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Partitioning of caseinomacropeptide in aqueous two-phase systems

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Abstract

This study evaluates the influence of type of salt and temperature on the partition coefficient of caseinomacropetide (CMP) to determine the best conditions for the recovery of CMP in aqueous two-phase systems (ATPS) composed by poly(ethylene glycol) (PEG) 1500 and an inorganic salt (potassium phosphate, sodium citrate, lithium sulfate or sodium sulfate). In all systems, CMP presented affinity for the PEG-rich phase. The PEG1500 + lithium sulfate showed the highest values of partitioning coefficient. In addition, thermodynamic parameters (ΔH°, ΔS°, ΔG°) as a function of temperature, were calculated for the system PEG1500-sodium citrate at different PEG concentrations and the results imply thermodynamic differences between partitioning of CMP in this system.
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Keywords: Aqueous two-phase systems; Caseinomacropetide; Hydrophobicity; Salt; Temperature; Thermodynamic parameters

1. Introduction

Caseinomacropeptide (CMP) or glycomacropeptide is formed by chymosin (or pepsin) cleavage of κ-casein between Phe105-Met106 during cheese-making [1]. CMP accounts for around 20% of the total protein content in whey protein products. In recent years, CMP has been the subject of growing interest, due to its beneficial biological and physiological properties [2], including the ability to bind cholera and *Escherichia coli* enterotoxins, inhibit bacterial and viral adhesion, modulate immune system responses, promote bifidobacteria growth, suppress gastric secretions and regulate blood circulation, as reviewed by Abd El-Salam et al. [3] and Thomä-Worringer et al. [1]. The most recent isolation techniques for the production of CMP are generally based on chromatography or ultrafiltration techniques using either chymosin-treated casein/caseinate or rennet whey as starting material. However, these techniques are usually expensive and difficult to scale up.

Aqueous two-phase systems (ATPS) have been successfully used for separation and purification of proteins due to their advantages over traditional methods. The ATPS are advantageous over other separation techniques due to the high water content of both phases, which means high biocompatibility and low interfacial tension, thus minimizing degradation of biomolecules. ATPS also provide good resolution, high yield, and a relatively high capacity. In addition, this system is easily scaled-up. ATPS could be a good alternative for a first purification step since such systems allow removal of several contaminants by a simple and low-cost process [4–9].

In this work, the effects of type of salt and temperature on the partition coefficient of CMP were studied aiming to improve the selectivity of the aqueous two-phase systems and to determine the best recovery conditions.

Abbreviations: CMP, caseinomacropetide; ATPS, aqueous two-phase systems; PEG1500, poly(ethylene glycol) with average molar mass of 1500

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2. Materials and methods

2.1. Chemicals

Poly(ethylene glycol) (PEG) with average molar mass of 1500 was obtained from Synth (Diadema, SP, Brazil). The polymer and salts were used without further purification. Caseinomacropeptide (CMP) was a kindly gift of Davisco Foods International (Eden Prairie, MN, USA), with purity higher than 83 mass%. All chemicals were of analytical grade and readily available commercial products. Ultrapure water for the experiments was obtained from a Milli-Q system (Millipore Inc., MA, USA).

2.2. Preparation of the aqueous two-phase systems

Biphasic systems were prepared by a mixture of PEG1500 and an inorganic salt (potassium phosphate, sodium citrate, lithium sulfate or sodium sulfate). To prepare ATPS, stock solutions of the phase components PEG1500 50 mass%, potassium phosphate, pH 7.0, 40 mass%, and water were mixed to obtain a total system composition. To obtain a stock solution of potassium phosphate, 40 mass% at pH 7.0, monobasic potassium phosphate (KH_2PO_4) and dibasic potassium phosphate (K_2HPO_4) were weighed and added in the proportion 1:1.82 (mono:di). Sodium citrate, lithium sulfate or sodium sulfate were employed by weighing the solid form; the pH of the PEG-sodium citrate aqueous biphasic system was adjusted to 7.0 and the buffer used was 0.1 M HCl; the pH values of the PEG-lithium sulfate and PEG-sodium sulfate biphasic systems were not adjusted since the systems presented pH 7.0. All the systems were prepared in graduated centrifuge tubes. The total weight of the phase system was 10 g. The amount of CMP added to the systems was 20 mg, and was the last component added to the systems. After 2 min of gentle stirring, the systems were centrifuged at room temperature and 2000 g for 20 min. The tubes were brought to equilibrium in a thermostatic bath according to the temperature used (278.15, 298.15, 308.15 and 318.15 K) and left overnight (at least 12 h). Following this treatment, it was observed a well-defined interface and clean, transparent bottom and top phases. To determine the concentration of proteins in each of the co-existing phases, samples from each phase were collected using a pipette for the upper phase and a long needle-syringe for the bottom phase. The volumes were determined in graduated centrifuge tubes. The molar mass of the polymer, salt and polymer concentration, temperature and pH were optimized to achieve the maximum partition coefficient for the CMP partitioning using ATPS.

2.3. Determination of the protein partition coefficient (K_p)

The partition coefficient (K_p) was defined as the ratio between $[P]_{top}$ and $[P]_{bottom}$, where $[P]_{top}$ and $[P]_{bottom}$ are the equilibrium concentrations of the partitioned protein in the PEG-(top) and salt-(bottom) enriched phases, respectively. This coefficient is used to quantify the degree of separation reached in an extraction process. The partition experiments were carried out in duplicate and the average results are the values reported in this work. To select the ATPS with the best capability of purifying CMP, it was also calculated the theoretical recovery (y, %) in the top phase by [4]:

$$y(\%) = \frac{100}{1 + (1/RK_p)} \qquad (1)$$

where R is V_T/V_B, V_B and V_T are the bottom and top phase volumes, respectively.

2.4. Determination of thermodynamic functions associated with protein partitioning

The partitioning coefficient was determined at different temperatures for the systems compounds for PEG1500-sodium citrate at different PEG concentrations. The sodium citrate was selected due to its environment safe characteristics. Thus, the thermodynamic parameters, the standard enthalpy change (ΔH°, $kJmol^{-1}$) and the standard entropy change (ΔS°, $Jmol^{-1}\,K^{-1}$), were obtained from the Van't Hoff plot by fitting $\ln K_p$ versus the reciprocal of the temperature by a non-linear regression analysis of data [10]. And the variation of Gibbs free energy (ΔG°, $kJmol^{-1}$) of solute transfer between two phases was expressed by

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \qquad (2)$$

2.5. Protein quantification

Protein quantification in the saline and polymeric phases was conducted using a chromatography ÄKTA Purifier® 10/100 system (Amersham Pharmacia Biotech, Uppsala, Sweden). The eluent was monitored by UV absorption UV-900 at 205 nm and conductivity measurements in a flow cell pH/C-900. Samples were injected by means of a loop of 50 μl. The column used was a Superdex® 75 HR 10/30 (Pharmacia Biotech, Uppsala, Sweden). The mobile phase was 0.1 M sodium phosphate monobasic, 0.1 M sodium phosphate dibasic, pH 6.0, with a flow rate of 0.65 mL min^{-1}. The maximum pressure of the column bed was 1.78 MPa. The polymeric phase was diluted before injection in a 2:1 (water:sample) ratio due to its high viscosity.

3. Results and discussion

3.1. Effect of phase-forming salt on protein separation

The change of the type and concentration of salt can alter the partition behavior of biological materials [5]. Besides, PEG-phosphate, PEG-citrate and PEG-sulfate ATPS are adequate for continuous large-scale purification of biological origin materials and allow the use of traditional liquid–liquid extraction equipment [11–14]. Thus, four PEG1500-salt systems were tested to investigate the effects of phase-forming salt on the separation of CMP. The final concentrations of PEG and salt in the different systems, the theoretical recovery and partition coefficients for the CMP in each system tested are shown in Table 1. The recoveries of CMP in the top phase were calculated by Eq. (1).

Table 1
Theoretical recoveries in the top phase and partition coefficients for the CMP in each system formed for PEG1500-salt, in different compositions of the assayed ATPS

System[a]	PEG (mass%)	Salt (mass%)	K_p	Recovery (%)
PEG1500-sodium citrate	14.00	14.89	8.85 ± 0.12	85.59 ± 0.28
	16.00	16.10	14.73 ± 0.15	92.00 ± 0.08
	18.00	17.28	18.56 ± 0.17	92.85 ± 0.07
PEG1500-potassium phosphate	14.00	10.52	3.29 ± 0.32	83.88 ± 1.36
	16.00	11.43	9.89 ± 0.08	93.95 ± 0.14
	18.00	11.45	10.61 ± 0.26	93.66 ± 0.09
PEG1500-lithium sulfate	10.00	13.03	10.91 ± 0.21	78.86 ± 0.31
	10.50	13.77	14.62 ± 0.36	88.33 ± 0.16
	11.00	14.52	22.25 ± 0.34	83.35 ± 0.34
PEG1500-sodium sulfate	10.00	9.52	2.02 ± 0.05	50.56 ± 0.21
	10.25	10.48	3.03 ± 0.10	59.59 ± 0.52
	10.50	11.87	6.04 ± 0.20	71.40 ± 1.13

[a] Each system is named with the PEG molar mass followed by the type of salt.

As shown in Table 1, the partition coefficient of CMP was higher than one ($K_p > 1$). The type and concentration of salt present a significant effect on the extraction efficiencies in ATPS. The salt influence on the partitioning is caused by the nonuniform distribution of the salt ions in the upper and lower phases, and by the difference of the electric current, which is generated by the inequality of the salt distribution [15]; in such case, ATPS may become negatively charged in the lower phase and positively charged in the upper phase, which improves the movement of the protein to the other phase by electrostatic repulsion effects. Thus, molecules which are negatively charged at neutral pH, like CMP with p*I* between 4 and 5 [1], move to the upper phase.

Except for the PEG1500-sodium sulfate system, CMP was recovered with high efficiency in the top phase, which could make the liquid–liquid extraction method suitable to be applied as an isolation method for this protein. The maximum percentage of recovery was 93.95% in ATPS containing 16 mass% PEG1500 and 11.43 mass% potassium phosphate.

In spite of the difficulty in developing a model that predicts the partition coefficient for the target molecules, many investigators have been searched for a mathematical relationship to the K_p values. Thus, Blázquez et al. [9] proposed the following simple linear relation to correlate protein partitioning in ATPS:

$$\ln K_p = A(w_{top} - w_{bottom}) \quad (3)$$

where w_{top} and w_{bottom} are the salt mass fractions in the top and bottom phase and A is a function of the interaction between salt and water. This simplified relationship could adequately describe the partition data of α-amylase.

Fig. 1 shows the partition data for CMP in different PEG1500-salt systems at increasing salt concentration difference (Δ[salt]) values, calculated as the difference between salt equilibrium concentration in the top and bottom phases, w_{top} and w_{bottom}, respectively. The trend observed is a linear relationship between $\ln K_p$ versus Δ[salt]. In all cases, higher values of the determination coefficients ($R^2 > 0.96$) were obtained. The increase of Δ[salt] for all types of salt induced an increment in the K_p value. Also, the slope values modify for each type of salt, suggesting the presence of salt–protein interactions.

Although partitioning depends on a number of factors such as hydrophobic properties, addition of salts, electrical potential between the phases, molecular size, and conformation of the molecules, the hydrophobic characteristics are considered to be the dominant factor when the influence of type of salts is analyzed. Hydrophobic effect is defined as an unfavorable interaction of nonpolar substances or moieties of molecules with water, which is responsible for their low solubility [16]. Moreover, the hydrophobicity of a substance could be described as a measure of the overall intensity of its total interactions with an aqueous medium (including hydrogen bonding, van der Waals, electrostatic interactions, etc.) [17].

When a protein is partitioned in PEG-salt ATPS, the type of salt and salt concentration have an effect on the hydrophobic interactions between the proteins and the hydrophobic media; the salt ions will interact with the oppositely charged groups in the protein to form a double layer of ionic groups. Thus, the protein will be dehydrated due to the hydration effect of salt molecules surrounding the protein, and the

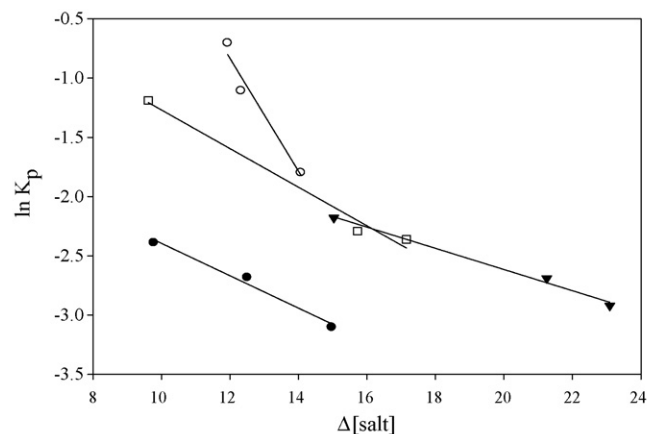

Fig. 1. Dependence of the salt concentration difference between the top and bottom phases for the CMP partition, sodium citrate (▼), sodium sulfate (◯), potassium phosphate (□), and lithium sulfate (●). Temperature: 298.15 K.

hydrophobic zones of the protein will be gradually naked, increasing with the salt concentration increment [18]. The influence of the salt ions on the protein behavior is usually related to their position in the Hofmeister series. The effectiveness of the Hofmeister series is given for anions by $SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^- > Br^- > I^- > SCN^-$, and for cations by $Li^+ > Na^+ > K^+ > NH^{4+} > Mg^{2+}$ [19]. At higher concentrations, the ions to the left of the series decrease protein solubility (salting-out effect) by increasing hydrophobic interaction and aggregation, and the hydration effect of the salt molecule surrounding the protein [20]. Thus, most proteins strongly favor the phase with lower salt concentration, increasing the interaction between the protein and the PEG molecules, improving the extraction to PEG-rich phase. According to Fig. 1, the PEG1500-lithium sulfate showed the highest values of coefficient partitioning, suggesting that the hydrophobic effect in ATPS was influenced by the Hofmeister series, and consequently by the type of salt.

3.2. *Effect of the temperature on protein partitioning*

As can be seen in Fig. 2 the temperature affected the partition behavior of CMP. Willauer et al. [21], studying the influence of temperature and type of salt on the lignin distribution using ATPS verified that the increase of the salt concentration of any salt is identical to the effect of increasing temperature on partitioning. Recent studies have shown that polymer-salt interactions are endothermic events, and the rise in temperature should be favorable to this reaction, which leads to an increase in the amount of salt necessary for phase splitting, enlarging the biphasic area and increasing the slope of the tie line [22,23]. Thus, it is expected in our study an increase in the phase divergence with the increase of the temperature. Consequently, the CMP will exhibit higher partitioning coefficients due to its preference for the PEG-rich phase, as it was found in this work in the systems with high salt concentration.

Fig. 2 shows the temperature effect on the partitioning coefficient, expressed as non-linear Van't Hoff plots. Changes in temperature and PEG concentration affected the partitioning coefficient of CMP. The increase of PEG concentration in the system increases the partitioning coefficient, but this behavior is in contrast with the excluded volume effect theory. This theory suggests that the increase of either the PEG concentration or the PEG molar mass induces a reduction of the protein solubility in the phase where the protein is placed. Spelzini et al. [24] reported that the PEG–protein–salt interaction prevails on the excluded volume effect. Furthermore, as the PEG concentration increases, the number of polymer units involved in the biomolecular partitioning also increases, and, hence, more protein molecules partition into the PEG phase, due to the hydrophobic interaction between the protein and PEG [14].

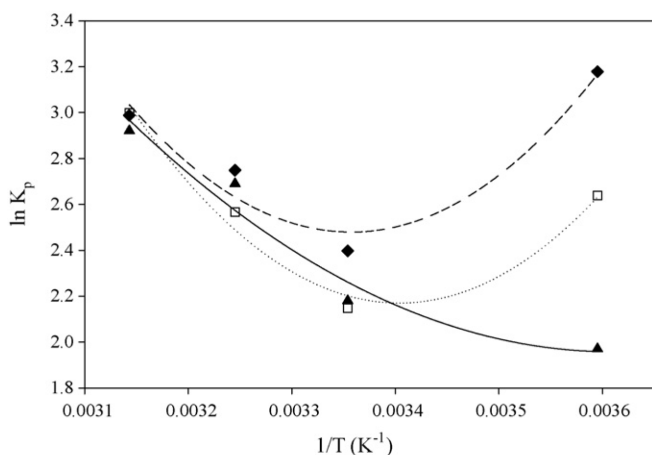

Fig. 2. Van't Hoff plots. Temperature effect of the partition equilibrium of CMP at different concentrations of PEG: 14 mass% PEG1500 (▲), 16 mass% PEG1500 (□), and 18 mass% PEG1500 (♦). pH 8.0.

Table 2
Adjusted parameters of the Van't Hoff equation

PEG (mass%)	Parameters			
	a	*b*	*c*	R^2
14.00	62.33	−33446.57	4632541.66	0.96
16.00	148.63	−86047.14	12638755.51	0.97
18.00	139.32	−81520.27	12141187.73	0.93

Fig. 2 also suggests that the enthalpic change associated to protein partition is temperature-dependent. The solids curves in the figure represent the predicted values using the quadratic form of Eq. (3). The adjusted parameters of the Van't Hoff equation obtained are presented in Table 2. In all cases, higher values of the coefficients of determination ($R^2 > 0.93$) were obtained.

The thermodynamic parameters for the partitioning of CMP were calculated based on Table 2 data and using Eqs. (1)–(3). The results are shown in Table 3 and Figs. 3–5.

These thermodynamic functions provide information on the molecular mechanism taking part in the protein transfer from the salt to the PEG-rich phase. The results presented demonstrate that protein partitioning enthalpy and protein partitioning entropy increase with temperature for CMP at all PEG concentrations. The partitioning process tends to be more enthalpic unfavorable with increase in temperature [22,23]; therefore, the transfer of CMP to the top phase was endothermic at the tem-

Table 3
Thermodynamic parameters for the partitioning of CMP in ATPS, at different PEG concentrations

Temperature (K)	PEG1500 (mass%)	ΔH° (kJmol^{-1})	ΔS° (Jmol^{-1} K^{-1})	ΔG° (kJmol^{-1})
278.15	14.00	1.14	20.38	−4.53
298.15		19.72	84.93	−5.61
308.15		28.10	112.60	−6.60
318.15		35.96	137.69	−7.85
278.15	16.00	−40.16	−122.50	−6.09
298.15		10.53	53.61	−5.46
308.15		33.40	129.09	−6.38
318.15		54.84	197.56	−8.02
278.15	18.00	−48.05	−146.42	−7.32
298.15		0.64	22.76	−6.15
308.15		22.61	95.26	−6.74
318.15		43.21	161.04	−8.03

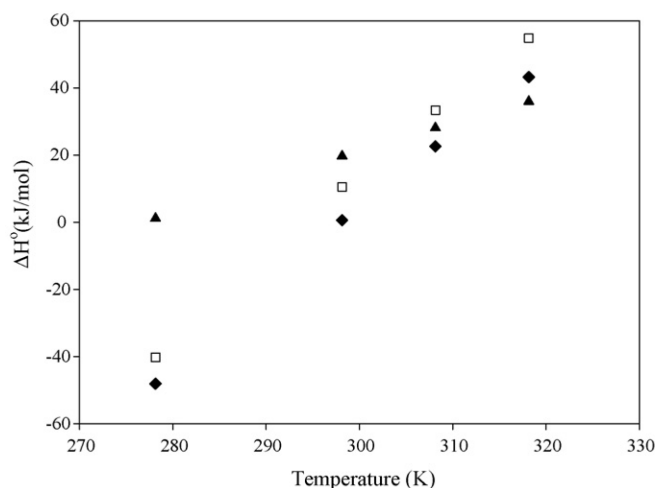

Fig. 3. Enthalpy change of the partition equilibrium of CMP at different concentrations of PEG: 14 mass% PEG1500 (▲), 16 mass% PEG1500 (□), and 18 mass% PEG1500 (♦). pH 8.0.

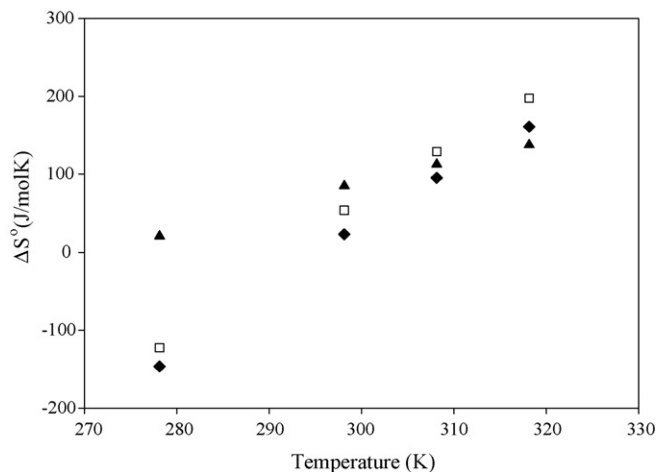

Fig. 4. Entropy change of the partition equilibrium of CMP at different concentrations of PEG: 14 mass% PEG1500 (▲), 16 mass% PEG1500 (□), and 18 mass% PEG1500 (♦). pH 8.0.

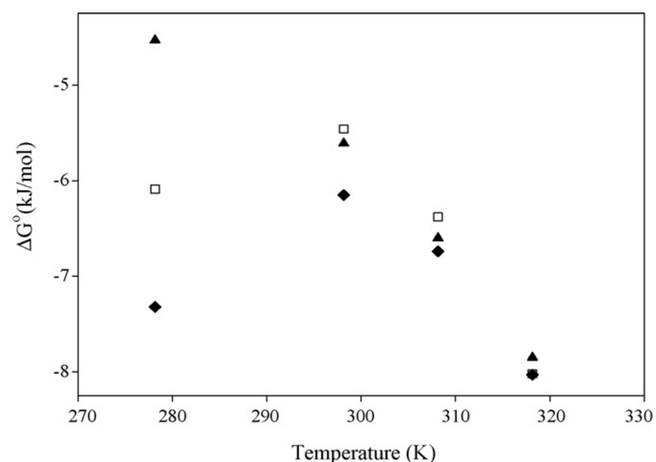

Fig. 5. Free energy change of the partition equilibrium of CMP at different concentrations of PEG: 14 mass% PEG1500 (▲), 16 mass% PEG1500 (□), and 18 mass% PEG1500 (♦). pH 8.0.

perature range 298.15–318.15 K, while at 278.15 K, an inversion of the sign in ΔH° was observed for concentrations of 16 and 18 mass% PEG1500. A similar inversion in the ΔS° sign was verified. In Figs. 3 and 4, a linear dependence of ΔH° and ΔS° on temperature was observed for all PEG concentrations. The partitioning process was found to be entropically driven as temperature increased [22,23]. The results also indicated that the change in Gibbs free energy is negative in all cases, a characteristic of spontaneous process, and becomes more negative as temperature is increased, in nearly all cases.

4. Conclusions

The influence of type of salt (potassium phosphate, sodium citrate, lithium sulfate or sodium sulfate) and temperature (278.15–318.15 K) on the CMP partition coefficient in the PEG/salt ATPS was studied. The CMP is a protein source of promising industrial potential. Thus, the influence of various factors on its partitioning was analyzed. Efficient and low-cost CMP extraction can be achieved by using PEG-salt biphasic system. The most favorable condition for recovery was found at 16 mass% PEG1500 + 11.43 mass% potassium phosphate + 72.57 mass% water, pH 7.0 and room temperature (278.15 K), being approximately 93.95% of CMP recovered. The effect of temperature on the partition behavior of CMP was also studied. The thermodynamic parameters (ΔH°, ΔS°, and ΔG°) as a function of temperature were calculated for the system PEG1500-sodium citrate at different PEG concentrations and the results explicit thermodynamic differences between partitioning of CMP. The ΔH°, ΔS°, and ΔG° values of the process studied have shown that this process is driven by entropy. Furthermore, CMP partitioning needs to be investigated in other polymer–polymer systems and the partitioning parameters must be determined, especially under a continuous mode of operation, to make the technique commercially viable.

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