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Characterization of chymotrypsin–ι-carrageenan complex in aqueous solution: A solubility and thermodynamical stability study

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The aim of this study is to report the results of research work on the molecular mechanism of complex formation between chymotrypsin and a negatively charged natural strong polyelectrolyte, ι-carrageenan, using spectroscopy techniques. The carrageenan–chymotrypsin complex showed a maximal non-solubility at pH around 4.50 with a stoichiometric ratio between 8 and 33 g of chymotrypsin per g of carrageenan. These values were depended on the enzyme concentration, pH and ionic strength medium. The insoluble complex was redissolved by modifying the pH and by a NaCl concentration around 0.2 M in agreement with a coulombic mechanism of complex formation. The non-soluble complex formation showed biphasic kinetics. A fast step was carried out around 10 s and a coulombic mechanism takes place, and a slower step of around 120 s, where participate only Van der Waals forces. The enzymatic activity of chymotrypsin was maintained even in the presence of carrageenan (0.005%, w/v).

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1. Introduction

Production of enzymes is a prime biotechnological application which includes upstream and, often more expensive, downstream processing steps to obtain the final product in the desired purified form. Bioseparation steps for the recovery of the final product can account for 50–80% of overall production costs. Most purification technologies employ precipitation of proteins as one of the initial operations aimed at concentrating the product for further downstream steps. Precipitation using salts, organic solvents and non-ionic polymers are well known and simple techniques for that purpose. However, these methods use a large amount of reagents that cannot be recycled or discarded into the environment, which makes these methodologies very expensive to be scaled.

A wide variety of polyelectrolytes can interact with globular proteins to form soluble or insoluble complexes. The non-soluble complex can easily be separated by centrifugation or simple decantation [1]. This is a suitable method for protein isolation because very low polyelectrolyte concentrations are used (0.1%, w/v). Besides, it offers high selectivity and the non-soluble complex can be redissolved by a pH change or by salt addition [2]. This technique offers the possibility of concentrating and purifying the target macromolecule at a low cost.

Carrageenan (Car) is a generic name for a family of polysaccharides obtained from certain species of red seaweeds. They are non-toxic, water soluble and widely used within the food, pharmaceutical, cosmetic, printing and textile industries. Car belongs to the family of the hydrophilic linear sulfated galactans. They mainly consist of alternating (1-3)-D-galactose-4-sulfate and β (1,4)-3,6-anhydro-D-galactose residues. There are different forms of these polymers: Iota (ι)-, Kappa (κ)-, Lambda (λ)-, Mu (μ)-, Nu (ν)- and Theta (θ)-carrageenan, the most commercially important being: Iota-, Kappa- and Lambda-carrageenan [3]. In this work ι-carrageenan was used.

Previous reports have demonstrated the usefulness of Car to isolate enzymes such as the obtainment of a protein from rice bran by precipitation using this polysaccharide [4]. However, there is no information about the molecular mechanism of the complex formation between this polyelectrolyte and different enzymes.

The main alkaline enzymes in pancreas are chymotrypsin, trypsin and elastase, all belonging to the serine-protease family. Serine proteases are of considerable interest, due to their activity and stability at alkaline pH and they are used in various industrial market sectors, such as detergent, food, and pharmaceutical. Chymotrypsin has a single polypeptidic chain of 324 amino acid residues and a molecular weight of 25.7 kDa, its optimum activity being at pH 8.2 and its isoelectric point 9.1.

Burton and Lowe [5] had designed ligands, which were attached to Sepharose CL-4B and purified pancreatic kallikrein 110-fold from

Abbreviations: Car, carrageenan; Chy, chymotrypsin; BTEE, N-benzoyl-L-tyrosine ethyl ester.

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a crude pancreatic extract. Other researchers purified Chy from viscera of sardine with a purification factor of 13 and 6% recovery after saline precipitation, gel filtration and ion exchange chromatography [6]. However, these methodologies are not suitable for scaling up in industry. Boeris et al. [7] developed a method for the isolation and purification of chymotrypsin by precipitation with polyvinylsulfonate from pancreas homogenate. However, the development of a new purification method of enzymes requires the use of reagents that can be discarded in the environment without having a negative impact.

This paper reports novel information about the mechanism of the interaction between Car and Chy and sets up both the best experimental conditions for Chy and Car non-soluble complex formation and the potential uses of this methodology to isolate Chy from its natural source. To deal with this question, we used spectroscopy techniques together with the determination of thermodynamical functions associated with complex formations.

2. Materials and methods

2.1. Chemical

Chymotrypsin (Chy), ι-carrageenan (Car) and N-benzoyl-L-tyrosine ethyl ester (BTEE) were purchased from Sigma–Aldrich and used without further purification. Buffers of different pH were prepared: 50 mM phosphate buffer, pH 7.0; 50 mM acetic acid/acetate buffer, pH 4.0, 4.5 and 5.0 and 200 mM Tris–HCl buffer, pH 8.2. The pH was adjusted with NaOH or HCl in each case.

2.2. Turbidimetric titration curves vs. Ph

Turbidity (absorbance at 420 nm) of solutions of 0.5 mg/ml of Chy with 0.005% (w/v) Car was measured and plotted against pH. The medium pH variations were obtained by adding NaOH or HCl aliquots and leaving the system to equilibrate before measuring the turbidity. The complex formation was followed in the absence and presence of different ionic strengths adding NaCl to the medium. A blank titration curve of polyelectrolyte alone was made in the same pH range assayed. These titration curves were made in order to estimate the pH range where the polymer–protein complex is soluble or insoluble as it was previously reported [2,8].

2.3. Chy turbidimetric titration curves with polymer

The formation of the insoluble polymer–protein complex was followed by means of turbidimetric titration. A fixed protein concentration in acid acetic/acetate buffer was titrated at 25 °C in a glass cell with the polymer solution as the titrant. To avoid changes in pH during titration, both protein and polyelectrolyte solutions were adjusted to the same pH value. The absorbance of the solution at 420 nm was used to follow the protein–polyelectrolyte complex formation and plotted vs. the total polymer concentration in the tube. The complex formation was followed in the absence and presence of different ionic strengths adding NaCl to medium.

The results were fitted with a 4-parameter sigmoidal function in order to determine the value of the minimal concentration of Car required to precipitate Chy. This parameter was calculated as the intersection of the tangent at the inflection point with the plateau of the plot. The [Chy]/[Car] ratio can be calculated as the rate between the Chy total concentration and the [Car] calculated. Absorbance solutions were measured using a Jasco FP520 spectrophotometer with constant agitation in a thermostatized cell of 1 cm of path length.

2.4. Kinetic of aggregation

In order to determine the kinetics of aggregation of Car with Chy, a fixed concentration of Chy and Car was mixed and the turbidity formation was followed using a Jasco FP520 spectrophotometer, measurements every 0.1 s were taken. The solution remained under continuous agitation during the measurements.

2.5. Analysis of size variations of the non-soluble Car–Chy precipitate

The changes in Car–Chy size complex were followed by the wavelength (λ) dependence on the turbidity (τ) for the suspensions, and expressed as:

$$\alpha = -\frac{\partial(\log\ \tau)}{\partial(\log \lambda)} \quad (1)$$

where α is a parameter which has an inverse relationship to the average size of the particles and can be used to detect changes in particles size. It was obtained from the slope of $\log \tau$ vs. $\log \lambda$ plots in the 420–700 nm range.

It has been shown that there is a good correlation between the α parameter and the size of the particles by using dynamic light scattering [9,10]. This technique allows us to determine the relative variation of particle size rather than its absolute size. Since our goal was to detect changes in the size of Car–Chy particles by a variation in the medium variables; α parameter is a simple and very useful parameter to follow this variation.

τ was measured as absorbance using a Spekol 1200 spectrophotometer with a diode arrangement detection system. The α values were calculated in the absence and presence of Car to analyze the medium condition on the non-soluble size particles of the complex. α determinations were the average of at least three replicates.

2.6. Determination of Chy activity

The Chy assay is based on the hydrolysis of benzoyl-L-tyrosine ethyl-ester (BTEE) [11]. The reaction rate was determined by measuring the absorbance increase at 256 nm, at 25 °C, which results from the hydrolysis of the substrate at 0.6 mM concentration in 200 mM Tris–HCl buffer, pH 8.2 – 200 mM $CaCl_2$. One Chy unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction and was calculated with the following equation: $U(\mu\text{mol/min}) = (\Delta\text{Abs}_{256\,\text{nm}}/\text{min}) \times 1000/964$, where 964 is the benzoyl-tyrosine molar extinction coefficient [6].

2.7. Protein thermal and chemical stability

Thermally induced unfolding was monitored by absorbance at 280 nm, as it was previously reported [12]. 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 absortivity coefficients of both protein states were different. The unfolded protein fraction was calculated from:

$$\alpha = \frac{A_i - A_D}{A_N - A_D} \quad (2)$$

where α is the unfolded protein fraction, A_N and A_D are the absorbances of the native and unfolded states respectively, A_i is the absorbance at a given temperature. Least squares were used to fit

the unfolded protein fraction vs. temperature data and the temperature at the mid-point of denaturation (T_m) was determined. The equilibrium constant, K, for the unfolding process was calculated from Eq. (3):

$$K = \frac{\alpha}{1-\alpha} \quad (3)$$

the free energy (ΔG°) was calculated as:

$$\Delta G^\circ = -RT \ln K \quad (4)$$

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

$$\Delta S^\circ = \left(\frac{\partial \Delta G^\circ}{\partial T}\right) \quad (5)$$

The enthalpic change was calculated from the equation:

$$\Delta H^\circ = \Delta G^\circ + T\,\Delta S^\circ \quad (6)$$

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

Chemical stability of the protein was assayed by measuring the native fluorescence emission of the protein at 340 nm (while exciting at 280 nm) in media of increasing urea and constant Chy concentration. We assumed that urea – a denaturant agent-induced unfolding due to the formation of a complex (U–D) between the denaturant agent (U) and the protein unfolded form (D) according to the previously demonstrated equilibrium equation (6):

$$N \Leftrightarrow D \quad (7)$$

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 fluorescence of both states were different. A non-linear least squares method has been used to fit the fluorescence vs. urea concentration data; the urea concentration at the mid-point of denaturation (C_m) was determined, and the unfolded protein fraction was calculated from:

$$\alpha = \frac{F_i - F_o}{F_D - F_N} \quad (8)$$

where α is the unfolded protein fraction, F_N and F_D are the fluorescence of the native (in absence of denaturant) and unfolded states (at high denaturant concentration) of the protein, respectively; and F_i is the fluorescence of the protein at i denaturant concentration. From Eq. (8), the equilibrium constant for the unfolded process can be calculated from:

$$K = \frac{\alpha}{1-\alpha} \quad (9)$$

the free energy (ΔG°) was calculated from Eq. (4).

3. Results

3.1. Solubility phase diagram of Chy–Car complexes as a function of pH and ionic strength

Generally, commercial ι-Car contains 32% (w/w) of sulfate [13] and such groups are relatively strong in acidity; therefore, they are dissociated at pH 1, being this polyelectrolyte soluble. In the presence of Chy, a basic protein with an isoelectrical pH of 8.2, the negatively charged group of Car interacts with the positively charged amine groups of Chy, forming a complex. As it has been demonstrated, the interaction of other negatively charged polyelectrolytes with this protein is mainly coulombic [7].

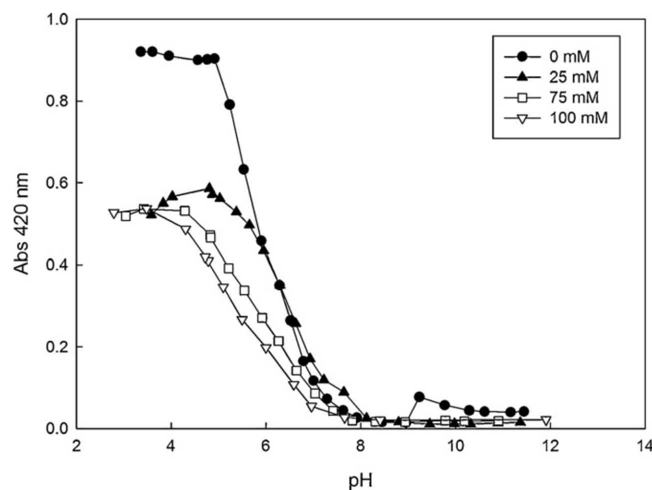

Fig. 1. Acid–base turbidimetric titration curve of Chy (0.5 mg/ml) in the presence of Car at different ionic strengths. Medium: 10 mM acetic acid/acetate–phosphate (1:1) buffer. Temperature 25 °C.

Fig. 1 shows the solubility of Car as a pH function in the presence of Chy. The blank titration curves of Car without Chy and the enzyme without Car were made in the same pH range, and no absorbance changes were observed (data not shown).

When pH decreased from 7 to lower values, a significant increase in the turbidity was observed, in agreement with formation of a non-soluble Car–Chy complex. The curves showed a sigmoidal behavior reached a maximum absorbance at a pH value lower than 5 and they were fitted using a 4-parameter sigmoid function.

Fig. 1 also shows the effect of increasing concentration of NaCl on the Car–Chy complex solubility. The presence of salt increased the solubility of the complex, as it is observed by the decrease of the turbidity. This is consistent with the fact that coulombic interactions drive the association between a polyelectrolyte and a protein of opposite charge.

It has been reported [14] that the formation of soluble protein–polyelectrolyte complexes is initiated at a specific pH, called the critical pH (pHc), which is a function of ionic strength, the protein isoelectric point, and the polyelectrolyte density charge. For polycations, pHc preceded the pH of visual phase separation, named pHϕ. This parameter is introduced as the pH at the half maximal value of turbidity is achieved. Since these non-soluble complexes can be considered a phase separation, the ionic strength dependences of both pHc and pHϕ, can be viewed as phase boundaries. These values were obtained from the solubility curves shown in Fig. 1. The pHc was calculated as the intersection of the tangent at the inflection point with the plateau of the plot while the pHϕ was obtained by fitting the data to a 4-parameter sigmoid function.

Fig. 2 shows the experimental values obtained. At pH values higher than 7.5, Coulombic attractive forces between the uncharged protein and the negatively charged polyelectrolyte are considerably diminished, which prevents the complex formation, and the protein and polymer molecules coexist as separate entities in the solution. At pH values lower than 7.4, a substantial increase in turbidity indicates the formation of a non-soluble complex. Fig. 2 shows that the increase in salt concentration does not influence the pHc values, which suggests that the zeta potential of Chy is not significantly influenced by ionic strength in this pH zone. However, the pHϕ value decreases steadily with the increase in salt concentration, suggesting that the presence of salt affects the amount of complex formed.

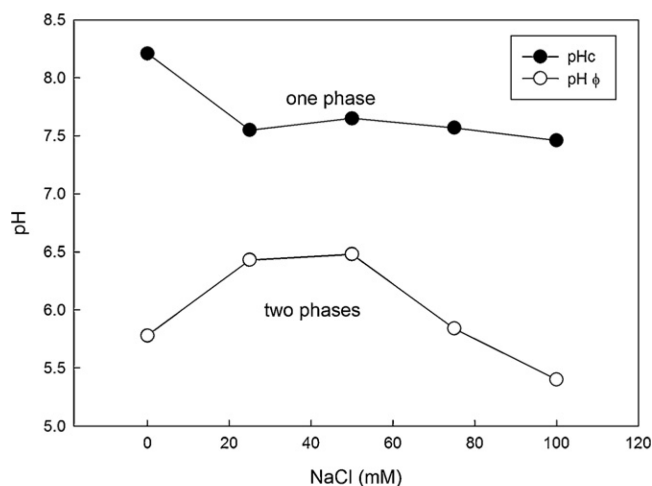

Fig. 2. Ionic strength effect on the pHc and pHϕ values. The data have been calculated from Fig. 1. All the other experimental conditions are same as Fig. 1.

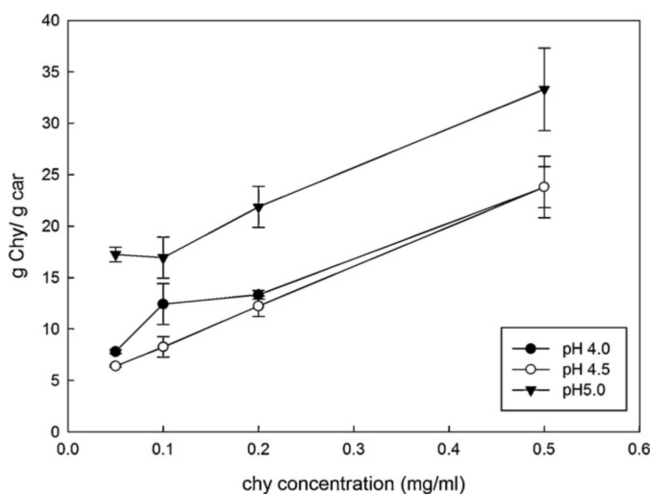

Fig. 4. Stoichiometry for the Chy–Car complex formation at different total concentration of Chy. Medium: 10 mM acetic acid/acetate buffer pH 4.0; 4.5 and 5.0. Temperature 25 °C. The data are mean of three independent measurements.

3.2. Turbidimetry titration curve of Chy with Car, pH and ionic strength effect on complex formation

Fig. 3 shows Chy turbidimetric titration curves with Car at pH 4.5 in the absence and presence of 200 mM NaCl. A similar behavior was observed for Chy at pH 4.0 and 5.0 (data not shown).

The non-soluble complex formation was dramatically affected by ionic strength as shown in Fig. 3, which is consistent with the presence of an important coulombic component during the process [15]. This finding may be interesting because it is the basis of the protein isolation method which allows precipitation using charged polymers, followed by the dissolution of the precipitate by the addition of salt. However, the presence of salt caused a loss of the sigmoidal behavior of experimental data, which showed a hyperbolic behavior with a saturation value higher than that obtained in the absence of salt. This finding should be associated with the presence of a non-coulombic component present in the complex formation and therefore it is not affected by the presence of salt. A similar mechanism has been reported for the interaction of other proteins with different polyelectrolytes [7,16] due to the presence of a hydrophobic effect produced by the loss of ordered water in the environment of the polyelectrolyte chain induced by the inclusion of the enzyme molecules into the polyelectrolyte chains.

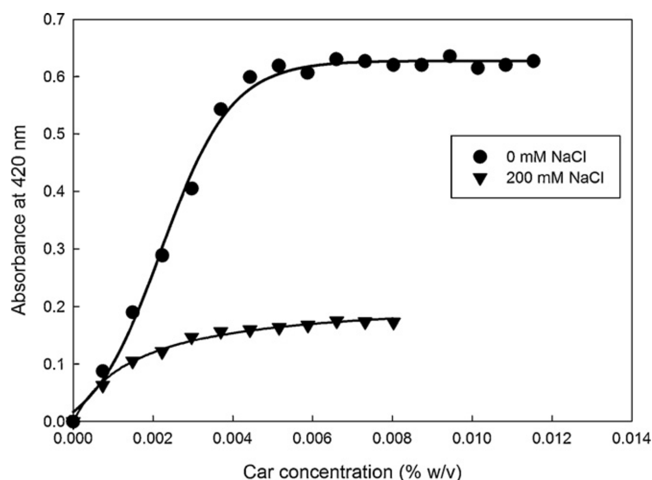

Fig. 3. Turbidimetry titration curve of Chy (0.5 mg/ml) with increasing concentrations of Car in the absence and presence of NaCl. Medium: 10 mM acetic acid/acetate buffer, pH 4.5. Temperature 25 °C.

Turbidimetry data at the three pHs and at increasing Car concentrations were fitted using a sigmoidal equation and from these curve fittings, the stoichiometry ratio Chy–Car was calculated. Fig. 4 shows that the stoichiometry ratio obtained was dependent on the enzyme total concentration which suggests that the final state in the complex formation is dependent on the initial enzyme concentration. This result is different from that obtained in the formation of other polyelectrolyte–enzyme complexes, where the ratio between the two macromolecules does not generally depend on enzyme concentration [17]. Thus, Fig. 4 shows that at pH 4.0, the increase by 10 times of Chy concentration induced an increase in the stoichiometry value. At pH 4.5, the same effect was observed; however, at pH 5.0, the stoichiometry ratio was not significantly influenced by the enzyme concentration variation.

Previous studies about protein–polyelectrolyte complex formation showed [18,19] that the mechanism of complex formation consist of two steps. The first step caused the formation of a soluble complex by the interaction of protein with the polyelectrolyte, while the second state is produced by interactions of the soluble complexes among them and is dependent on the initial concentration of the soluble complex.

When pH increased from 4.0 to 5.0, an increase in the enzyme bound to polyelectrolyte was observed. However, a decrease in the enzyme–polyelectrolyte interaction is to be expected, due to a smaller number of positive electrical charges present in Chy as a consequence of a decrease in proton with an increase in pH.

We suppose that at lower proton concentration, there is a competition between the NH_3^+ of Chy for the SO_3^- groups of Car, which results in an increase in the amount of enzyme bound to the polyelectrolyte.

3.3. Kinetics of the Car–Chy complex formation

Previous reports have demonstrated that the time required for the formation of the polyelectrolyte–protein complex is from several seconds to a few minutes. Fig. 5 shows the turbidity variation as a function of time, expressed as: $(\text{Abs}_{(\infty)} - \text{Abs}_{(t)})$, where $\text{Abs}_{(\infty)}$ is the absorbance of the medium when all the protein is forming the non-soluble complex and $\text{Abs}_{(t)}$ is the absorbance at any time. The kinetic curves were obtained at pHs 4.0; 4.5 and 5.0 at different Chy/Car ratios in the presence and absence of NaCl. Fig. 5 only shows the curves obtained at the three pHs assayed and at constant Chy (0.5 mg/ml) and Car (0.005%, w/v) concentrations. The fitting of

Fig. 5. Kinetic of the turbidity formation vs. time. Chy (0.5 mg/ml), Car (0.005%, w/v), medium: 10 mM acetic acid/acetate buffer. Temperature 25 °C. All the other conditions are shown in the figure.

Fig. 6. Change in the size of the precipitate particles with Car concentration in the absence and presence of NaCl. The data has been expressed as α vs. Car concentration. Medium: 10 mM acetic acid/acetate buffer, pH 4.5. Temperature 25 °C.

the experimental data, showed that the experimental point follows a double exponential curve, of the type shown in Eq. (10):

$$y = ae^{-bx} + ce^{-dx} \quad (10)$$

in agreement with the presence of two kinetic processes, a fast process with a life span around 0–10 s and a second slow process which ends at around 120 s. Table 1 summarizes the experimental kinetic constants obtained for the complex formation.

The kinetic constant for the fast process (b), increases its value as pH decreases. On the other hand, in the presence of NaCl 100 mM, a significant decrease in its value was observed, which suggests that an important electrostatic component take part in the first step of the non-soluble complex formation.

The presence of salt induced a slightly decrease in the kinetic constant for the slow process (d), suggesting that the presence of salt does not significantly affect this step.

3.4. Change in the size of the precipitate particles

Fig. 6 shows the dependence of the α coefficient with Car concentration in the absence and presence of NaCl. It is seen that α values significantly decreased as Car increased its concentration, they reached a minimum value at a Car concentration of 0.005% (w/v), and eventually increased at higher Car concentrations. This finding suggests that in the first step of the complex formation, Car binds the molecules of Chy and the neutralization of the electrical charges induces an increase of the complex size. At higher Car concentrations, there is probably a greater interaction between the soluble complex particles which is evidenced in a decrease in the complex size. In the presence of NaCl, the Car concentration required to reach the minimum complex size decreases significantly, around 0.002%P/V, suggesting a smaller formation of complex particles through inhibition of the Coulombic forces between Chy and Car by salt.

Table 1
Effect of pH and salt on the kinetic of the turbidity formation. All the measurements were performed in 10 mM acid acetic–acetate buffer, 0.5 mg/ml of Chy and 0.005% (w/v) of Car in the absence and presence of salt (100 mM NaCl) (values in bold).

Parameter	pH			
	4.5		4.0	
B	1.05	**0.83**	1.30	**0.33**
D	$4.35e^{-2}$	$\mathbf{8.14e^{-3}}$	$1.71e^{-2}$	$\mathbf{1.65e^{-3}}$

3.5. Car effect on the Chy activity

Previous reports have demonstrated that polyelectrolytes influence the enzymatic activity because they induce a modification of the secondary and tertiary protein structure [20,21]. Chy at constant concentration (0.5 mg/ml) was incubated for 30 min in media of increasing Car concentrations (from 0 to 0.02%, w/v) at the pH of the enzyme maximal activity (8.2). Under these conditions, the Chy–Car complex remains in the soluble form. The enzymatic activity of the Chy was determined with respect to a Car free medium.

Table 2 summarizes the data of the activity obtained. When comparing the Chy activity in the different media, it was found that Car induced a slight decrease in the enzyme activity. The loss in the Chy enzymatic activity is around 10% compared to the Chy enzymatic activity in a free Car media. This is observed in media containing up to ten times the Car concentration necessary to quantitatively precipitate Chy.

3.6. Thermal and chemical stability of Chy in the presence of Car

The interaction between a flexible chain polymer and a protein may induce conformational changes in the tertiary and secondary structure of the protein, with a loss of its thermodynamical stability and biological activity. The thermal unfolding of Chy was analyzed by measuring the protein absorbance change at 280 nm with increasing temperatures. Fig. 7 shows the dependence of the Chy unfolded fraction vs. the temperature in the absence and presence of Car. The presence of the polyelectrolyte changes the shape of the curve inducing a decrease in the middle zone (around the T_m value). Car also induced a slightly thermodynamical stabilization of Chy since an increase in the T_m value was observed as shown in Table 3. It is well known that entropic and enthalpic changes are positive for the proteins thermal denaturation process.

Table 2
Car effect on the Chy activity. The values are the mean of three independent measures.

Car concentration (%, w/v)	Activity recovered (%)
0.0	100 ± 1
1.4e−3	90 ± 3
2.4e−3	95 ± 1
3.1e−3	92 ± 1
4.4e−3	90 ± 1
5.2e−3	93 ± 4

Table 3
Thermodynamic function associated to the thermal and chemical Chy unfolding.

Condition	Parameter					
	ΔS° (cal/K mol)	ΔH° (kcal/mol)	T_m (K)	C_m (M)	ΔG_{H_2O}	m (kcal/mol M
Chy	345.06	113.03	330.7	4.97 ± 0.86	1.74	0.35
Chy–Car 0.03%	81.10	26.78	332.8	6.06 ± 0.20	4.39	0.72
Chy–Car 0.06%	79.26	26.24	334.9	5.88 ± 0.15	3.25	0.55

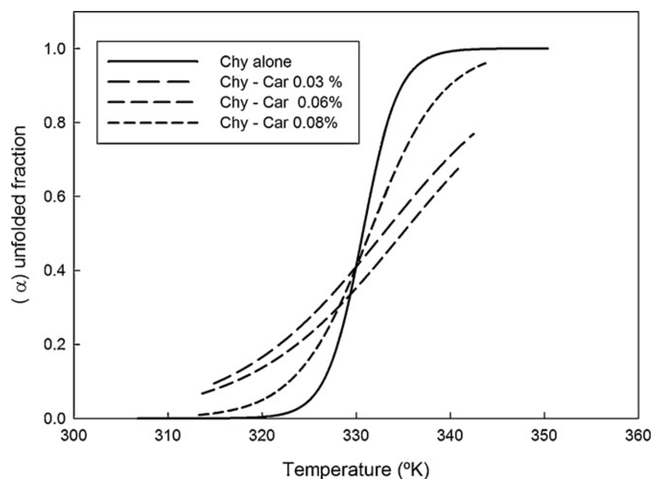

Fig. 7. Unfolding thermal shape of Chy (0.5 mg/ml) in the absence and presence of Car. The data has been expressed as unfolding protein fraction vs. temperature. Heating rate: 1 °C/min. Medium: 10 mM acetic acid/acetate buffer, pH 4.0.

These positive entropic and enthalpic changes were associated with the loss of ordered water molecules around the hydrophobic moieties of Chy and with a change in its tertiary structure. In the presence of Car, both an increase in Chy T_m value and a significant decrease of the unfolding entropic change were observed, which suggests that the Chy–Car complex is more stable than Chy alone in the initial state previous to the thermal unfolding. In addition, the decrease of endothermicity in the Chy unfolding process in the presence of Car is consistent with a previous stabilization of the Chy initial state. Fig. 7 also shows a significant change in the cooperativity of the unfolding process; the presence of Chy induced a decrease in the $(\partial\alpha/\partial T)$ value calculated at the T_m ($\alpha = 0.5$) in agreement with a loss of the Chy unfolding trend.

Also, we assayed the chemical stability of Chy in the presence of Car by obtaining the stability chemical curve described in Section 2 (data not shown). Table 3 shows a significant increase in C_m value in the presence of Car, which suggests an increase in the chemical stability of Chy. Pace [22] have found a linear relationship between the unfolding free energy change (ΔG_U) and urea concentration following the equation:

$$\Delta G_U = \Delta G_{H_2O} - m\,[\text{urea}] \tag{11}$$

where ΔG_{H_2O} is the protein free energy change of unfolding at zero urea concentration and m is the dependence of free energy on the denaturant concentration ($\partial\Delta G_U/\partial$[urea]), which is proportional to the difference in the solvent-exposed surface area between the denatured and native states (ΔA). The m value depends on the number and type of groups which are exposed to the solvent when the protein unfolds and ΔG_{H_2O} is a measurement of the solvent unfolding capacity on the protein.

By plotting ΔG_U values, calculated from Eqs. (8), (9) and (4), vs. urea concentration, a linear behavior was observed (data not shown); from the slope of the strength line the m value was calculated (see Table 3). Car significantly increased the ΔG_{H_2O} value, suggesting a greater protein stability due to the complex formation which includes the enzyme in a more hydrated environment.

Alonso and Dill [23] developed a statistical mechanical theory for the effects of denaturing agents on protein stability. They reached a physical interpretation of the slope of the unfolding free energy change (ΔG) vs. denaturant agent concentration curve (ΔG/[denaturant]) according to the following equation:

$$\frac{\partial\ \Delta G}{\partial c} = \Delta A \frac{\partial \chi}{\partial c} kT \tag{12}$$

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 temperature and on the chemical composition of the solution. It also depends slightly on protein structure, while A depends largely on protein structure because it is related not only to the number of amino acids 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 second term of Eq. (12) remains constant because it depends on the amino acid 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. Car increases m value, which suggests an increase of the surface contact area of the enzyme to the solvent by polyelectrolyte interaction.

4. Conclusion

Protein precipitation provides an effective method for raising the concentration of a protein in a dilute solution. Precipitation plays an important role in the downstream processing in biotechnology.

Proteins interact strongly with both synthetic and natural polyelectrolytes. There are numerous synthetic polyelectrolytes proposed as protein precipitants. However, most of them are toxic and difficult to be removed from the final product. Car, a common food ingredient, was found to be effective in precipitating Chy, a strategic enzyme widely used in different biotechnological industrial processes. The advantage of precipitating using this polysaccharide is that greater amounts of protein can be recovered from the solution while using a safe precipitant agent.

Previous reports [4] have demonstrated the capacity of Car to form non-soluble complexes with protein; however, few studies have been reported to date. A better knowledge of the molecular mechanism of precipitation by which the complexes are formed allows the interval values of the experimental variables to be exactly determined. Different techniques have been used to study the complex formation, but the most widely used is turbidimetry which is a simple method.

This study showed that Chy could interact with Car and form either soluble o insoluble complexes depending on the medium pH values, ionic strength, etc. The main mechanism of Car–Chy complex formation was coulombic, as it was reported by the formation of other protein–polyelectrolyte complexes. One parameter determined is the stoichiometry ratio for protein-polymer complex formation. The values obtained were in the order of 6–33 g of Chy

per gram of Car, depending on the conditions of the medium. The high Chy–Car ratio observed in the non-soluble form of the complex may be useful to precipitate the protein as very low concentrations of polymer are required.

It was found that the presence of Car thermodynamically stabilizes Chy with a minimum loss of its biological activity, favoring its storage and transportation.

In this paper we have used a simple, previously shown technique to obtain information about the kinetics and size of the Car–Chy complex formation a field where no previous work has been carried out. By lowering the pH values, negatively charged carboxylic groups are protonated losing their net electrical charge, thus decreasing their repulsion with negatively charged SO_3^- groups of Car. This facilitates the formation of protein–polyelectrolyte complexes, allowing positive net charges of Chy and the negative changes of Car to interact. In the presence of NaCl, there is a shielding effect on the Chy negatively charged carboxylic groups, so that when pH decreases, the repulsion between the SO_3^- and carboxylic groups is partially canceled, favoring the interaction between NH_3^+ and SO_3^-, leading to an increase in the kinetics.

We have performed the experiments present in this work using pure Chy to address which is the molecular mechanism of interaction between this enzyme and Car. Our goal is apply these data for the development of a Chy purification method from fresh pancreas homogenate. However, when fresh pancreas homogenate is treated with Car at pH 4.5 the precipitate obtained contains different enzymes (like trypsin, elastase) Chy being the main protease present in it, because it account for the major percentage of pancreatic proteases. However, the precipitation method is useful as a primary method of concentration and pre-purification of this enzyme, because this is carried out in only one step.

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