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Extraction of Ascorbate Oxidase from *Cucurbita maxima* by Continuous Process in Perforated Rotating Disc Contactor Using Aqueous Two-Phase Systems

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Abstract The ascorbate oxidase is the enzyme used to determine the content of ascorbic acid in the pharmaceutical and food industries and clinics analyses. The techniques currently used for the purification of this enzyme raise its production cost. Thus, the development of alternative processes and with the potential to reduce costs is interesting. The application of aqueous two-phase system is proposed as an alternative to purification because it enables good separation of biomolecules. The objective of this study was to determine the conditions to continuously pre-purify the enzyme ascorbate oxidase by an aqueous two-phase system (PEG/citrate) using rotating column provided with perforated discs. Under the best conditions (20,000 g/mol PEG molar mass, 10% PEG concentration, and 25% citrate concentration), the system showed satisfactory results (partition coefficient, 3.35; separation efficiency, 54.98%; and purification factor, 1.46) and proved suitable for the pre-purification of ascorbate oxidase in continuous process.

Keywords Ascorbate oxidase · ATPS · Continuous extraction · *Cucurbita maxima* · PRDC

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Introduction

The determination and monitoring of vitamin C (ascorbic acid) has been used as a fundamental objective marker for defining the commercial value of fruit and vegetable products. The measurement is usually based on the total conversion of ascorbic acid to dehydroascorbic acid by ascobate oxidase [1].

The enzyme ascorbate oxidase (AO; EC 1.10.3.3) has been extracted from various plants, such as melons, squash, cucumber, grape, orange, pepper, tomato, pepper, and starfruit. Studies of purification, characterization, and gene expression were performed; however, its biological function is still little known [2].

In general, protein recovery from the plant material includes extraction, clarification, protein capture, purification, and polishing. The overall production cost is mainly determined by the efficiency of the initial capture and purification steps [3]. Therefore, the establishment of efficient primary recovery procedures for the recovery of recombinant proteins from transgenic crop is needed. In this context, aqueous two-phase systems (ATPS) are an attractive alternative to facilitate the adoption of bioprocesses based on plants as production systems and proved to have great potential for the recovery and purification of biological compounds [4].

The main advantages of purifying biomolecules by ATPSs are the easiness in scaling up, rapid mass transfer, low energy requirements to achieve balance thanks to mechanical mixture, use of room temperature, high rapidity and selectivity, and low operating costs compared to other separation processes [5].

Statistical design of experiments is widely used for optimization and control of ATPS. It is a convenient method to study the effects of the variables involved in the purification because it allows estimating their significant effects on the selected responses, as well as their possible interactions [6, 7].

Liquid–liquid extraction equipments are widely used in biotechnology process for extraction of proteins [8, 9], enzymes [8, 10–12], toxin [7], etc. The rotating disc contactor, consisting of a vertical shell equipped with a series of rotor discs and stators, can be used for this process [13]. Extractors and columns of liquid–liquid extraction have basically two functions: (1) to establish a contact between the two liquid phases present in the system, usually by continuously dispersing one phase (in the form of drops) in the other, with the aim of improving the mass transfer and (2) to separate the two liquids after the extraction [14].

Another tool that has been recently successfully applied to liquid–liquid extraction with ATPSs is the perforated rotating disc contactor (PRDC). This type of extraction apparatus shows greater efficiency and better operational flexibility than the more conventional types [10].

The PRDC has several advantages compared to other types of liquid–liquid extraction equipments such as packed, spray, and pulsed columns because of its simplicity in construction, high throughput, relatively low power consumption, and high efficiency owing to circular motion of the rotating discs, which increase the turbulence in the contacting liquid phases [15]. The main advantage of the column extraction method is that the phases separate easily and quickly by gravity, without the need of a centrifuge. In addition, the process is simple and easy to operate in continuous mode [11]. The PRDC was applied only to a few aqueous two-phase PEG/salt systems, but no study is reported in the literature, to the best of our knowledge, on its use for the PEG/citrate one.

The objective of this study was to select the conditions to extract and pre-purify the AO from pumpkin (*Cucurbita maxima*) by liquid–liquid extraction in ATPSs in continuous process using PRDC.

Materials and Methods

Materials

AO (EC 1.10.3.3) from crude extract of *Curcubita maxima* was used. The enzyme was extracted following the procedure of Carvalho et al. [16]. Polyethylene glycol (PEG) with molar mass of 3,350, 8,000, and 20,000 g/mol was obtained from Sigma Chemical (St. Louis, MO, USA). All other chemicals were analytical grade reagents.

Preparation of the Phase System

The phase system (700 g) was prepared from stock solutions of 50% (*w*/*w*) PEG and 30% (*w*/*w*) sodium citrate/citric acid buffer. The latter solution consisted of a mixture of sodium citrate salt and citric acid at pH6.0. The system, prepared by mixing PEG and citrate stock solutions so as to give desired final compositions (Table 1), was stirred for 4 h at 25°C, and the phases were separated by funnel overnight. After separation, the phases were introduced into the column by means of peristaltic pumps.

Perforated Rotating Disc Contactor

The PRDC was made of a Perspex column with 32-mm internal diameter and 160-mm height. Six perforated discs with 30-mm diameter and drilled with 20 holes with 2-mm diameter (disc free area of about 9%) were mounted on a central shaft and equally separated. The column was maintained at 25±1 °C throughout all the experiments, and the disc rotational speed was set at 80 rpm by means of a shaft stirrer. The dispersed phase and continuous phase flowrates were 2 and 1 mL/min, respectively.

Experimental Design

The influence of the PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), and citrate concentration (C_{CIT}) on the four responses was evaluated from the results of experiments performed according to a 2^3 factorial experimental design [17] plus two central points (Table 1). All statistical and graphical analyses were carried out using the Statistica 8.0 software [18].

Experimental Procedure

The column was initially filled with 90 mL of the citrate phase containing enzyme extract, with final protein concentration of 0.5 mg/mL. The dispersed phase (inlet PEG solution)

Table 1 Variable levels of the 2^3 experimental design for the continuous extraction of ascorbate oxidase in PRDC.

Variables	Levels		
	Low (−1)	Central (0)	High (+1)
M_{PEG} (g/mol)	3,350	8,000	20,000
C_{PEG} (%)	10	15	20
C_{CIT} (%)	15	20	25

and continuous phase (inlet citrate solution containing AO) were fed upward and downward, respectively, while the extracted phase (outlet PEG solution enriched with AO) and the raffinate phase (outlet exhaust salt solution) were withdrawn at the top and bottom of the column, respectively. The column was operated counter-currently in continuous mode for 1 h to reach steady-state conditions.

Analytical Methods

The enzymatic activity was determined in continuous, raffinate, and extracted phases according to method described by Carvalho et al. [16], using ascorbic acid (50 μM) as substrate. The concentration of total proteins in these phases was determined by the Bradford method [19].

Definition of Process Parameters

The definitions of process parameters evaluated for the extraction by ATPS in continuous mode, and the related equations were already described by several authors [7, 10, 11, 20].

To determine the fractional disperse phase "hold up" (H), the total volume of the ATPS (V) and the volume of dispersed phase (V_D) were measured after achieving the steady state. This was performed by simultaneous stop of the inlet and outlet streams and agitation, followed by removal of the extractor content and volume measurement of the two phases. H was measured 30 min after interruption and was calculated according to Eq. 1:

$$H = \frac{V_{\mathrm{D}}}{V} \tag{1}$$

The mass transfer coefficient ($K_{\mathrm{D}}a$) was expressed according to Eq. 2:

$$K_{\mathrm{D}}a = \frac{F_{\mathrm{D}}}{V} \ln \frac{C_{\mathrm{Di}} - KC_{\mathrm{C}}}{C_{\mathrm{Df}} - KC_{\mathrm{C}}} \tag{2}$$

where C_{Di} and C_{Df} are the initial and final protein concentrations in the dispersed phase (mg/mL); C_{C} is the protein concentration in the continuous phase (mg/mL); and K is the partition coefficient (dimensionless).

The purification factor (P_{f}) of AO was defined as the ratio between the AO specific activities after extraction by ATPS in the PRDC and the crude extract. The partition coefficient (K) was defined as the ratio between total enzyme concentration in the top and bottom phases, respectively.

The separation efficiency (E_{S}) was determined under steady-state conditions using the Eq. 3:

$$E_{\mathrm{S}} = \left(\frac{C_{\mathrm{C\,inlet}} - C_{\mathrm{C\,outlet}}}{C_{\mathrm{C\,inlet}}} \right) \times 100 \tag{3}$$

where $C_{\mathrm{C\ inlet}}$ and $C_{\mathrm{C\ oulet}}$ are the protein concentrations in the continuous phase (inlet) and raffinate phase (outlet), respectively.

Results and Discussion

The results of previous batch tests of AO extraction by aqueous two phase systems demonstrated that the AO partitioned to the PEG phase, which was then selected as the

dispersed phase in this study and were used as a starting basis to perform the continuous extraction in the PRDC. To this objective, we used the PEG molar mass (M_{PEG}), PEG concentration (C_{PEG}), and citrate concentration (C_{CIT}) as the independent variables, and partition coefficient (K), hold up (H), the mass transfer coefficient (K_Da), purification factor (P_f), and separation efficiency (E_S) as the responses (Table 2).

A positive effect of M_{PEG} and C_{CIT} and a negative effect of C_{PEG} on the response K were estimated (Table 3), which means that the highest level of M_{PEG} and C_{CIT} and the lowest level of C_{PEG} provided the highest value of K (3.35). Low PEG concentration associated with high citrate concentration and PEG molar mass favored the transfer of the enzyme to the disperse phase, thus increasing the partition coefficient. Some authors [3–6] proposed that, when the system has high M_{PEG}, a remarkable volume exclusion effect might have taken place, hence suggesting that there was no space enough for the enzyme in the PEG phase of the system; however, this phenomenon was not observed in the present study.

Figure 1 shows the interaction between the three variables. PEG molar mass of 20,000 g/mol, PEG concentration of 10% (*w*/*w*), and citrate concentration of 25% (*w*/*w*) were the best conditions that favored the enzyme partition to the disperse phase, rich in PEG (run 6). This behavior was expected by the fact that PEG 20,000 (g/mol) ensured the best performance of purification of *C. maxima* AO in batch extraction tests (data not shown).

Statistical analysis for the purification factor was done (Table 3), but all the independent variables were not significant. The results shown in Table 2 confirmed that PEG 20,000 (g/mol), PEG concentration of 20% (*w*/*w*), and citrate concentration of 15% (*w*/*w*) were the best conditions also for the purification factor (2.49). They are similar to those obtained for the extraction of AO in pulsed cap column (PEG 1,500/fosfato; P_f=1.34) [8] and alpha toxin in PRDC using PEG/phosphate ATPS (P_f=2.4) [7].

Experiments based on H measurements were initially performed to investigate the hydrodynamics of the PRDC. This parameter expressed the amount of solvent actually available to remove the desired product from the feed [7, 10, 11]. It corresponded, in the particular case under investigation, to the percentage of extracted (PEG) phase able to extract AO from the continuous phase (salt phase). Thus, measurements of hold up can be used to evaluate the mass transfer capability of an extraction unit under defined conditions. The hold up decreased with increasing the levels of PEG molar mass and concentration, owing to the consequent increase in viscosity. In addition, in a more viscous phase, the

Table 2 Results of the 2^3 design for the continuous extraction of AO in PRDC using PEG/Citrate systems.

Runs	M_{PEG} (g/mol)	C_{PEG} (%)	C_{CIT} (%)	K	P_f	K_Da	E_s (%)	H
1	3,350	10	15	0.214	1.99	0.0041	16.86	0.61
2	20,000	10	15	0.162	2.14	0.0025	10.65	0.55
3	3,350	20	15	0.439	2.36	0.0063	24.57	0.64
4	20,000	20	15	0.224	2.49	0.0163	51.60	0.52
5	3,350	10	25	0.381	0.88	0.0172	53.31	0.56
6	20,000	10	25	3.356	1.46	0.0586	54.98	0.40
7	3,350	20	25	1.667	0.95	0.0143	46.61	0.60
8[a]	20,000	20	25	–	–	–	–	–
9 (C)	8,000	15	20	0.364	0.51	0.0110	39.45	0.71
10 (C)	8,000	15	20	0.265	0.18	0.0136	37.24	0.55

[a] No phase formation

Table 3 Effects calculated from the responses of the 2^3 design for the continuous extraction of AO in PRDC using PEG/Citrate systems.

Effects	K	P_f	$K_D a$	E_s	H
(1) M_{PEG}	5.28	1.57	6.82	4.41	−2.91
(2) C_{PEG}	−9.06	−0.21	−8.78	−9.34	−1.09
(3) C_{CIT}	22.19[a]	−6.64	11.75	12.70	−2.38
1×2	−24.42[a]	−2.31	−8.50	−7.35	−1.51
1×3	8.01	−1.62	3.60	2.68	−1.79
2×3	−11.98	−4.11	−14.92[a]	−17.33[a]	−1.08
1×2×3	−22.19[a]	−2.78	−12.96[a]	−10.08	−1.21

[a] Statistically significant values (at the 95% confidence level).

formation of large drops is known to favor the reduction of the hold up [11]. The highest hold up (0.71) was observed using PEG with 8,000 g/mol molar mass (Table 2), a value no so far from that (0.80) reported by Cavalcanti et al. [7] in PRDC.

The study of mass transfer inside the liquid–liquid extraction columns is of fundamental importance to the knowledge of the limits of operation. In agreement with the literature [10, 21], the mass transfer coefficient increased with decreasing the PEG concentration and reached a maximum value of 0.0586 min^{-1}. These results are in agreement with those obtained by Porto et al. [10] for AO by PEG/phosphate in PRDC, using the same dispersed phase flowrate (2 mL/min).

The results of the statistical analysis of the mass transfer coefficient are presented in Table 3. Only the interactions between two variables (PEG and citrate concentrations) and three variables (PEG molar mass, PEG concentration, and citrate concentration) were significant. Figure 2 shows the effect of the interaction among the three variables. The mass transfer coefficient increased with decreasing PEG concentration and increasing PEG molar mass and citrate concentration. This behavior can be explained by the increased difference in the density, viscosity, and interfacial tension between the phases. The interfacial tension in fact is known to increase with increasing polymer concentration, and the total effect of

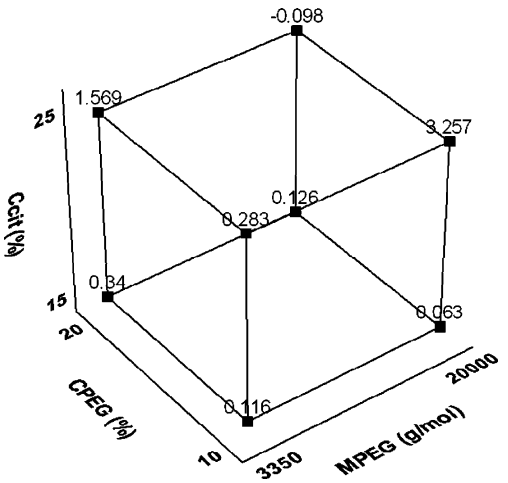

Fig. 1 Cubic plot of the effects on the partition coefficient obtained according to the design of Table 2

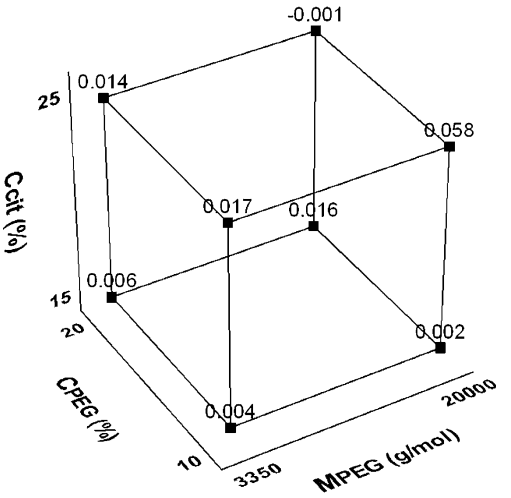

Fig. 2 Cubic plot of the effects on the mass transfer coefficient obtained according to the design of Table 2

the viscosity results in a decrease of the protein diffusibility, thus affecting the mass transfer [9, 21].

The interaction between the concentrations of PEG and citrate significantly influenced the separation efficiency (Table 3), and because one variable interfered with the effect of the other, they were assessed together. The highest value of this parameter (54.98%) was obtained with the system consisting in 20,000 g/mol PEG molar mass, 10% (*w*/*w*) PEG concentration and 25% (*w*/*w*) citrate concentration (Table 2). The separation efficiency showed the same profiles as the partition coefficient and mass transfer coefficient.

Figure 3, which illustrates the interaction between these two variables and their effects on E_S, clearly shows that the system with 10% (*w*/*w*) PEG concentration and 25% (*w*/*w*) citrate concentration ensured the highest value of the separation efficiency (E_S=54.9%). This result is in agreement with the observations of Porto et al. [10].

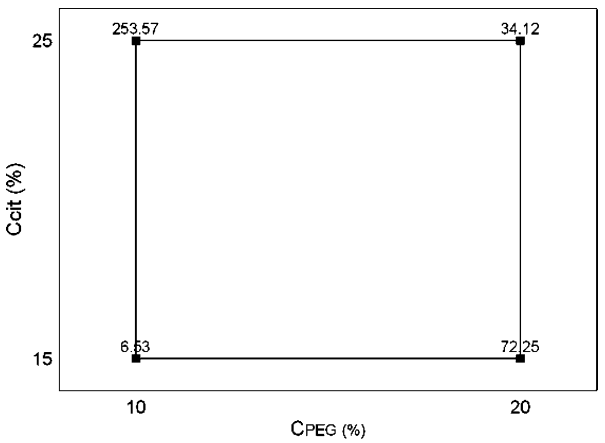

Fig. 3 Separation efficiency as a function of citrate and PEG concentrations

Conclusions

This study selected the conditions to extract and pre-purify the AO from pumpkin (*C. maxima*) by liquid–liquid extraction in ATPSs in continuous process using PRDC. The results demonstrated that the aqueous two-phase PEG/citrate system using PRDC had excellent performance and operational stability in the extraction of AO under the conditions investigated. The best results (2.49 purification factor) were obtained at high PEG molar mass, high PEG concentration, and low citrate concentration. The results presented and discussed in this study did not allow selecting the optimum conditions, but the continuous extraction with PRDC has been used successfully to extract AO by PEG/citrate ATPS. These results will be used in successive optimization and hydrodynamic investigations of the process.

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