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Liquid–Liquid Extraction of GSH Using DEHAP/Octanol Reverse Micelles

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Abstract: In this study, di-(2-ethylhexyl) ammonium phosphate (DEHAP)/octanol reverse micellar extraction was investigated and demonstrated to be an effective method to separate GSH from yeast fermentation broth. The effect of several important factors i.e., pH, DEHAP concentration, and type & concentration of cations on both extraction and strip-extraction was investigated. The optimum operating conditions for both extraction and strip-extraction were obtained by two groups of orthogonal experiments. At the optimum operating conditions, the yield of GSH can reach 68%. In the lyophilized product, the purity of GSH was higher than 75%, all the proteins can be separated, and only a small portion of amino acids were left.

Keywords: DEHAP reverse micelles, GSH, reverse micellar extraction

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INTRODUCTION

GSH (Glutathione), a kind of tri-peptide composed by glutamic acid, cysteine, and glycin, is the most abundant low-molecular-weight thiol compound in living plants or animal cells. GSH participates in many important biological processes (1–5) and is significantly in demand for therapeutic purpose because of its multiple biomedical functions (maintaining the integrity of membrane of red blood cells, protecting the function of mitochondria and kidney (6–8), keeping Vitamin C and E at a reduced state, and participating in the synthesis of DNA, RNA, protein, and other macromolecules (9–11). The producing method of GSH includes yeast fermentation (12–15), enzyme synthesis (16,17), and chemical synthesis (18), etc. Among them, the method of yeast fermentation is most extensively studied and has been industrialized to mass-produce GSH. However, the low concentration of GSH and the existence of multiple proteins, saccharide, and amino acids in the fermentation broth make the separation and purification of GSH very difficult. Moreover, the sulfhydryl group of GSH is very active and susceptible to be oxidized, which prefers to separate and purify GSH under mild conditions. During the past two decades, considerable efforts have been made to the study of all kinds of separation methods in the purification of GSH. The investigated methods include two-phase aqueous extraction (19), capillary electrophoresis (20) ion exchange (21,22), hot/boiling water extraction (23,24), nanofiltration (25), etc. However, all the methods have their limitations such as time or energy consumption, being limited to small-scale production, etc. A new method is highly needed to produce GSH of high purity in a large-scale and more effectively.

Reverse micellar extraction has been widely used in bio-separation due to its advantages in the ease of scale-up, reusable surfactant and solvent, high extraction efficiency, and continuous separation of biological molecules from fermentation mixtures, etc. Moreover, owing to the mild operating condition, the properties of bi-molecules are not affected during reverse micellar extraction. Therefore, the application of reverse micellar extraction in the separation and purification of protein, amino acid, and DNA purification has attracted a lot of attention (26–34). In reverse micelles, the hydrophilic tails of the surfactants arrange outward and contact with the organic solvent, whereas the hydrophobic heads arrange inward to form ''water pools,'' which serve as carriers to solve the biomolecules. For the ''water pool'' in the reverse micelles can be adjusted, reverse micellar extraction has been proved to be an effective method not only for separation and purification of biological macromolecules, but also bioactive material with small molecules (35).

In this paper, we attempted to extract GSH from the yeast fermentation broth using DEHAP/octanol reverse micelles. The effect of several important factors including pH, DEHAP concentration, and the types and concentration of cations was investigated. The aim is to continue the effort to extend the application of reversed micellar extraction to the purification of natural pharmaceuticals with small molecules.

MATERIALS AND EXPERIMENTS

Materials

Reduced GSH (Chromatographic reagent grade) is purchased from Shanghai Bo'ao Technology Ltd. (shanghai, China), dry yeast (biochemical reagent grade) from Harbin Mali Yeast Ltd. (Harbin, China), di-(2-ethylhexyl) ammonium phosphate (DEHAP, Industial reagent grade) from Shanghai Rare Earth Chemical Ltd. (Shanghai, China), Octanol (analytical reagent grade) from Tianjin Tiantai Fine Chemical Ltd. (Tianjin, China), and NKA-12 Resin from Nankai Uninersity Chemical Factory (Tianjin, China).

Procedures of Experiments

Preparation of Deoxidized D.I. Water

Double-distilled water in an airtight bottle was vacuumed at $-0.08 \sim -0.09$ MPa for 1 h. The bottle was then filled with high-purity nitrogen and sealed before the water was being used.

Preparation of the Pre-Extraction GSH Solution

The yeast mud of saccharomyces cerevisiae after aerobic fermentation was acquired by centrifuging the fermentation mixtures. Then, in a container, dimethylbenzene was added to break the microzyme in the yeast mud. After the container was sealed and oscillated in a temperature-controlled oscillator at 120 RPM for 24 hours, deoxidized D.I. water was added followed by oscillating for 6 more hours. After the oscillation, the supernatant was gathered by centrifuging and de-colored using NKA-12 resin followed by being filtered by using 0.65 μme micro-membrane to acquire the pre-extraction GSH solution. The solution was conserved at low temperature before being used and GSH

concentration was measured by using DTNB (5′-5-Dithiobis(2-nitrobenzoic) acid, Ellman reagent) method (36).

Preparation of DEHAP/Octanol Reverse Micelles

DEHAP is a kind of anionic surfactant and DEHAP/sulfonic kerosene reverse micelles has been successfully employed to extract amino acids from aqueous solution of NaCl in high concentration (37). In this study, DEHAP/octanol was selected as the extraction solution after the investigation of several reverse micellar systems.

To prepare the DEHAP/octanol reverse micelles, a known quantity of DEHAP was first added to octanol. After mixing thoroughly, a known amount of deoxidized D.I. water was then added slowly with stirring. The expected DEHAP reverse micelles were obtained when the system became transparent. In this study, the DEHAP concentration ranges from 20 to 200 mmol/L, the water/DEHAP molar ratio (W_o) from 2 to 20.

Extraction of GSH Using DEHAP Reverse Micelles

In a purpose-built extraction container, the pre-extraction GSH solution was mixed with the DEHAP reverse micelles at a stirring speed of 200 rpm. The temperature was controlled at $25 \pm 0.2^{\circ}C$ and other operating condition (pH, ion concentration) was also specified. During the extraction, the extraction liquid was sampled and centrifuged in a timely manner to separate the extract and the raffinate phase. The GSH concentration in the raffinate phase was then measured to calculate the partition coefficient and extraction efficiency as Eqs. (1) and (2).

$$\text{partition coefficient(D)} = \frac{\text{GSH concentration in extract phase}}{\text{GSH concentration in raffinate phase}} \quad (1)$$

$$\text{extraction efficency(E,\%)} = \frac{\text{Quantity of GSH in extract phase}}{\text{Total quantity of GSH in pre-extraction solution}} \times 100 \quad (2)$$

Strip-Extraction Experiments

In the purpose-built extraction container, the extract phase (DEHAP reverse micellar solution with GSH) obtained from the extraction experiment was mixed with a strip-extraction liquid (aqueous phase of different

pH with anions such as Ca^{2+}) for strip-extraction at $25 \pm 0.2°C$ with stirring. After the strip-extraction, the strip-extraction liquid was centrifuged to separate the extract phase and the raffinate phase, the GSH concentration in the extract phase was then measured to calculate the strip-extraction partition coefficient as Eq. (1) and the strip-extraction efficiency as Eq. (3).

$$\text{strip-extraction coefficient}\,(\%) = \frac{\text{Quantity of GSH in extract phase of strip-extraction}}{\text{Quantity of GSH in reverse micellar solution before strip-extraction}} \times 100 \quad (3)$$

RESULTS AND DISCUSSION

Factors Affecting the DEHAP Reverse Micellar Extraction of GSH

Effect of pH

The molecular structure and dissociation equilibrium of DEHAP is shown as Fig. 1.

The molecular structure of reduced GSH was showed as Fig. 2. In aqueous solution the nitrogen atom in α-amino groups can bind with a H^+ ion to become positively charged, the two carboxylic groups and the sulfhydryl group can, respectively, dissociate to lose a H^+ ion, and become negatively charged. Figure 3 shows the dissociation equilibrium of GSH in aqueous solution.

Where pK_1, pK_2, pK_3, and pK_4 are four equilibrium constants of the dissociation reactions. The reported isoelectric point of GSH is around 5.93 (25). As mentioned in the introduction, the extraction mechanism for the reverse micelles formed by ionic surfactant is the electrostatic interaction between the surfactant and the oppositely charged solute.

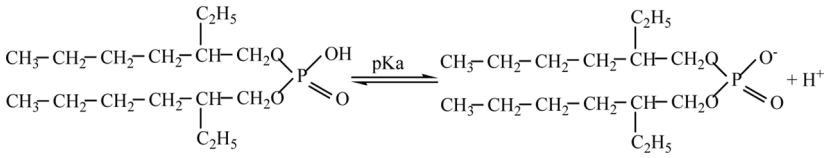

Figure 1. Molecular structure and dissociation equilibrium of DEHAP. Where pKa is the dissociation constant, which is equal to 2.12.

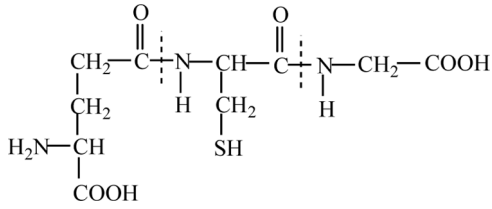

Figure 2. Molecular structure of GSH.

Since DEHAP is negatively charged, GSH has to be positively charged in order to be extracted, which means that the pH must be under 5.93.

Since the dissociation and the charge status of both DEHAP and GSH is a function of H^+ concentration as Figs. 1 and 3, the pH of the solution would surely affect the extraction. Figure 4 showed the experimental partition coefficient for reverse micellar extraction of GSH versus pH of the aqueous GSH solution. The results showed that the partition coefficient first increases with an increase in pH until reaching a maximum value when the pH of the aqueous phase is around 2.5, then decreases with a further increase in pH. The reason for this result is the different affect of pH on the dissociation equilibrium of DEHAP and

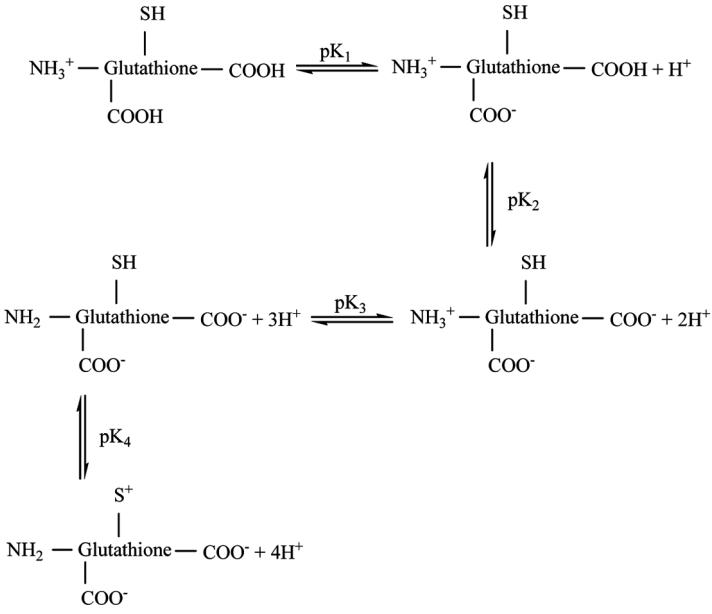

Figure 3. The dissociation equilibrium of GSH.

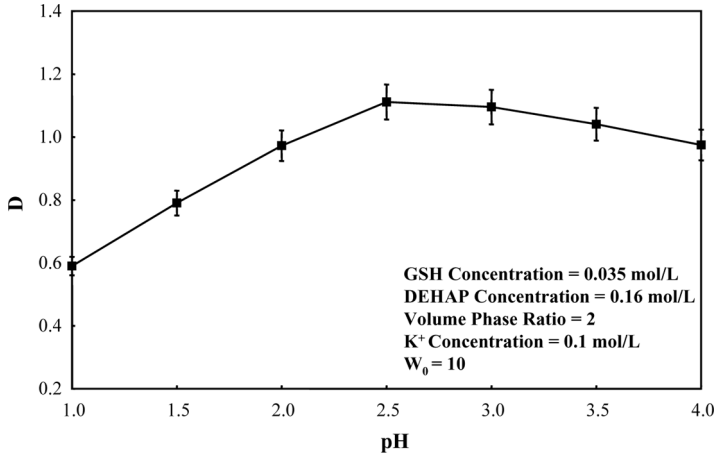

Figure 4. Experimental partition coefficient versus pH value of GSH solution.

GSH. With an increase of the pH, the decrease of H^+ concentration drives the dissociation of DEHAP to generate more DEHAP anions, which benefit the formation of $DEHAP^-GSH^+$ complexes and increases the partition coefficient. For the dissociation constant of DEHAP to be 2.12, the pH has to be larger than 2.12 for the DEHAP to take effect. However, for the GSH to be extracted by $DEHAP^-$, GSH must be positively charged, which requires the pH to be smaller than the isoelectric point of GSH (5.93). Moreover, GSH^+ concentration increases with a decrease in pH and higher pH is beneficial for the extraction from the viewpoint of GSH^+ concentration. Thus, it can be seen that the effect of pH on the extraction has two sides. Low pH is in favor of the formation of GSH^+, whereas high pH increases the concentration of $DEHAP^-$. The interrelation of this two-side effect causes an optimum pH for the reverse micellar extraction.

The Affect of DEHAP Concentration

The DEHAP reverse micellar extraction of GSH is a process that GSHs transfer from the aqueous phase into the "water pools" of the reverse micelles due to the electrostatic interaction between GSHs with the DEHAP surfactants. Therefore, the partition coefficient of the extraction is surely affected by DEHAP concentration. Figure 5 shows the experimental partition coefficient versus DEHAP concentration when other operating conditions and the amount of water in the reverse micelles were

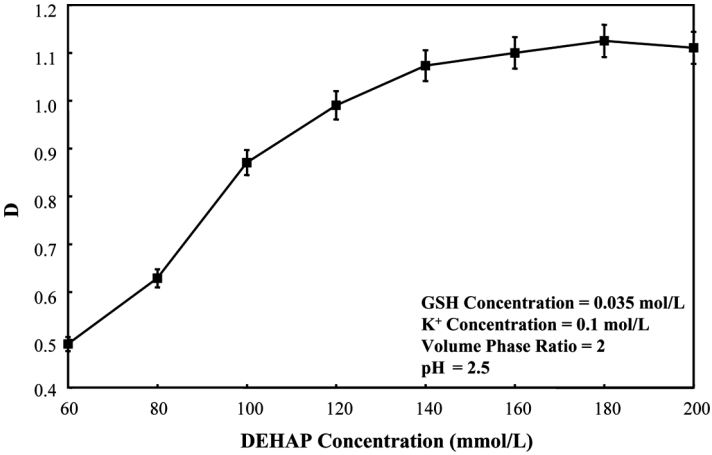

Figure 5. Experimental partition coefficient versus DEHAP concentration.

fixed. The results show that the partition coefficient first increases with an increase of DEHAP concentration, then reaches a plateau value when the concentration of DEHAP is around 160 mmol/L. The water in the "water pools" can be categorized into two classes: the "free" water and the "binding" water that is bound with the surfactants (38–42). The properties of the "binding" water are different from that of the "free" water. The extraction capability of the reverse micelles is mainly dependent on the total amount of "binding" water. With the concentration of the DEHAP increases, a larger portion of the water inside the reverse micelles is bound with the surfactants and becomes "binding" water, which increases the "capability" of "water pools" to solve GSHs and improve the extraction. However, with the amount of "free" water decreases, the acquirement of "free water" becomes more and more difficult with the increase of DEHAP concentration. When all the water inside the reverse micelles becomes the "binding" water, the increase of the concentration of the DEHAP would not affect the extraction any further.

To verify this result, the partition coefficient of the extraction at different water concentration was also studied with DEHAP concentration being fixed. The result was shown in Fig. 6 by plotting the partition coefficient versus W_0. The partition coefficient first increases, then reaches a plateau value with an increase in the amount of water. At low concentration, the added water can be bound with DEHAP and becomes "binding water," which increases the "solving capability" of the reverse micelles. When the water concentration reaching a critical

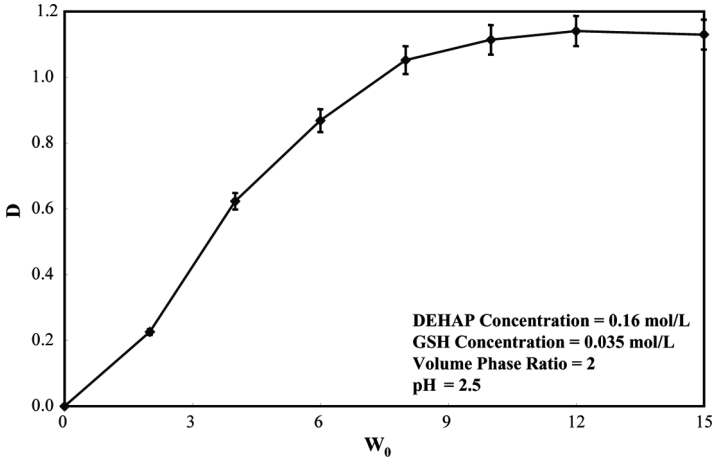

Figure 6. Experimental partition coefficient versus W_0.

point, all the surfactants were bound with water, and a further increase of water concentration would not increase the "solving capability," the partition coefficient reaches the plateau value. The critical W_0 for both Figs. 5 and 6 is around 10.

The Affect of Ions

In the reverse micellar extraction with electrostatic force as the driving force, the existence of ions in the system always affects the extraction significantly because ions can influence the electrostatic interaction between surfactants and solutes in several ways. Figure 7 shows the effect of K^+, Na^+, and NH_4^+ on the partition coefficient of GSH extraction. At low concentration, the partition coefficient increases with an increase in cation concentration until reaching a maximum value. It then decreases with a further increase in cation concentration.

The optimum anion concentration for all the three anions studied is all around 0.1 mol/L.

The existence of ions in the system can affect the reverse micellar extraction in several possible ways and the affecting mechanism of ions on reverse micellar extraction of proteins was thoroughly studied (43). Whereas at this stage, only a possible explanation can be given about the influence of ions on reverse micellar extraction of biomaterials with small molecules. The existence of ions can improve the dissociation of the GSH in the aqueous phase due to the so-called salting-in effect, thus

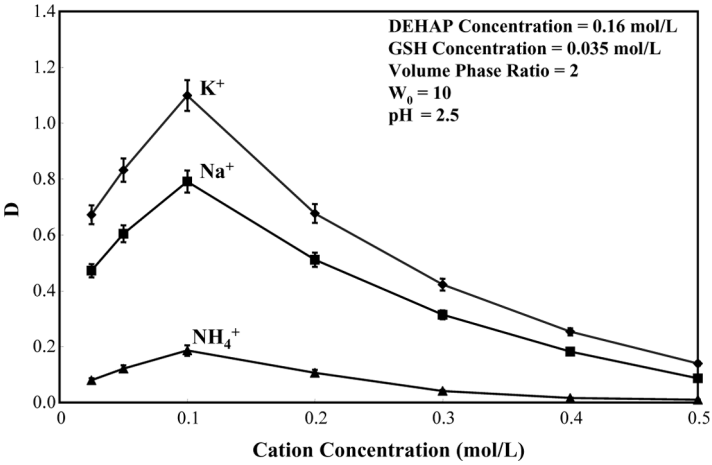

Figure 7. Experimental partition coefficient of GSH extraction versus concentration of different anions.

improving the extraction. However, the cations can form cation-$DEHAP^-$ complexes, which screens the binding of GSH^+ with $DEHAP^-$ and decreases the partition coefficient. At low anion concentration, the salting-in effect dominates; at higher cation concentration, the screening effect prevails, instead. Therefore, the experimental results show that the partition coefficient of GSH first increases with an increase in anion concentration until reaching a maximum, then decreases with a further increase in anion concentration. Figure 7 also shows that the partition coefficient for K^+ in the system is higher than that for Na^+ and NH_4^+. The reason is probably because of the different binding strength of the cations with DEHAPs. The binding strength of the three complexes that the cations forms with DEHAP is: $K^+DHAP^- < Na^+DEHAP^- < NH_4^+DEHAP^-$. Because the strength of K^+DEHAP^- is the weakest, which means that K^+ $DEHAP^-$ can dissociate more easily, the screening effect of K^+ is the weakest among the three cations, the partition coefficient with K^+ in the system is, therefore, higher than that with Na^+ and NH_4^+ in the extraction system.

The Kinetics of DEHAP Reverse Micellar Extraction

During the extraction of GSH, GSH first diffuses from the bulk of the aqueous phase to the interface. At the interface, GSH is encapsulated in a reverse micelle. The filled reverse micelle, then, diffuses from the

interface into the bulk organic phase. At the same time, some of the filled reverse micelles also diffuse from the bulk organic phase to the interface, where GSHs are released and diffuse back to the aqueous phase. The process reaches a kinetic equilibrium at the end of the extraction. Changing the operating conditions of the aqueous phase (pH, types, and the strength of ions, etc), the kinetic equilibrium will be shifted to another level. The implement of strip-extraction is based on this principle.

Because the interfacial resistance resulting from the formation of the GSH-filled reverse micelles plays an important role in the transfer of GSH, the mass transfer of GSH during the extraction can be described by the two-film theory combined with the interfacial resistance. Thus, the overall mass transfer coefficient, K_0, can be expressed as (44,45,46)

$$\frac{1}{K_0} = \frac{1}{k_{aq}} + \frac{1}{Dk_{org}} + \frac{1}{k_i} \tag{4}$$

where k_{aq} is the mass transfer coefficient in the aqueous film, k_{org} is the mass transfer coefficient in the organic film, and k_i is the mass transfer rate due to the formation of GSH-filled reverse micelles.

During the extraction, the total mass balance of GSH gives

$$C_0 = C_t + RC_{mt} \tag{5}$$

where C_0 is the initial concentration of GSH in aqueous phase, R is the phase volume ratio. C_t is the concentration of GSH in aqueous solution at time t. C_{mt} is the concentration of GSH in reverse micelles at time t.

The mass transfer rate during the extraction can be described by (44,45,46)

$$\frac{dC_t}{dt} = -K_0 a(C_t - C_t^*) \tag{6}$$

where a is the specific surface area. K_0a is the total volumetric mass transfer coefficient, C_t^* is the concentration of GSH in aqueous phase at equilibrium with $C_{mt} \cdot C_t - C_t^*$ stands for a driving force due to the concentration gradient. Assuming that the partition coefficient, D is constant at the concentration range studied,

$$C_t^* = C_{mt}/D \tag{7}$$

Integration of Eq. (6) gives

$$C_t/C_0 = \beta(1 + RDe^{-\alpha t}) \tag{8}$$

where

$$\beta = \frac{1}{DR + 1} \tag{9}$$

$$\alpha = K_0 a(DR + 1)/(RD) \tag{10}$$

Because R is related to the experimental design and D can be measured by experiments. In the kinetic Eq. (8), only α or $K_0 a$ is unknown. Therefore, $K_0 a$ can be calculated by fitting the experimental kinetic data to Eq. (8).

Figure 8 compared the experimental GSH concentrations in aqueous phase with those predicted by Eq. (8) with $K_0 a = 0.209$. The maximum $K_0 a$ obtained by Lye et al. (44) were 0.145 for the AOT reverse micellar extraction of lysozyme with electrostatic interaction as the driving force. In the study of affinity extraction of protein using CB-modified lecithin reverse micelles by Sun et al. (45), $K_0 a$ were calculated around $1.5 \sim 2.4 \times 10^{-3}$. Therefore, the mass transfer rate of the present extraction is much faster than that of the affinity-based extraction of protein, whereas at the same level as that of electrostatic force-based reverse micellar extraction of protein.

Strip-Extraction of GSH

As the extraction, strip-extraction is a process that solutes transfer back from the organic phase to the aqueous phase by changing the

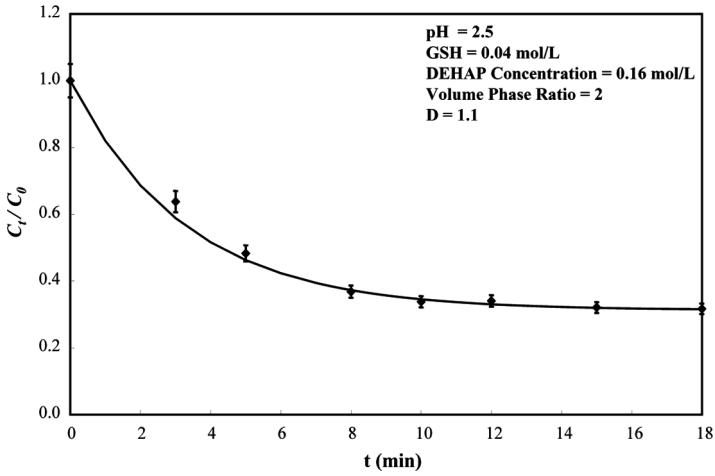

Figure 8. The GSH concentration in aqueous phase versus extraction time. The line is the prediction by Eq. (8) with $K_0 a = 0.209$.

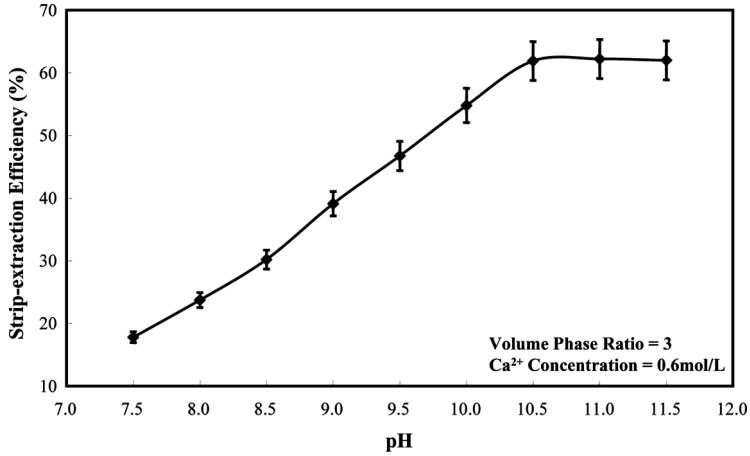

Figure 9. Experimental strip-extraction efficiency versus pH value.

operating conditions of the aqueous phase (pH, types, and the strength of ions in, etc).

Figure 9 shows the strip-extraction efficiency versus the pH of the strip-extraction liquid at a range from 7 to 11. The strip-extraction efficiency increases with an increase in pH until reaching a plateau value at pH = 10.5. Figure 10 shows the change of the strip-extraction efficiency with the concentration of Ca^{2+} in strip-extraction liquid. It is

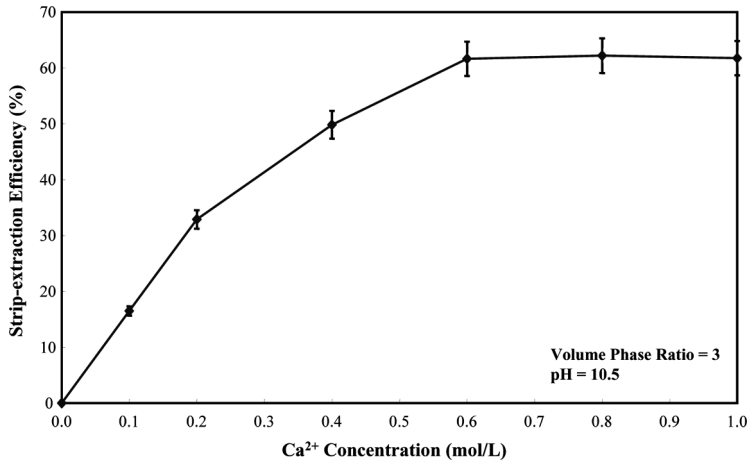

Figure 10. Experimental strip-extraction efficiency versus Ca^{2+} concentration.

seen that the strip-extraction efficiency increases with an increase in Ca^{2+} concentration before reaching a maximum at the concentration of 0.6 mol/L. Ca^{2+} can significantly screen the interaction of $DEHAP^-$ with GSH^+ because it forms stable $DEHAP^-Ca^{2+}$ complex. Therefore, the existence of Ca^{2+} increases the strip-extraction efficiency, remarkably.

The Optimum Operating Condition

On the basis of single-factor experiments, the optimum conditions for GSH extraction from the fermentation broth were investigated via a group of orthogonal experiments (L_{16}, 4^6). The optimum operating conditions for the extraction are: pH of the aqueous phase is 2.5, the concentration of DEHAP is 0.16 mol/L, W_0 is 10, and the ratio of the organic phase to the aqueous phase is 2:1 (V/V), the concentration of K^+ is 0.1 mol/L, and the extraction time is 15 min. At the optimum operating conditions, with the concentration of GSH in the pre-extraction solution of 0.035 mol/L, the single extraction efficiency can reach 68.2%. After two extractions, the total extraction efficiency can reach 85.1%. The optimum conditions for strip-extraction via a group of orthogonal experiments (L_9, 3^4) are: pH of the aqueous phase is 10.5, Ca^{2+} concentration is 0.6 mol/L, the ratio of the organic phase to the aqueous phase is 1:3 (V/V), the strip-extraction time is 45 mins. At the above condition, the single strip-extraction efficiency can reach 62.2%. After two strip-extractions, the total strip-extraction efficiency can reach 81%. After strip-extraction, the strip-extraction extract phase was concentrated and lyophilized. The composition of the lyophilized products was determined. Moreover, the anti-oxidation capability was tested and compared with commercial GSH. The results showed that the lyophilized products had comparable anti-oxidation capability with commercial GSH. Reverse micellar extraction does not affect the biological activity of GSH.

Except GSH, the yeast fermentation broth also contains multiple proteins and amino acids. After extraction and strip-extraction, the composition of the lyophilized products is: GSH 75.6%, amino acids 12.9%, and water and other inorganic salts 11.5%. It can be seen that DEHAP reverse micellar extraction can separate all the proteins and most of the amino acids from GSH.

CONCLUSION

Reverse micellar extraction has been widely used in the purification of macromolecules such as protein and DNA, whereas the previous application of this technology in the purification of biomaterials with

small molecules is only constrained to amino acids. As an effort to extend the application of reversed micellar extraction to the purification of natural pharmaceuticals with small molecules, the DEHAP/octanol reverse micelles extraction was studied and demonstrated to be an effective method for separating GSH from the yeast fermentation broth. At optimum operating conditions, the yield of GSH can reach 68.5%. In the lyophilized product, the concentration of GSH is 75.6% (wt) and that of amino acids is 12.9%. No proteins were found in the final product. The experimental studies showed that the factors affecting the extraction include pH, DEHAP concentration, and types and concentration of cations.

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