Liquid-Liquid Equilibrium and Partitioning Features of Bovine Trypsin in Ucon 50 HB5100 /Sodium Citrate Aqueous Two Phase Systems

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Abstract

The phase diagrams of Ucon 50-HB-5100, a non-ionic random copolymer of ethylene oxide and propylene oxide (EOPO) and sodium citrate aqueous two-phase systems were determined at different pHs (5.20 and 8.20) and temperatures (5, 20 and 40º C). The binodal curves were determined by refractive index and enzymatic assay of the solution and described using a four-parameter sigmoidal equation, the reliability of the measured tie line compositions was ascertained by correlation equations given by Othmer Tobias and Bancroft. The two-phase area was expanded by increasing both pH and temperature. The partitioning of bovine trypsin and α-chymotrypsin, proteases of similar physico-chemical properties was investigated in order to evaluate the applicability of partitioning as a putative method to isolate from pancreas and to obtain any information about their partitioning mechanism. The effect of different factors such as pH, tie line length and the presence of an inorganic salt on the protein partition coefficient were analyzed.

Resumen

Se caracterizaron las curvas binomiales correspondientes a los sistemas bifásicos acuosos formados por Ucon 50-HB-5100, un copolímero al azar de óxido de etileno y óxido de propileno (EOPO) y citrato de sodio a diferentes pHs (5,20 y 8,20) y temperaturas (5, 20 y 40º C). Las curvas binomiales se obtuvieron por determinación del índice de refracción y ensayos enzimáticos de las soluciones correspondientes; las composiciones de las líneas de unión se corroboraron por las ecuaciones propuestas por Othmer Tobias y Bancroft. El aumento del pH y la temperatura condujeron a un aumento del área bifásica. También se ensayó el comportamiento de reparto de dos proteasas tripsina y α-quimotripsina con el objetivo de emplear los principios de partición como método de aislamiento y purificación de Tripsina a partir de páncreas bovino y de obtener información acerca del mecanismo de partición de la misma. Se analizó el efecto del pH, longitud de la línea de unión y presencia de sales inorgánicas sobre el coeficiente de partición de las enzimas.

1. Introducion

Bovine trypsin is an enzyme that is widely used for commercial purposes to digest or process other proteins, including some therapeutic proteins. Besides, with a high purity grade is employed with researching purposes in protein sequenciation. Another well-known pancreatic serine protease, ?-chymotrypsin, represents the principal contaminant since both proteases exhibit chemical similarities. Their separation is achieved after different chromatographic steps which are long and require expensive materials [1].

The liquid-liquid extraction principle applied to aqueous two-phase systems (ATPSs) offers a method for purification of biologically active materials since they allow the separation of these substances in biocompatible surroundings [2]. In laboratory scale separations the most commonly used systems are composed by the polymers polyethyleneglycol (PEG) and dextran while for large scale enzyme extraction, PEG/salt systems are used. These systems are attractive because of their low cost and rapid phase disengagement. Previous studies [3] have demonstrated that replacing the inorganic salts by other biodegradable and non-toxic ones such as citrates could be considered a good alternative, since citrates can be discharged into biological wastewater treatment plants. Recently, the use of thermo-separating polymers in ATPSs has been introduced [4]. When such polymers are heated above a lower critical solution temperature (LCST), the solubility of the polymer will decrease and a system composed of water and a polymer phase is formed. This makes it possible to perform temperature induced phase separation whereby a target protein can be separated from the polymer and recovered in the water phase.
The general mechanism governing the partition of biological material in aqueous two-phase systems is still not well understood; therefore the ATPS which provides the optimal separation conditions can only be selected after an experimental work. In this work, Ucon 50 HB-5100, an EOPO random copolymer of 50 % ethylene oxide and 50 % propylene oxide (mass) with an average molecular mass of 3900 was selected to form ATPSs with NaCit at pH 5.20 and 8.20. The corresponding phase diagrams were determined and the effect of temperature on the binodal curves was also studied. Then we describe the partitioning features of bovine trypsin and ?-chymotrypsin in the characterize Ucon/NaCit ATPSs in order to evaluate the ability of these systems of separating both proteins.

2. Experimental

2.1. Chemicals: Trypsin (TRP), α-chymotrypsin (ChTRP) from bovine pancreas, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-Benzoyl-L-tyrosine ethyl ester (BTEE) and Citric acid were purchased from Sigma Chem. Co. and used without further purification. Enzymes for citrate determination were obtained from Boehringer Ingelheim, Germany. Ucon 50 HB-5100 of average molecular mass of 3900 was obtained from Union Carbide (NY). All the other reagents were of analytical quality. Stock solutions of UCON of (40-50 % w/w) and NaCit (25 % w/w), of a given pH, were prepared by weighing known quantities of the polymer and citric acid respectively.

2.2. Phase diagram determination: A phase diagram is constituted of a binodal curve and tie lines. The determination of the binodal curve was carried out by a turbidimetric titration method [5]. The system temperature was maintained constant and controlled by immersing the glass tube and the stock solutions in a thermostatic bath.
For the determination of the tie lines, a series of ATPSs of at least three different known total compositions were prepared. When phases were separated, citrate and Ucon equilibrium concentrations were determined by an enzymatic assay described by Mollering and Gruber [6] and refractive index measurements [7] using a refractometer ABBE, NAR 3T (Atago Japan).

2.3. TRP and ChTRP enzymatic activity determination: Trypsin activity was determined with the substrate BAPNA using a method modified from Gildberg and Overbo [8]. The α-chymotrypsin assay is based on the hydrolysis of BTEE [9]. Both enzyme assays were performed at constant temperature of 22 °C. The activities were calculated from the initial linear portion of the absorbance vs. time curve.

2.4. Preparation of the aqueous biphasic system: To prepare the biphasic aqueous systems, stock solutions of the phase components of a given pH were mixed according to the binodal diagram previously obtained. Low-speed centrifugation was used after a thorough gentle mixing of the system components to speed up phase separation, and then each phase was mixed to reconstitute several two-phase systems in which the protein partition was assayed. Three different tie lines were assayed numbered (from 1 to 4) according to their increasing TLL.

2.5. Determination of the partition coefficient (Kp): Partitioning behaviour of TRP and ChTRP was analysed by the method given in ref 5. The partition coefficient was defined as:

$$Kp = \frac{[P]_T}{[P]_B} \qquad (1)$$

where $[P]_T$ and $[P]_B$ are equilibrium concentrations of the partitioned protein in the Ucon and NaCit-rich phases, respectively. In our case, the Kp for TRP and ChTRP was calculated by the ratio of the enzyme activities in each phase. A correction factor was calculated as the ratio between the activities of reference solutions (of known concentration) of the enzyme in ach phase. All the measurements were developed by triplicate and constant temperature.

2.6 Selection of ATPS with the best separating capability: To select the ATPS with the best separating capability, the theoretical recovery and purity percentages of TRP in the top phase ($R_{TRP,T}$ and $P_{TRP,T}$) after a first extraction step, were calculated according to:

$$P_{TRP,T}(\%) = \frac{R_{TRP,T}}{R_{TRP,T} + R_{ChTRP,T}} 100 \qquad R_{TRP,T}(\%) = \frac{Kp_{TRP}\frac{V_T}{V_B}}{1 + Kp_{TRP}\frac{V_T}{V_B}} 100 \qquad (2)$$

where V_B and V_T are the bottom and top phase volumes. The following assumptions were made: - similar concentrations of TRP and ChTRP in the starting sample, - the V_T/V_B ratio equal to be one, -similar Kp values for each protein in the mixture and alone.

3. Results and Discussion

3.1. Binodal curve: The total system compositions and tie line length determined for the studied systems are shown in Table 1. From visual inspection of the parameters and the corresponding determination coefficients (R^2), it is possible to conclude that of a sigmoidal equation is the most suitable to fit the binodal data since their R^2 values are closer to one than those obtained from literature expressions. Fig.1 summarizes the binodal data corresponding to ATPSs of Ucon/NaCit at different pHs. Binodal curves show similar shapes for the several pH values and the two-phase area is found to be expanded when pH is increased. At 20ºC, binodal curves corresponding to the different pHs tend to superimpose at high concentrations of Ucon or NaCit, thus indicating that either the exclusion or the salting out effect respectively prevails in phase-separation processes. When

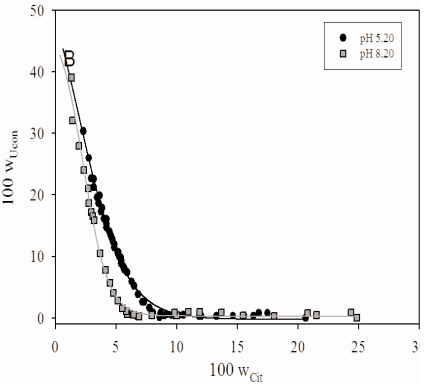

Figure 1. Effect of pH on binodal curve of Ucon/NaCit aqueous two-phase systems at 5 °C.

Ucon and NaCit concentrations adopt intermediate values, a smaller concentration of NaCit is needed for two-phase formation at basic pHs (with higher ratios between trivalent and divalent citrate ions). This effect is also observed in binodal curves at 5°C, even at high Ucon concentrations (low NaCit concentrations) at which no superimposition of binodal curves is observed. At low temperatures, the EO and PO units in the copolymer, are known to be strongly hydrated with two or three water molecules [10]. Similarly, ionic species in solution are known to be hydrated and the extent of hydration depends upon the ion valency.

Table 1. Phase Compositions for Ucon/NaCit ATPSs.

		total compositions		top phase		bottom phase						
pH	Temp	$100w_{NaCit}$	$100w_{Ucon}$	$100w_{NaCit}$	$100w_{Ucon}$	$100w_{NaCit}$	$100w_{Ucon}$	STL*	σ**	TL	100TLL	Kp***
5.20	5º C	7.97	8.96	3.22	22.31	10.97	0.51	-2.81	0.11	1	23.14	3.4
		8.99	8.98	2.81	25.88	12.12	0.39	-2.74	0.08	2	27.14	4.3
		10.06	9.00	2.48	28.82	13.37	0.34	-2.61	0.13	3	30.49	5.4
		10.99	8.98	2.22	30.96	14.44	0.32	-2.51	0.08	4	32.99	6.5
	20º C	5.70	10.65	3.32	21.40	7.28	3.48	-4.53	0.14	1	18.35	2.2
		5.99	11.02	2.84	25.21	7.87	2.52	-4.51	0.09	2	23.24	2.8
		6.22	17.08	1.89	33.40	10.64	0.42	-3.77	0.08	3	34.12	5.6
		6.54	17.87	1.64	35.53	11.38	0.21	-3.63	0.11	4	36.64	6.9
	40º C	6.23	17.15	1.19	43.75	9.32	0.78	-5.28	0.21	1	43.73	7.8
		6.48	17.81	0.97	46.43	9.87	0.19	-5.20	0.16	2	47.09	10.1
		7.09	19.49	0.49	52.51	11.15	0.09	-4.94	0.15	3	53.49	22.6
8.20	5º C	6.45	9.44	1.95	22.73	9.62	0.09	-2.95	0.09	1	23.90	4.9
		7.48	9.37	1.50	26.78	10.71	0.19	-2.89	0.12	2	28.14	7.1
		8.47	9.40	1.12	30.39	11.80	0.19	-2.83	0.11	3	32.03	10.5
		9.41	9.37	0.74	33.90	12.78	0.18	-2.81	0.14	4	35.81	17.2
	20º C	4.32	14.88	2.16	26.45	6.93	0.93	-5.35	0.21	1	25.96	3.2
		4.49	16.33	1.93	29.37	7.58	0.61	-5.09	0.15	2	29.31	3.9
		5.00	17.70	1.63	33.33	8.74	0.33	-4.64	0.14	3	33.76	5.4
		5.49	19.18	1.41	36.24	10.02	0.22	-4.18	0.17	4	37.03	7.1
	40º C	1.99	15.85	0.87	26.95	3.35	2.31	-9.94	0.40	1	24.76	3.8
		2.20	16.39	0.82	28.80	3.83	1.75	-8.97	0.18	2	27.22	4.7
		2.64	20.21	0.42	41.69	5.03	0.04	-9.03	0.27	3	41.90	12
		3.13	23.36	0.41	46.06	5.79	1.12	-8.35	0.25	4	45.26	14.1

*STL tie line slope, **σ Standard deviation, ***Kp Fraction of NaCit retained in the bottom phase divided by the NaCit in the top phase

Thus, triply charged citrate can be expected to be more effective than doubly charged citrate in salting out the copolymer because of competition for water. Therefore, trivalent ions are more efficient than divalent ions in promoting the phase separation [11]. However, this effect does not seem to be significant at low NaCit concentration at 20°C, since binodal curves corresponding to the different pHs overlap. In this case, the breakdown of structured water molecules around the EO/PO-chains associated with an increase in temperature is probably the predominant cause of phase separation.

The effect of temperature on phase-separation processes is given in Fig. 2. An increase in temperature increase from 5ºC to 20ºC induced a slight increase in the biphasic area, while a significant expansion of the two-phase region was observed when the temperature was raised up to 40º C. This trend was also observed for other polymer/salt systems such as PEG/NaCit [12]. According to the model proposed by Kjellander and Florin [2], the entropically unfavourable structuring of water produced by Ucon at low temperatures is overcome owing to the large decrease in enthalpy (due to the energetically favourable and highly directional interactions, such as hydrogen-bonding, between unlike molecules). At higher temperatures, provided that the structure of water in the Ucon hydration shell does not break down too rapidly with increasing temperature [11], the unfavourable entropy contribution becomes prominent and the system phase separates itself. In addition by increasing the temperature the magnitude of the tie line slopes increases (see Table 1), indicating the increase of the asymmetry of the diagrams. Similar results were obtained for other polymer/salt systems from literature [13].

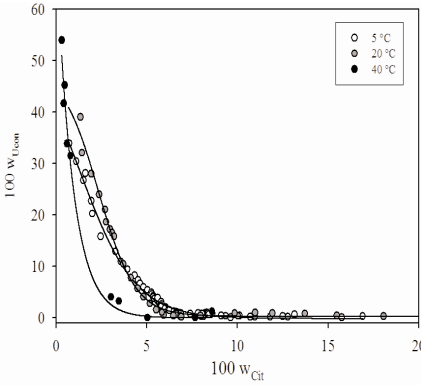

Figure 2. Effect of temperature on binodal curve of Ucon/Cit pH 8.20 aqueous two-phase system.

3.2. Tie lines: In Table 1 it also lists the value of the partition coefficient (Kp) of the salt, defined as the fraction of salt retained in the bottom phase divided by the salt in the top phase. High values of Kp were obtained (from 2.2 to 22.6) which is an indication of the separation obtained by adding an amount of Ucon to a given brine solution.

For most of the assayed systems, the tie lines became steeper for total compositions in the vicinity of the critical point. An increase in the STL magnitude indicates an increase in the difference between the polymer concentrations at a given difference in the salt concentrations in the same phase. This implies a decrease in the mutual solubility of the aqueous polymer- and salt-containing media. Empirical equations have been proposed to ascertain the reliability of calculated tie line data in traditional liquid-liquid extraction, being the most widely used those of Othmer-Tobias and Bancroft [14]. Linearization of both equations produced acceptable consistency in the results.

3.3. Partition behaviour of TRP and ChTRP in Ucon/sodium citrate ATPSs: The effect of medium pH on the TRP and ChTRP partition coefficients (Kps) was analyzed. The increase in pH from 5.2 to 8.2 produced an increase of the Kp value for ATPSs formed by Ucon. This behaviour could be satisfactorily explained on the basis of the Albertsson equation [2], which takes into account an electrostatical and a non-electrostatical term. In these systems, the interfacial potencial assumes positive values (since the bottom phase is enriched in the citrate anion); therefore, the electrostatic term will assume the opposite sign to the net protein charge. At pH.5.2 and 8.2 media, TRP and ChTRP are positively charged since their isoelectrical points are 9.1 and 10.5 respectively. When pH raises from 5.2 to 8.2, the net protein charge

decreases, thus increasing both protein Kp values. Protein partitioning behaviour showed to be sensitive the surface. Protein partitioning behaviour showed to be sensitive the surface hydrophobicity [15]. Partition equilibrium for TRP showed to be more displaced to the top phase than ChTRP for assayed ATPSs.

3.4. Influence tie line length and pH on the TRP and ChTRP partitioning: The effect of TLL and pH medium on these proteins partitioning are describes in Fig. 3. The increase in the TLL is accompanied by a decrease in the Kp value. Previous reported data [16] showed that the partition coefficient became more one sided when the tie line length was increased due to the increase in the difference between both the polymer and salt concentrations in the top and bottom phases. For those systems where proteins exhibit a great affinity for the bottom phase, (Kp <1) the partition equilibrium displaced to the bottom phase as the TLL increases while in ATPSs where proteins prefer the polymer rich-phase (Kp >1) the opposite behaviour was observed. Another system variable such as the pH medium was studied. For most of the assayed systems an increase in the Kp values was observed when pH increased from 5.20 to 8.20 in accordance with the Albertsson equation [2]. These findings showed that both TLL and pH would be able to be manipulated in order to separate TRP from ChTRP.

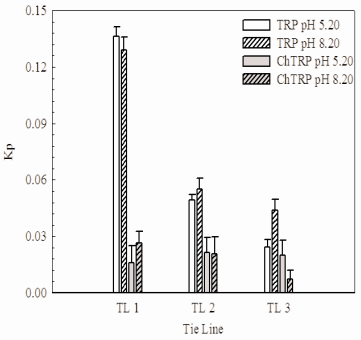

Figure 3: Effect of tie line and pH on the Kp for TRP and ChTRP Ucon/NaCit aqueous two phase system.

Addition of certain salts, such as NaCl, has been reported [4] to displace the partitioning equilibrium to the top phase thus enhancing the protein yield in this phase. Fig. 4 shows that the NaCl presence induces a transfer of trypsin to the top phase. Besides, TRP partitioning showed to be sensitive to salt presence (Kp increases) thus leading to an enhancement of the ATPS selectivity. Our results demonstrate that NaCl addition is a successful tool to improve protein yield and lead to a significant purification factor enhancement but not enough to obtain a satisfactory result.

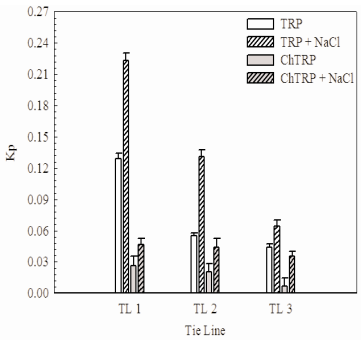

Figure 4: Effect of tie line and NaCl on the Kp for TRP and ChTRP. Ucon/NaCit aqueous two phase system.

3.5 Selection of optimal separating conditions: According to literature [17], the ratio between the target and contaminant protein partition coefficients ($Kp_{target}/Kp_{contaminant}$) can be considered as a measure of system selectivity, those systems with higher ratio being the most selective ones. In this study we included additional calculations in order to make the decision more appropriate. The theoretical recovery (R) and purity (P) percentages of TRP in the top phase after one extraction step were calculated according to equation (2) in order to select the ATPS with the best capability of separating TRP from ChTRP. From a visual inspection of Fig. 5, it is evident that Ucon/NaCit ATPSs would lead to high TRP purity values (R>70%). This fact is in agreement with the predicted effect of Kp on the R value according to equation (2). In contrast, conduce to lower TRP yields values. At this point, a non-trivial topic to be decided is if either the recovery or the purity should be optimised. The answer depends on the source availability and final application of the target protein.

4. Conclusions

The phase diagrams of Ucon/NaCit ATPSs were determined. Reliable and complete data on the composition and properties of these systems were not available at present, being this information necessary for the design of an extraction process. The phase formation proved to be both temperature and pH-dependent. Much lower amounts of polymer and salt than conventional systems are required to form the two phases, thus reducing the environmental impact. In addition, thermo-separating properties of Ucon can be used to recycle this polymer from the polymer rich-phase. These characteristics and several additional advantages such as biodegradability of citrate anion, low cost and rapid phase separation make Ucon/NaCit ATPSs a promising, versatile and attractive system in the field of bio-separation. TRP and ChTRP partitioning behaviour in Ucon/NaCit ATPSs showed to be sensitive to medium pH and tie line length. In spite of their similar physicochemical properties such as molecular weight and isoelectrical point, both proteins showed different partitioning behaviour. ChTRP is more partitioned to the bottom phase (citrate-riched) than TRP for most assayed systems. The presence of NaCl 3 % enhances the separation capability of Ucon/NaCit ATPSs for the separation of both proteins. Although further work needs to be done to choose the most adequate ATPS, these findings suggest that Ucon/NaCit ATPSs could be employed as a viable and potentially useful first step procedure for the separation of TRP and ChTRP.

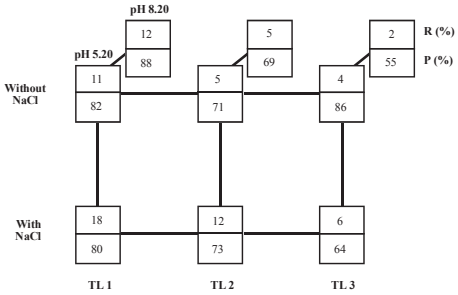

Figure 5: Effect of polymer molecular mass, TLL and pH on the theoretical recovery (R) and purity (P) percentage of TRP at the top phase after one extraction step and employing equal top/bottom phase volumes.

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