nm) as a function of protein concentration (inset Fig. 4). The fluorimetric

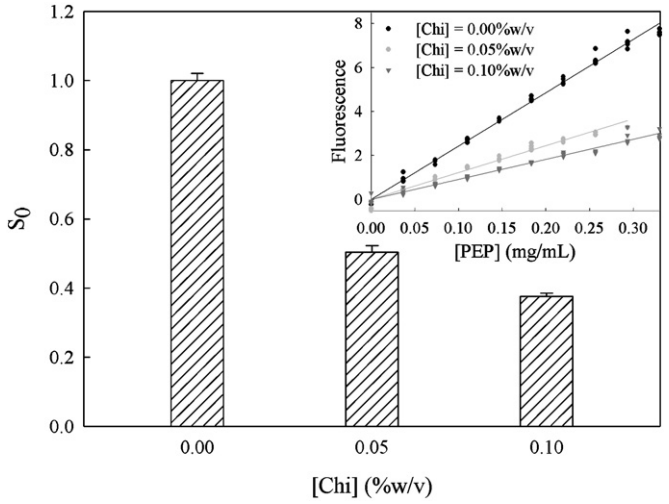

Fig. 4. Effect of Chi on PEP surface hydrophobicity (S_0) determined by the ANS. Medium acetic 50 mM pH 3.7. Temperature 25 °C. PEP concentration 0.25 mg/mL.

Table 3
Temperature of melting and thermodynamic functions associated with PEP thermal unfolding at 25 °C. PEP concentration 0.25 mg/mL.

	T_m (°C)	ΔG^0 (kcal/mol)	ΔS^0 (cal/mol K)	ΔH^0 (kcal/mol)
PEP	51.6 ± 0.1	11.1 ± 0.2	376 ± 5	123 ± 3
PEP + Chi 0.1% (w/v)	52.0 ± 0.1	11.3 ± 0.3	382 ± 6	125 ± 4

titration was obtained in the absence and presence of a constant Chi concentration. It has been demonstrated that the slope value of the straight line obtained is proportional to the surface hydrophobicity of the protein (Cardamone & Puri, 1992). The presence of Chi induced a significant decrease in S_0. Taking into account the dramatic enhancement of the ANS fluorescence when it is placed in a non-polar environment such as the hydrophobic sites of a protein, these results agree with the fact that the presence of an atmosphere of Chi molecules in the surroundings of the PEP hydrophobic surface prevents ANS from binding. This last finding is in agreement with an interaction between the enzyme hydrophobic zones exposed to the solvent and Chi.

3.6. Thermal stability of PEP in the presence of Chi

To analyze the stability of PEP in the presence of Chi from a thermodynamical point of view, a thermal pattern of the enzyme was obtained by heating it and calculating its unfolded fraction versus temperature (data not shown). Then, by non-linear fitting of the data, the middle point temperature of the transition and the thermodynamic functions were calculated (as shows Table 3). The presence of Chi did not modify neither the shape of the curve nor the value of the temperature at the middle point of the transition. Table 3 shows the ΔG°, ΔS° and ΔH° values associated with the thermal unfolding process. No changes in these values were induced by the presence of Chi, which is consistent with the fact that Chi does not make the process more cooperative.

3.7. Chemical stability of PEP in the presence of Chi

Protein unfolding is an equilibrium process where the compact native form of a macromolecule exposes its buried hydrophobic residues to the solvent and becomes an extended random coil molecule. The protein chemical denaturation may be explained on the basis of an unfolding equilibrium displacement towards the unfolded state due to the binding of the denaturant agent (urea) to peptide bonds, which are more exposed when the protein unfolds. Pace has found a linear relationship between the unfolding free energy change ($\Delta G^0{}_U$) and urea concentration following the equation:

$$\Delta G^0 = \Delta G^0 - m \ [\text{urea}] \tag{12}$$

where $\Delta G^0{}_{\text{water}}$ is the protein free energy change of unfolding at zero urea concentration and m is the dependence of free energy on the denaturant concentration ($\partial \Delta G^0{}_U/\partial[\text{urea}]$). The m value depends on the number and type of groups which are exposed to the solvent when the protein unfolds. Fig. 5 shows a non-linear fitting of the obtained values of protein unfolded fraction dependence with the urea concentration in the absence and presence of different Chi concentrations. By plotting $\Delta G^0{}_U$ values, calculated from Eq. (8), versus urea concentration, linear behaviors were observed. From the slope of the straight line, the m value was calculated (see Table 4). $\Delta G^0{}_{\text{water}}$ is a measurement of the solvent unfolding capacity on the protein, the observation of the yielded values shows that Chi does not modify the unfolding equilibrium on PEP. Alonso and Dill (1991) have developed a statistical mechanical theory for the denaturing agents effects on protein stability. They reached a physical interpretation of the slope of curve of the unfolding free energy change ($\Delta G^0{}_U$) versus denaturant agent concentration

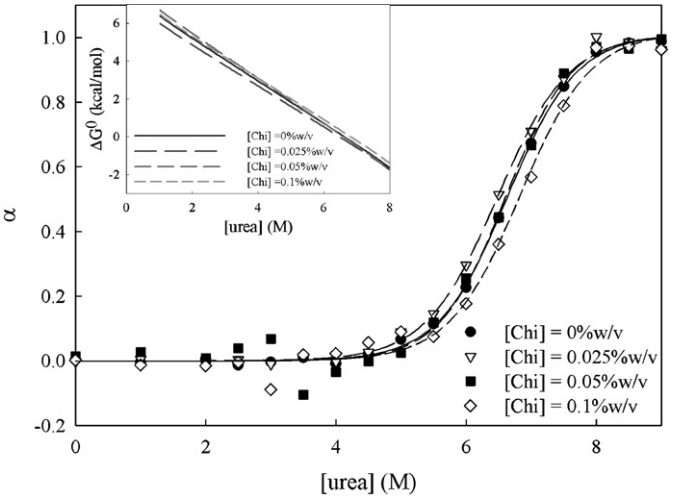

Fig. 5. Unfolded fraction of PEP versus urea concentration in the presence and absence of different concentrations of Chi. Medium acetic 50 mM pH 3.7. Temperature 25 °C. PEP concentration 0.25 mg/mL.

Table 4
Thermodynamic parameter for the chemical denaturation of PEP (concentration 0.25 mg/mL).

[Chi] (%, w/v)	C_m (M)	$\Delta G^0{}_{\text{water}}$ (kcal/mol)	m (M^{-1} kcal/mol)
0	6.63 ± 0.04	7.49 ± 0.03	−1.134 ± 0.004
0.025	6.48 ± 0.08	7.05 ± 0.06	−1.092 ± 0.009
0.05	6.60 ± 0.07	7.86 ± 0.06	−1.194 ± 0.007
0.1	6.82 ± 0.07	7.54 ± 0.05	−1.110 ± 0.006

($\partial \Delta G^0{}_U/\partial[\text{urea}]$) according to the following equation:

$$\frac{\partial \Delta G^0}{\partial[\text{urea}]} = \Delta A \left(\frac{\partial \chi}{\partial[\text{urea}]} \right) kT \tag{13}$$

where k is the Boltzman constant, T the absolute temperature, and χ is the transfer free energy necessary to move an average hydrophobic residue from an aqueous medium to a denaturant solution. It has been shown that χ depends on both, the temperature and the chemical composition of the solution. It also depends slightly on the protein structure, while A depends largely on the protein structure because it is related not only to the number of aminoacids per protein molecule but also to the change in the hydrophobic residue fraction on the protein surface, associated with the unfolding process. For a protein, the term $(\partial \chi/\partial[\text{urea}])kT$ of Eq. (13) remains constant because it depends on the aminoacid composition; therefore, the variation of m values will be directly related to the modification of the area exposed to the solvent when the protein is unfolded by urea. Chi does not affect the m value significantly, i.e., Chi does not affect the change in the area exposed during the denaturation process.

4. Conclusion

Chi is the only natural basic polyelectrolyte and its use in drug delivery, bioremediation and downstream processing has increased dramatically in the last years. In a previous report (Boeris et al., 2009a), we demonstrated the validity and efficiency of this

polymer to precipitate proteins with isoelectrical point below 5. In addition, we have used Chi to obtain PEP from its natural source (Boeris et al., 2009b). Our present finding suggests that PEP keeps its secondary structure and its biological activity. Chi does not modify change in the denaturation process of PEP, i.e., the polymer does not affect the thermal and chemical thermodynamical stability of the enzyme. Chi did not modify the thermodynamic functions associated with the thermal denaturation of PEP; also no significant changes were observed in the functions that characterize the process of chemical denaturation of the enzyme: T_m, C_m and ΔG^0_{water}. This suggests that when PEP forms the complex with Chi, the integrity of the enzyme remains unchanged. The decrease in the size of the soluble complexes support the idea of the electrostatic interaction between PEP and Chi. However, some hydrophobic component is also responsible for the interaction PEP–Chi, since the TRP environments are modified by the Chi presence and the hydrophobic surface also. The results reported here open up the possibility to use this natural and non-expensive polyelectrolyte for the purification of acidic enzymes by a simple precipitation method due to its non-aggressive interaction with the protein.

Acknowledgements

This work was supported by a grant from FonCyT PICT 508/2006 and CONICET PIP 0196/09-011. We thank María Robson, Geraldine Raimundo, Mariana De Sanctis and Marcela Culasso for the language correction of the manuscript.

References

Alizadeh-Pasdar, N., & Li-Chan, E. C. Y. (2000). Comparison of protein surface hydrophobicity measured at various pH values using three different fluorescent probes. *Journal of Agricultural and Food Chemistry*, *48*, 328–334.

Alonso, D. O. V., & Dill, K. A. (1991). Solvent denaturation and stabilization of globular proteins. *Biochemistry*, *30*, 5974–5985.

Boeris, V., Farruggia, B., Nerli, B., Romanini, D., & Picó, G. (2007). Protein–flexible chain polymer interactions to explain protein partition in aqueous two-phase systems and the protein–polyelectrolyte complex formation. *International Journal of Biological*, *41*, 286–294.

Boeris, V., Spelzini, D., Peleteiro Salgado, J., Picó, G., Romanini, D., & Farruggia, B. (2008). Chymotrypsin–poly vinyl sulfonate interaction studied by dynamic light scattering and turbidimetric approaches. *Biochimica et Biophysica Acta*, *1780*, 1032–1037.

Boeris, V., Romanini, D., Farruggia, B., & Picó, G. (2009). Interaction and complex formation between catalase and cationic polyelectrolytes: Chitosan and Eudragit E100. *International Journal of Biological*, *45*, 103–108.

Boeris, V., Spelzini, D., Farruggia, B., & Picó, G. (2009). Aqueous two-phase extraction and polyelectrolyte precipitation combination: A simple and economically technologies for pepsin isolation from bovine abomasum homogenate. *Process Biochemistry*, *44*, 1260–1264.

Cardamone, M., & Puri, N. K. (1992). Spectrofluorimetric assessment of the surface hydrophobicity of proteins. *The Biochemical Journal*, *292*, 589–593.

Chatterjee, S., Chatterjee, S., Chatterjee, B. P., & Guha, A. K. (2004). Clarification of fruit juice with chitosan. *Process Biochemistry*, *39*, 2229–2232.

Chen, L., & Tianqing, L. (2008). Interaction behaviors between chitosan and hemoglobin. *International Journal of Biological*, *42*, 441–446.

Chow, R. B., & Kassell, B. (1963). Bovine pepsinogen and pepsin. *The Journal of biological chemistry*, *243*, 1718–1724.

Fan, M., Hu, Q., & Shen, K. (2009). Preparation and structure of chitosan soluble in wide pH range. *Carbohydrate Polymers*, *78*, 66–71.

Greene, R. F., & Pace, C. N. (1974). Urea and guanidine hydrochloride denaturation of ribonuclease, lysozyme, α-chymotripsin, and β-lactoglobulin. *The Journal of Biological Chemistry*, *249*, 5388–5393.

Horn, D., & Heuck, C. C. (1983). Charge determination of proteins with polyelectrolyte. *The Journal of Biological Chemistry*, *254*, 1665–1670.

Lakowicz, J. R. (1983). *Principles of fluorescence spectroscopy*. New York: Plenum Press.

LeHoux, J. G., & Dupuis, G. (2007). Recovery of chitosan from aqueous acidic solutions by salting-out: Part 1. Use of inorganic salts. *Carbohydrate Polymers*, *68*, 295–304.

Li, J., Jiang, Z., Wu, H., Liang, Y., Zhang, Y., & Liu, J. (2010). Enzyme–polysaccharide interaction and its influence on enzyme activity and stability. *Carbohydrate Polymers*, *82*, 160–166.

Montilla, A., Casal, A., Moreno, F. J., Belloque, J., Olano, A., & Corzo, N. (2007). Isolation of bovine β-lactoglobulin from complexes with chitosan. *International Dairy Journal*, *17*, 459–464.

Pace, C. N., & Vanderburg, K. E. (1979). Determining globular protein stability: Guanidine hydrochloride denaturation of myoglobin. *Biochemistry*, *18*, 288–292.

Pace, N. C., Laurents, D. V., & Thomson, J. A. (1990). pH dependence of the urea and guanidine hydrochloride denaturation of ribonuclease A and ribonuclease T1. *Biochemistry*, *29*, 2564–2572.

Picó, G. (1997). Thermodynamic features of the thermal unfolding of human serum albumin. *International Journal of Biological*, *20*, 63–73.

Rangel-Mendeza, J. R., Monroy-Zepedab, R., Leyva-Ramosb, E., Diaz-Floresa, P. E., & Shirai, K. (2009). Chitosan selectivity for removing cadmium (II), copper (II), and lead (II) from aqueous phase: pH and organic matter effect. *Journal of Hazardous Materials*, *162*, 503–511.

Rungsardthong, V., Wongvuttanakul, N., Kongpien, N., & Chotiwaranon, P. (2006). Application of fungal chitosan for clarification of apple juice. *Process Biochemistry*, *41*, 589–593.

Singh, P. K., & Gupta, M. N. (2008). Simultaneous refolding and purification of a recombinant lipase with an intein tag by affinity precipitation with chitosan. *Biochimica et Biophysica Acta*, *1784*, 1825–1829.

Sinha, V. R., Singla, A. K., Wadhawan, S., Kaushick, R., Kumria, R., Bansal, K., et al. (2004). Chitosan microspheres as a potential carrier for drugs. *International Journal of Pharmaceutics*, *274*, 1–33.

Sperber, B. L. H. M., Schols, H. A., Cohen Stuart, M. A., Norde, W., & Voragen, A. G. J. (2009). Influence of the overall charge and local charge density of pectin on the complex formation between pectin and β-lactoglobulin. *Food Hydrocolloids*, *23*, 765–772.

Subramanian, A., Rau, A. V., & Kaligotla, H. (2006). Surface modification of chitosan for selective surface–protein interaction. *Carbohydrate Polymers*, *66*, 321–332.

Tubío, G., Nerli, B., & Picó, G. (2004). Relationship between the protein surface hydrophobicity and its partitioning behaviour in aqueous two-phase systems of polyethyleneglycol–dextran. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *799*, 293–301.

Wibowo, S., Velazquez, G., Savant, V., & Torres, J. A. (2005). Surimi wash water treatment for protein recovery: Effect of chitosan–alginate complex concentration and treatment time on protein adsorption. *Bioresource Technology*, *96*, 665–671.