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Complex formation between protein and poly vinyl sulfonate as a strategy of proteins isolation

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ABSTRACT

The complex formation between the basic protein trypsin and the strong anionic polyelectrolyte poly vinyl sulfonic acid was studied by using turbidimetric and isothermal calorimetric titrations. The trypsin–polymer complex was insoluble at pH lower than 5, with a stoichiometric ratio polymer mol per protein mol of 1:136. NaCl, 0.5 M inhibited the complex precipitation in agreement with the proposed coulombic mechanism of complex formation. The protein structure and its thermodynamic stability were not significantly affected by the presence of the polyelectrolyte. The enzymatic activity of trypsin increases throughout time, even in the presence of the polymer.

1. Introduction

The ability of synthetic and natural polyelectrolytes to interact with globular proteins forming stable protein–polyelectrolyte complexes (PPC) is well known [1,2]. The protein–polymer interactions result in the formation of soluble or insoluble complexes [3,4].

The main conclusions derived from previous studies may be summarized as follows: (i) PPCs are formed mainly through electrostatic forces; (ii) in salt-free systems, at least, protein molecules are complexed with flexible polyelectrolytes through the stoichiometric formation of ion pairs (or salt linkages) between oppositely charged groups [5,6]; (iii) the ion pairs between the polyelectolytes and protein molecules are very labile and may be severed by a change in pH as well as by the addition of small ions and polyions [7] and (iv) there is an appreciable retention of biochemical functions in the resultant complexes, therefore, changes in the three-dimensional conformations are not so large as to cause a loss of original functions.

When PPC is specifically formed with one of the protein in the crude extract followed by a phase separation, the process could be used as a convenient strategy for the isolation and purification of the target protein.

Precipitation finds a place in most protein purification protocols and has been traditionally applied as a simple and rapid technique for protein concentration at the beginning of the downstream processing. The various precipitation methods include the use of salts organic solvents, non-ionic polymers and polyelectrolytes [8–10]. Precipitation as a product concentration step offers several advantages in that it is easy to scale up, uses simple equipment and can be based on a large variety of alternative precipitants [11].

Trypsin is a serine protease found in the digestive system. It is used for numerous biotechnological processes. It is secreted into the intestine, where it acts to hydrolyse proteins into smaller peptides or amino acids. This is necessary for the uptake of protein in the food.

Trypsin is produced in the pancreas in the form of inactive zymogen, trypsinogen. It is then secreted into the small intestine, where the enzyme enterokinase activates it into trypsin by proteolytic cleavage [12]. The resulting trypsins themselves activate more trypsinogens (autocatalysis), so only a small amount of enterokinase is necessary to start the reaction.

We have used spectroscopic and calorimetric techniques to obtain information about the molecular mechanism of interaction between a basic model protein (trypsin) and a negatively charged polyelectrolyte (poly vinyl sulfonate) with the aim of applying this

Abbreviations: TRP, trypsin; PVS, poly vinyl sulfonate; ITC, isothermic tritation calorimetry; PPC, protein–polyelectrolyte complex.

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information to the polyelectrolyte–protein complex formation as a tool for protein separation.

2. Materials and methods

2.1. Chemical

Trypsin (TRP) from porcine pancreas was purchased from Sigma Chem. Co. (USA) and the polymer poly vinyl sulfonic acid, sodium salt (PVS) 25% (w/w) sol. in water, molecular average mass 170 kDa, were purchased from Aldrich and used without further purification. Phosphate buffer solutions of different pH were prepared at concentration of 50 mM. They were adjusted with NaOH or HCl in each case.

2.2. Trypsin turbidimetric titration curves with polymer

The formation of the insoluble polymer–protein complex was followed by means of turbidimetric titration [13]. Buffer sodium phosphate solutions (10 mL) with a fixed protein concentration were titrated at 20 °C in a cubic 1 cm path-length glass cell with the polymer solution as the titrant. Unless otherwise noted, the concentrations of protein and polymer were 70 μM and 0.25% (w/w), respectively. To avoid changes in pH during titration, both protein and polyelectrolyte solutions were adjusted to the same pH value. The complex formation was followed through a plot of absorbance at 420 nm vs. polymer–protein molar ratio. Therefore, polymer–protein complex with three different selected stoichiometry around the plateau region of the above cited plot are titrated with alkali and acid, the absorbance at 420 nm being plotted vs. pH.

2.3. Isothermal titration calorimetry (ITC)

Measurements were performed at 20 °C by using a VP-ITC titration calorimeter (MicroCal Inc., USA). The sample cell was loaded with 1.436 mL of trypsin solution (70 μM) and the reference cell contained Milli-Q grade water. Tritation was carried out using a 0.3 mL syringe filled with polyelectrolyte solutions. The experiments were performed by adding 30–100 aliquots of 3 μL of polymer solutions 0.25% (w/w) to the cell containing the protein solution. The heat associated in the interaction between protein and polymer was calculated by discounting the heat of protein dilution and polymer, which was determined by the titration of a protein solution into buffer and the buffer into polymer solution, respectively [14]. The resulting data were fitted to a single set of identical binding sites models using MicroCal ORIGIN 7.0 software supplied with the instrument and the intrinsic molar enthalpy change for the binding, ΔH°, the binding stoichiometry, n, and the intrinsic binding constant, K, were thus obtained. The intrinsic molar free energy change, ΔG°, and the intrinsic molar entropy change, ΔS°, for the binding reaction were calculated by the fundamental thermodynamic equations:

$$\Delta G^{|\circ} = -RT \ln K_b \quad (1)$$

$$\Delta S^\circ = \frac{\Delta H^\circ - \Delta G^\circ}{T} \quad (2)$$

2.4. Enzyme assays

Trypsin activity was determined with the substrate α-*N*-benzoyl DL-arginine-*p*-nitroaniline (BAPNA) using a method modified from Gildberg and Overbo [15]. BAPNA was used in the assay at a final concentration of 0.85 mM in 100 mM buffer phosphate pH 7.0. The reaction is followed by measuring the absorbance of the released reaction product, *p*-nitroanilide, which absorbs at 400 nm (molar absorptivity of 10,500 M^{-1} cm^{-1}) for 5 min. The activities were calculated from the initial linear portion of the absorbance vs. time curve.

The enzyme assays were performed at a constant temperature of 20 °C in the presence and absence of PVS. Three different PVS–TRP molar ratios were chosen from the plateau of tritation curves.

In order to evaluate the activity of the enzyme in presence of the polymer, TRP was incubated in PVS and the activity was measured during 24 h.

2.5. TRP precipitation with PVS

A solution of PVS and TRP to a relation 1:66 mol of polymer per mol of protein in buffer Pi 50 mm of pH 3.0 was prepared. The precipitate formed was incubated for 30 min at 20 °C and centrifuged at 1000 × *g* during 10 min. The supernatant of the precipitate was separated. Then, the precipitate was redissolved in two different ways: by the addition of phosphate buffer with NaCl 1 M and by the addition of phosphate buffer solution of pH 7.0. The enzymatic activity in the supernatant and in the redissolved precipitate was measured.

3. Results

3.1. Trypsin turbidimetric titration curves with PVS

Fig. 1 shows typical titration curves of TRP with PVS, from which, two important characteristics were observed. At low polymer–protein ratios, absorbance increases with an increase in the polymer total concentration and, at high polymer–protein ratio, there is a plateau which depends on the medium pH.

The stoichiometric protein–polymer ratio which corresponds to the situation where the protein has been precipitated as an insoluble complex was calculated from the intersection of a straight line which corresponds to the prolongation of the linear zone of the curve (at low polymer concentration) with a line which gives a plateau.

Table 1 shows the molar protein–polymer ratio which corresponds to the stoichiometry of the complex formation calculated from the titration curves for the different experiments. These values are important because they allow us to calculate the minimal polymer amount necessary to precipitate the protein in a complete form. The data have been expressed as the number of TRP moles

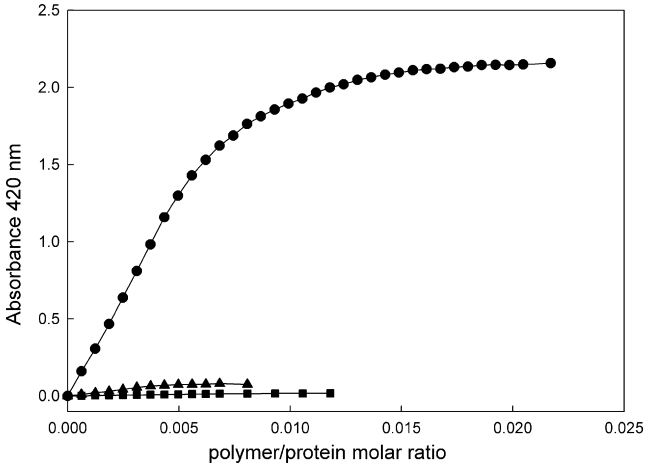

Fig. 1. Turbidimetric titration curves of TRP (70 M) solution with PVS (0.25% w/w) in a medium with phosphate buffer 50 mM, pH 3.0 (●), 5.5 (▲) and 7.0 (■). Temperature 20 °C.

Table 1
Stoichiometric of TRP/PVS complex at different pH

pH	Protein–polymer molar ratio
3.00	136 ± 3
5.50	228 ± 21
7.00	147 ± 12

bound per polyelectrolyte mol. Despite the fact that these values were similar, turbidity is much higher at pH 3.0 which suggest a major size of the precipitate particle.

3.1.1. pH effect on the complex formation

Trypsin is a basic protein with 19 amino residues, an isoelectrical pH between 11.0 and 11.4 and a molecular mass of 14.3 kDa. Therefore, at the pHs where the turbidimetry titration was assayed, the protein has a net positive electrical charge. Formation of TRP–PVS complex was observed to be influenced by the medium pH. Fig. 2 shows the pH variation effect on the insoluble complex formation obtained for different PVS–TRP molar ratios. The increase of pH above 6 induced a dramatic decrease in the maximum absorbance values which suggested a minor amount of complex formation. However, the absorbance maximum value was observed to be greater at pH 2 than pH 3, which can be due to the loss of the native structure of this protein by influence of the acid medium. These curves determine the optimum pH interval in which the protein complex is insoluble.

3.1.2. Ionic strength effect of the complex formation

Fig. 3 shows the ionic strength effect on the complex formation, given by NaCl (0–0.5 M). The PVS–TRP complex was dramatically affected by ionic strength 0.25 or higher; in this case, not formation of the insoluble complex was found. The inhibition of the precipitate formation was directly proportional to the salt concentration, consistent with the presence of an important coulombic component in the insoluble complex formation [16,17].

Due to the fact that the molecular mechanism of the TRP–PVS interaction is electrostatic in nature, the turbidimetric titrations at pH 3.0 were performed in a medium of different ionic strength such as shown in Fig. 3. For this system, a NaCl concentration of 0.5 M is not enough to avoid the formation of the insoluble protein–polymer complex. This finding may be interesting because it is the bases of protein isolation method. Allowing in a first step the precipitation by the polymer and second the precipitate may be dissolved by the addition of a NaCl solution at low concentration. The low ratio polymer–protein values found suggested that one polymer molecule is bounded to many protein molecules according to the model proposed by Kokufuta and co-workers [18] where one polymer molecule has the capability to interact with many protein molecules.

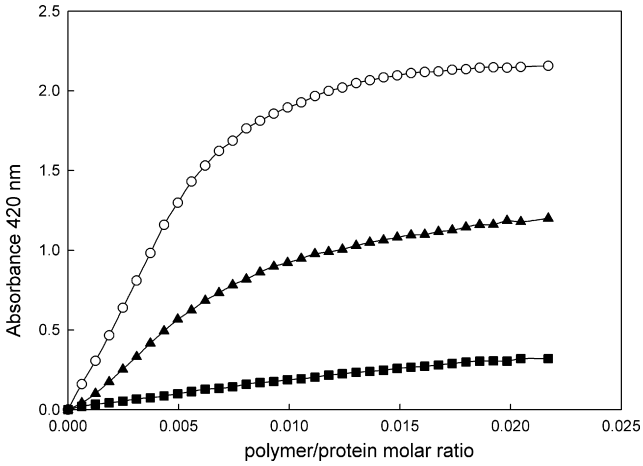

Fig. 3. Salt effect on the turbidimetric titration curves of TRP with PVS, pH 3.0, NaCl concentration: (○) 0 M, (▲) 0.25 M and (■) 0.5 M. Temperature 20 °C.

3.2. ITC isothermal calorimetric titration of TRP with polymers

The complex formation between TRP and poly vinyl sulfonate was investigated by isothermal titration calorimetry. The direct curves obtained showed negative heat associated with the titration, and the polyelectrolyte dilution in the buffer (in the absence of protein) was associated with a large heat release (Fig. 4), being the heat of dilution of the protein by the buffer negligible. The heat associated with the interaction polymer–protein (ΔH_i) was calculated by subtraction using the equation:

$$\Delta H_i = \Delta H_t - \Delta H_d \quad (3)$$

where ΔH_t is the polymer–protein titration heat and ΔH_d is the heat of dilution of the polymer in the buffer in the absence of the protein. Fig. 4 shows the calorimetric titration curve of TRP with PVS

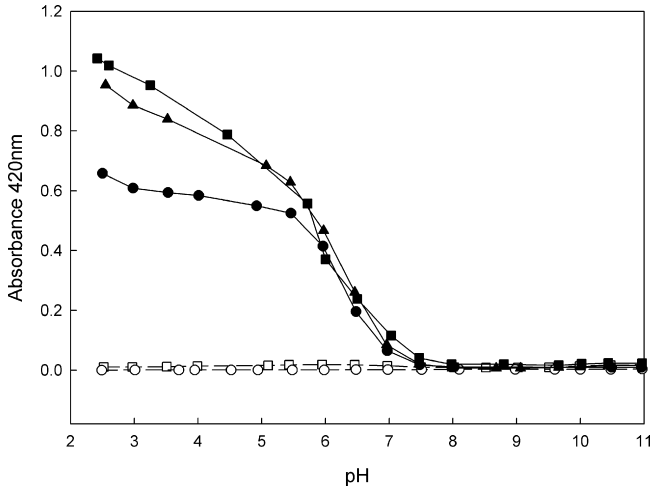

Fig. 2. Dependence of the absorbance at 420 nm vs. the medium pH at a constant protein–polymer molar ratio of TRP–PVS: (●) 1:157, (▲) 1:87, (■) 1:66. Temperature 20 °C.

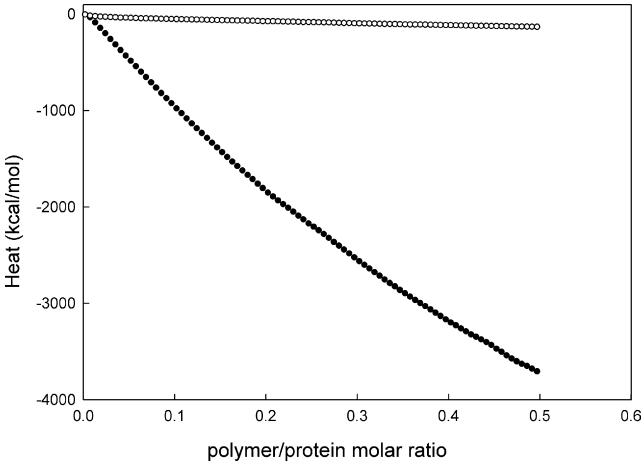

Fig. 4. Binding curve for the calorimetric titration of TRP with PVS (0.25%, w/w). ΔH_d (○) and ΔH_t (●). Protein concentration 70 μM. Medium sodium phosphate buffer 50 mM, pH 3.0. Temperature 20 °C.

Table 2
Thermodynamic and binding parameters of the interaction TRP–PVS from ITC experiments

Parameters	TRP–PVS
n (protein/polyelectrolyte)	46.9 ± 0.4
K (M^{-1})	2.2×10^3
ΔH° (kcal/mol)	−35.8
ΔS° (e.u.)	−106.8

The enthalpic change is expressed per mol of protein bound.

in a 50 mM, pH 3.0 medium phosphate buffer. By non-linear fitting of these data, the affinity constant (K) for the protein binding to the polymer and the number of protein molecules (n) bound per polymer molecule was calculated using the software provided by the instrument.

The mathematical model equation selected to fit the ITC data was derived from a model that assumes that the polymer molecule binds to several protein molecules, all with the same intensity; in other words, the polyelectrolyte was considered as a macromolecule having *n independent and equivalent sites*, all of which have the same affinity constant, K, for the ligand (trypsin):

$$r = \frac{nKc}{1 + Kc} \quad (4)$$

where c is the free protein concentration and r is the bound protein concentration over polymer concentration.

A better quantitative analysis of heat associated to the protein–polyelectrolyte complex formation can be made if the n values are expressed as protein mol per polymer mol in the same way as in the turbidimetry titration. The values obtained are shown in Table 2. Values of 47 protein mol per polymer mol were found for TRP–PVS complex formation. The ΔH was normalized per mol of protein, therefore, heat value of −35.8 kcal/protein mol is yielded. This heat of interaction is expected in the order of magnitude for interactions of electrostatic nature published in similar systems [13,19].

The ΔS value found was negative as a result of the formation of a more ordered structure by protein–polymer complex formation.

3.2.1. TRP biological activity in the absence and presence of PVS

Fig. 5 shows the biological activity of TRP in the absence and presence of polymers. It can be seen that at three concentrations of PVS, the enzyme activity is practically the same either in the absence or presence of the polymer. This shows that the formation of TRP–PVS complex does not significantly alter the catalytic ability of the enzyme when there is excess of polymer.

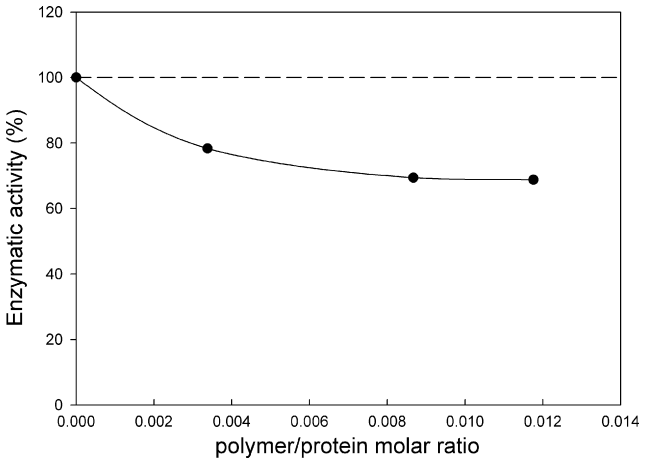

Fig. 5. Percent of biological activity of TRP (2.5 μM) in the absent and the presence of PVS. Temperature 20 °C.

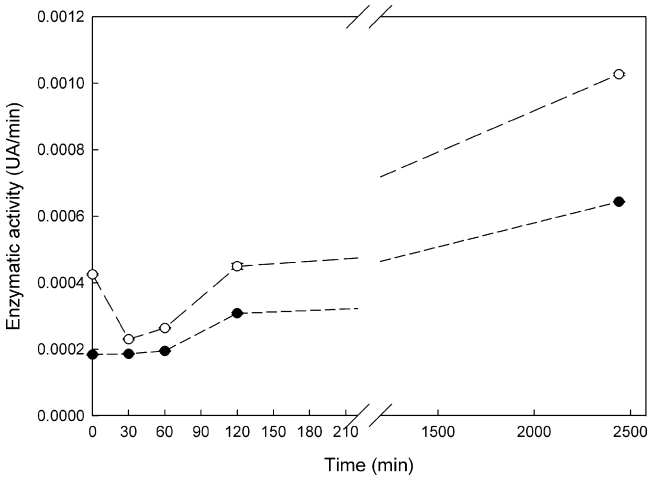

Fig. 6. Biological activity of TRP (2.5 μM) in the absent (○) and the presence (●) of PVS, molar ratio PVS–TRP 1:85. Temperature 20 °C.

3.2.2. TRP biological activity through time, in the absence and presence of PVS

Fig. 6 shows the biological activity of TRP in the presence of PVS at a polymer–protein ratio of 1:85. It can be seen that the enzyme activity remains at a temperature of 20 °C for about 24 h. This high polymer–protein ratio was chosen to verify the polymer presence effect on trypsin through time, however, in the following experiments a lower ratio was assayed.

3.3. TRP precipitation by PVS–protein insoluble complex formation

A medium containing TRP (70 μM) in sodium phosphate, 50 mM at pH 3.0, was precipitated adding PVS. The precipitate formed was incubated at 20 °C for 30 min and centrifuged at 1000 × g during 10 min. The precipitate was dissolved in two different ways: by the addition of NaCl 1 M and in pH 7.0 phosphate buffer. The trypsin activity was determined in the supernatant and in the dissolved precipitate. This activity is a proportional parameter to the protein concentration. Moreover, the enzymatic activity is an evidence of the conformational state of the enzyme. In this case, it is more important to evaluate the enzymatic activity than the concentration of the enzyme. Control and a test curves were also carried out. Table 3 shows the TRP recovery for both systems.

The recovery was considered to be around 84% when the precipitate was redissolved with NaCl. On the other hand, the precipitate activity of TRP was significantly lower than the previous case when it was redissolved in phosphate buffer pH 7.0. In both cases, the complex can be seen interact with the positively charged TRP, which resulted in a better recovery of the enzyme.

4. Discussion

Protein precipitation provides an effective method for raising the concentration of a protein in a dilute solution. Precipitation

Table 3
TRP activity in buffer pH 7.0 and after precipitation

	TRP activity (UA/min)	TRP activity (%)
Phosphate buffer (pH 7)	4.29×10^{-3}	100
Supernatant	9.87×10^{-5}	23.02
Precipitate (NaCl 1 M)	3.60×10^{-3}	83.93
Precipitate (buffer phosphate, pH 7)	1.22×10^{-3}	28.45

plays an important role in the downstream processing in biotechnology. A variety of charged precipitating agents can be used to precipitate proteins with a high industrial value using insoluble complex formation.

The isothermal tritation calorimetry (ITC), turbidimetry and the enzymatic activity provide useful quantitative information about the interaction of trypsin and poly vinyl sulfonate in aqueous solution.

Oppositely charged polyelectrolytes such as carboxymethyl cellulose, poly acrylic acid and poly methacrylic acid have been used to selectively precipitate proteins from an aqueous mixture on the basis of different affinities [20,21]. Although protein precipitation and colloid flocculation with polyelectrolytes have been studied for many years, few theoretical studies have been directed toward understanding the mechanism of precipitation [22].

This study showed that TRP could interact with poly vinyl sulfonate and form either soluble or insoluble complexes depending on the solution pH, ionic strength, etc. Insoluble complexes were formed at pH values where the protein and polymer had opposite electrical charges (pH around 3) because of strong electrostatic attraction between two biopolymers [23].

ITC experiments have given us the direct heat associated with the complex formation and thermodynamic parameter of the complex formation. We have found that 47 TRP molecules bind to one polymer molecule (stoichiometry of complex) and we also detected a high negative heat value of interaction between them.

In the case of turbidimetry it has been shown a stoichiometry of 136. The presence of high ionic strength of 0.5 M could not redissolve completely the complex. For that reason we used a more concentrated solution of salt (1 M). On the other hand the precipitate was redissolved by adding phosphate buffer pH 7.0.

The results showed that the enzymatic activity of TRP slightly decreased in the presence of PVS but the enzyme kept an 84% of the activity in the excess of polymer. Moreover, the enzymatic activity was kept through time when it was incubated in the polymer presence. However, the decrease in the enzymatic activity could be attributed to a conformational change in the active site tertiary structure of the enzyme by the binding to the polymer.

TRP precipitation with PVS at previously studied conditions showed a high efficiency of PVS to the precipitation. This method could be suitable for the precipitation of the protein from its natural source. Unlike other traditional isolation protein methods, it has the advantage on enabling the concentration of the sample.

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References

[1] B. Mattiasson, A. Kumar, I.Yu Galaev, J. Mol. Recognit. 11 (1998) 211.
[2] S. Nath, C.S. Patrickios, A. Hatton, Biotechnol. Progr. 11 (1995) 99.
[3] C.S. Patrickios, L.R. Sharma, S.P. Armes, N.C. Billingham, Langmuir 15 (1999) 1613.
[4] F. Hilbrig, R. Fretag, J. Chromatogr. B 790 (2003) 79.
[5] D. Takahashi, Y. Kubota, K. Kokai, T. Izumi, M. Hirata, E. Kokufuta, Langmuir 16 (2000) 3133.
[6] C.L. Cooper, P.L. Dubin, A.B. Kayitmazer, S. Turksen, Curr. Opin. Colloids Interface Sci. 10 (2005) 52.
[7] A. Tsuboi, T. Izumi, M. Hirata, J. Xia, P.L. Dublin, E. Kokufuta, Langmuir 12 (1996) 6295.
[8] E. Kokufuta, H. Shimizu, I. Nakamura, Macromolecules 15 (1982) 1618.
[9] V. Gupta, S. Nath, S. Chand, Polymer 43 (2002) 3387.
[10] A. Lali, N. Aruna, J. Roshnnie, T. Devika, Process. Biochem. 35 (2000) 777.
[11] M. Vikelouda, V. Kiosseoglou, Food Hydrocolloids 18 (2004) 21.
[12] R. Beynon, J.S. Bond, Proteolytic Enzymes, Practical Approach, 2001.
[13] D. Romanini, M. Braia, R. Giatte Angarten, W. Loh, G. Picó, J. Chromatogr. B 857 (2007) 25.
[14] N.L. Almeida, C.L.P. Oliveira, I.L. Torriani, W. Loh, Colloids Surf. B 38 (2004) 67.
[15] K.D. Johnson, A. Clark, S. Marshall, Comp. Biochem. Physiol. B 131 (2002) 431.
[16] T. Menkhaus, S. Eriksson, P. Whitson, C. Glatz, Biotechnol. Bioeng. 7782 (2002) 148.
[17] A. Manzur, D. Spelzini, B. Farruggia, D. Romanini, G. Picó, J. Chromatogr. B 860 (2007) 63.
[18] S. Azegami, A. Tsuboi, T. Izumi, M. Hirata, P.L. Dubin, B. Wang, E. Kokufuta, Langmuir 15 (1999) 940.
[19] K. Henzler, B. Haupt, M. Ballauff, Anal. Biochem. 378 (2008) 184.
[20] Y. Wang, J. Gao, P.L. Dubin, Biotechnol. Progr. 12 (1996) 356.
[21] R. Agarwal, M.N. Gupta, Protein Expr. Purif. 7 (1996) 294.
[22] F. Fornasiero, J. Ulrich, J.M. Prausnitz, Chem. Eng. Process. 38 (1999) 463.
[23] T. Harnsilawat, R. Pongsawatmanit, D.J. Mc Clements, Food Hydrocolloids 20 (2005) 577.