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System Establishment of ATPS for One-Step Purification of Glutamate Decarboxylase from *E. coli* After Cell Disruption

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Abstract The partition of glutamate decarboxylase (GAD) from *Escherichia coli* in polyethylene glycol (PEG) and sodium sulfate aqueous two-phase systems (ATPS) has been explored with the purpose of establishing a phase system for the purification of GAD after cell disruption. The results showed that the partitioning of GAD was slightly influenced by PEG molecular weight (MW) but depended on the tie line length (TLL) and NaCl and loading sample concentrations. The optimum system obtained for GAD purification was composed of a PEG MW of 4,000, TLL of 63.5%, a volume ratio of 2.31, a loading sample concentration of 0.4 g/mL, which produced a GAD recovery of 90% with the purification fold of 73. Furthermore, the feasibility of directly purifying GAD from the cell disrupts using ATPS was evaluated. The established ATPS for GAD purification exhibited an efficient integrated purification process compared to the reported purification process in terms of purification efficiency and recovery.

Keywords Aqueous two-phase system · One-step purification of GAD · Molecular weight · Tie line length · Sample concentration

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Introduction

Glutamate decarboxylase (GAD, EC 4.1.1.15) is the unique enzyme catalyzing the conversion of L-glutamic acid (Glu) to γ-aminobutyric acid (GABA). Due to the growing effort in studying the physiological functions of GABA in recent years, there is an increasing demand for a large amount of GAD to meet the increasing market demand for GABA. In addition, since GAD has been implied as the primary autoantigen involved in the autoimmune destruction of b-pancreatic islets in insulin-dependent diabetes mellitus (type I diabetes) [1], insufficient quantities of purified GAD have prevented further study of its potential therapeutic benefits. Owing to its biological role and beneficial applications, numerous attempts have been made to obtain sufficient quantities of purified, enzymatically active GAD for comprehensive studies on its structure, function, regulation, and therapeutic use [2]. However, most of the methods used involve several steps and are typically developed in the laboratory with milligram quantities of product, and the scale-up of these methods for commercial applications is difficult and expensive. In that regard, further investigation is needed to develop a simple but efficient method for separation and purification of the enzyme.

The aqueous two-phase system (ATPS) is one of the novel techniques to provide secured separation and purification of biomolecules [3]. ATPS functions when two polymers or one polymer and one salt are mixed together at appropriate concentrations. In comparison with conventional methods for isolation and purification of proteins, ATPS has various advantages such as the short time required for reaching equilibrium, biocompatibility, nontoxic, high capacity and yield, ease of scale-up, and the potential for polymer recycling [4–6]. Some successful applications of ATPS at large/industrial scale have been demonstrated [7–10]; however, it was only exploited for the recovery of biological products as a single purification technique without focusing on the use of the developed ATPS for removal of cell debris to purify biomolecules, which could present another promising application by establishing an integrated process to enhance purification. In an integrated purification process of ATPS, the selectively desired enzymes or proteins are partitioned to one phase and the contaminant proteins to a separate phase, not only purifying the enzymes but also concentrating them into different phases [11, 12]. In doing so, contaminants other than the target proteins are removed from the sample, which enhances the purity of the desired enzymes as compared to precipitation and chromatography that are used for partial purification. Thus, the process integration with ATPS will exclude the contaminant protein and concentrate the desired protein (GAD in this case) to subsequently achieve the high purity GAD in a concentrated form, making it easy to recover GAD using simple, low-cost, and time-saving methods.

There are two ATPS systems available for study, i.e., PEG–salt ATPS and PEG–dextran systems, with the former having certain advantages over the latter, such as low viscosity and lower cost, which thus is more suitable for the extraction of biological materials [13, 14]. In a specific system, the selective partition of ATPS constituents may be affected by different factors such as the nature and size of the biocompound, the molecular structure and chain size of the polymer, the tie line length (TLL), salts concentration, pH, and so forth [6, 15, 16]. To our knowledge, few reports are available in the literature regarding purification of GAD by an ATPS system. Thus, the goal of this work is to examine the technical feasibility of direct purification of GAD from the cell disrupts after ultrasound sonication using a PEG–sodium sulfate system based on evaluating parameters related to enzyme activity recovery such as polymer molecular

weight, TLL, NaCl addition, and sample loading, which affect the partitioning behavior of the protein. The performance of one-step purification by ATPS was evaluated by comparing with the chromatography results in terms of purification efficiency and enzyme recovery.

Materials and Methods

Microorganism, Media, and Culture Conditions

The *Escherichia coli* utilized in this study was a gift from the Northeast Agricultural University in Harbin, China. The strain was cultivated in the Luria–Bertani medium (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) at 37 °C, 200 r/min for 24 h prior to use.

Preparation of Cell Disrupts

After cultivation, the *E. coli* cells were collected by centrifugation at 5,000 rpm for 20 min. The harvested cell pellets were resuspended in sodium phosphate buffer (pH 5.0). Disruption of cells was performed using an ultrasonic homogenizer (Brandson Ultra 250, Danbury, USA). The cell suspension with a biomass concentration of 0.5 g/mL was sonicated for a predetermined duration (30 min), suspension volume (20 mL), and acoustic power (90 W). The samples were placed in an ice bath with the disruption period being at 30 s at 60-s intervals to prevent overheating.

Preparation of ATPS for System Establishment

To prepare ATPS, stock solutions of PEG 50% (*w*/*w*), sodium sulfate, and water were mixed thoroughly to obtain a total system composition required for the experiments. All systems were prepared in graduated centrifuge tubes with a total phase system mass of 10 g on a *w*/*w*% basis. The amount of the cell disrupts added to the systems was 4 mL, which was the last added component. For the systems with sodium chloride, the powdered salt was directly dissolved into the systems to achieve concentrations from 0 to 1 M. Each system was centrifuged at 2,000 rpm for 20 min and allowed to settle for 1 h at room temperature to reach equilibrium.

After reaching equilibrium, measurements of the volumes of top and bottom phases were made in graduated centrifuge tubes. In order to determine the concentration of proteins in each of the coexisting phases, separate samples from both the top and bottom phases were collected using a syringe and analyzed.

Analysis of ATPS

The tie line length (TLL), which represents the length of the line that connects the composition of the top and bottom phase, was calculated according to Eq. (1):

$$\mathrm{TLL} = \sqrt{\Delta\mathrm{PEG}^2 + \Delta\mathrm{Salt}^2} \tag{1}$$

where ΔPEG and ΔSalt were the differences between the concentration of PEG and salt in the top and bottom phases expressed as a percentage (*w*/*w*).

The partition coefficient determined the extent of separation of the protein in the polymer phase, which was influenced by the molecular weight of polymer, salt, and polymer concentration, temperature, and pH [3].

The partition coefficient for GAD activity in the aqueous two-phase systems was defined as

$$K_{\mathrm{p}} = \frac{\mathrm{Activity}_{\mathrm{topphase}}}{\mathrm{Activity}_{\mathrm{bottomphase}}} \tag{2}$$

Recovery of enzyme in the top phase was tabulated as percent yield. A formula calculating percent yield of enzyme in the top phase was

$$Y_{\mathrm{topphase}} = \frac{\mathrm{Enzyme}_{\mathrm{top}}}{\mathrm{Totalenzyme}} \times 100 = \frac{\mathrm{CtVt} \times 100}{\mathrm{CtVt} + \mathrm{CbVb}} = \frac{100}{1 + mR} \tag{3}$$

where R was the ratio of the volume of top phase to the volume of bottom phase (yt/yb) and m was the partition coefficient of enzyme (Ct/Cb).

The purification factor reflected the purity degree of GAD by ATPS and was defined as

$$\mathrm{PF} = \frac{\mathrm{Specificactivity}_{\mathrm{top}}}{\mathrm{Specificactivity}_{\mathrm{total}}} \tag{4}$$

where specific activity represented the ratio of the enzyme activity to the protein concentration in a sample.

The experimental results presented were the mean values of at least three measurements of activity (the accuracy was considered to be 5%) on a minimum of three replicates (each an independent experiment) for every partition data point.

Enzyme and Protein Assay

Protein concentrations in various preparations were assayed by the method of Bradford [17]. The reaction mixture (pH 5.2) consisted of 200 μl of 50 mM sodium phosphate, 100 mM L-glutamate, 0.05 mM pyridoxal 5-phosphate monohydrate, and 100 μl of enzyme liquid and was incubated at 40 °C for 60 min with periodic shaking. The reacted solution (1 mL, pH 8.7) was mixed with 1 mL of dabsyl chloride (1 mg/mL, in acetone) and reacted at 65 °C for 10 min, the pH of which was kept at 9 by adding 1 M $NaHCO_3$ solution. After that, the reaction was stopped by an ice bath, and then, the dabsyl sample was filtered through a 0.45-μm nylon filter membrane [18, 19]. The filtrate was analyzed for its GABA content by HPLC, with a spectrophotometric detector at 440 nm. A reversed-phase column (Hypersil ODS, 250×4.6 mm) coupled with a C18 cartridge was used. The column temperature was maintained at 40 °C, and the flow rate was 1 mL/min. The composition of the optimized mobile phase was kept at 40% of acetonitrile and 60% of 0.006 M CH_3COONa (pH 4). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of GABA in 1 h.

Statistical Analysis

Results were calculated as the mean of the triplicate experiments. The significance of the results was statistically analyzed by a one-way analysis of variance (ANOVA) with Tukey's multiple comparison for pairwise comparison with SPSS (Chicago, IL). Statistical significance was set at $P<0.05$.

Results and Discussion

Effect of Molecular Weight of PEG on GAD Purification

ATPS experiments were carried out at four different molecular weights (MWs) of PEG, i.e., 1,000, 4,000, 6,000, and 8,000. The results presented in Table 1 indicated that the performance of PEG–$(NH_4)_2SO_4$ system in terms of GAD activity recovery and purification fold is slightly influenced by the PEG molecular mass based on the p value. The specific activity of GAD in the upper phase did not exhibit a significant trend with the increase of MW of PEG, and the purification fold is around 60.

The partition coefficient (Kp) determines the extent of separating the protein in the polymer phase, and the results showed that the partition coefficient was the highest (6.75) when the molecular weight of PEG was 6,000, but there were no significant differences in partition coefficient among different MWs ($p>0.05$). The recovery of top phase also showed no regular tendency and was similar in all cases. In addition, the results by pairwise comparsion exhibited that the mean differences between every two treatments were insignificantly different ($p>0.05$) for all the responses.

Theoretically, the effect of polymer molecular weight is usually attributed to the excluded volume effects that increase with increasing polymer molecular weight. Thus, the increase in the MW of PEG induces a reduction of the protein solubility in the phase in which the protein is located [11, 14, 20–22]. However, the general tendency observed was not in line with the expected results that an increase of the partition coefficients and top phase yield would occur as the PEG MW decreased. The reason for that may be explained as changing the polymer MW will alter the polymer concentration, which influences the partition coefficients in the opposite direction [23]. In this study, since PEG 4,000 showed the best performance among all, it was chosen for the remaining experiments.

Effect of TLL on the GAD Purification

In order to select the ATPS in which the target product (GAD) and cell debris concentrate in opposite phases (preferentially the target product in the top phase to facilitate further processing), the partitioning behavior was studied using systems comprising a stock solution of cell disruption from a paste rich in the product of interest.

ATPS experiments were carried out at different TLLs ranging from 36.85% to 85.13%. Table 2 presents the effect of TLL on the performance of the ATPS for GAD purification. From the p values in Table 2, it can be observed that the partitioning of GAD in PEG–

Table 1 Influences of MW on the purification performance of ATPS for GAD (top phase)

MW	Volume ratio	Total enzyme unit (U)	Enzyme activity (U/mL)	Specific enzyme activity (U/mg)	Partition coefficient (k_p)	Recovery (%)	Purification fold
1,000	1.50	19,698.59	984.93	895.26	6.31	85.65	59.68
4,000	1.01	19,858.09	992.90	918.82	6.75	86.34	61.25
6,000	0.90	19,429.90	971.50	910.90	6.54	84.48	60.73
8,000	0.89	19,777.83	988.89	971.95	6.63	85.99	60.80
p value	–	–	–	–	–	–	–

– represent insignificant differences between different treatment groups by ANOVA ($p>0.05$)

Table 2 Effect of TTL on the purification performances of ATPS for GAD (top phase)

TTL (%)	Volume ratio	Total enzyme unit (U)	Enzyme activity (U/mL)	Specific enzyme activity (U/mg)	Partition coefficient (k_p)	Recovery (%)	Purification fold
36.85	1.28	16,856.70	842.84	591.10	2.74	73.29	32.74
43.07	0.82	17,325.90	866.30	624.05	3.05	75.33	42.27
48.14	0.78	17,958.40	897.92	650.00	3.56	78.08	50.00
59.26	1.32	18,903.70	945.19	769.85	4.03	82.19	57.99
62.36	1.94	19,598.30	979.92	814.85	5.76	85.21	60.99
63.68	4.82	20,601.10	1,050.06	957.50	8.58	90.57	70.50
65.42	1.42	18,597.80	929.89	824.45	7.22	80.86	61.63
66.23	1.41	17,482.30	874.12	732.20	6.15	76.01	55.48
85.13	1.32	17,500.71	875.04	602.75	6.10	76.09	46.85
p value	*	*	*	*	*	*	*

* represent significant differences between different treatment groups by ANOVA ($p<0.05$)

sodium sulfate system is statistically dependent on the TLL. When the TLL was increased from 36.85% to 63.68%, an increase in the Kp values for the studied PEG MWs was observed. For all the ATPS studied in Table 2, more than 70% of GAD could be potentially recovered from the upper phase, and the TLL of 63.68% exhibited the best recovery of GAD from the top phase (i.e., 90.57%). Increasing TLL from 36.85% to 63.68% facilitated the top phase recovery of GAD in ATPS in conformance with the rise of the molecular mass of PEG used. Similar trends for total enzyme units and enzyme activities were exhibited with the increases of 22.2% and 24.58%, respectively. Meanwhile, increasing TLL from 36.85% to 63.68% caused a significant increase (62.9%) in the specific activity of GAD in the top phase recovery (from 591.0 to 957.50), ending up with the maximum purification fold of 70.5. The results demonstrated that the target product was partitioned preferentially to the top phase, while the opposite for cell debris, when the TLL increased within the range discussed above, which proved that high TLLs of ATPS benefited the separation of cell debris from GAD. The effect of TLL on a protein partition observed above can be attributed to two mechanisms, i.e., (1) a balance is achieved between the high ionic strength caused by the increment of salt concentration and the binding through the hydrophobic surface of the protein and PEG [14, 24] and (2) the free volume of the top phase of ATPS rises when the TLL is increased, thus increasing the space available to allocate the solutes [25, 26].

However, further increases of TLL from 63.68% did not lead to continuous increases of Kp, purification fold, and recovery. Although ANOVA test showed statistical significance in the responses of Kp, purification fold and recovery by various treatments of TLL, pairwise comparisons (Tukey, $p<0.05$) demonstrated the nonsignificant differences in purification fold among TLL of 62.36%, 65.36%, and 66.23%. Meanwhile, pairwise comparisons revealed the significant differences in Kp and recovery between TLL of 66.23% and 85.13%.

Such partition behavior may be explained by changes in the free volume and density of the phases [27] because the free volume in the upper phase would decrease when the TLL was increased from 63.68%, which was evidenced by the decrease of volume ratio. As a result, the solutes in the upper phase may be promoted to partition to the bottom phase, leading to decreasing top phase recovery of GAD accompanied by increases in TLL. The

observations from this study clearly indicate that, for an extraction system, a PEG 6,000–ammonia sulfate system with a TLL of 63.68% (PEG 20%, $(NH_4)_2SO_4$ 10%) will be the most favorable to maximize the partitioning of the product of interest and cell debris contaminants to opposite phases, which is essential for the potential application of ATPS processes in recovery of GAD from *E. coli*.

Effect of Salts on GAD Purification

The effects of NaCl addition on partition of GAD in PEG–(NH4)2SO4 systems were shown in Table 3. Salt concentration has been reported to alter protein partition in the aqueous two-phase systems [3, 14, 28, 29]. In this study, a significant decrease in the partition coefficient with the increase of the NaCl content in the system was observed (Table 3, $p<0.05$), with the maximum partition coefficient being at 0 M NaCl concentration of the system. The occurrence of the substantial variance among treatments (pairwise comparison, $p<0.05$) may be due to the increased net charge of the protein by binding the ions (Na^+, Cl^-), which could increase the electrostatic free energy of the protein, thus leading to its instability [14, 30].

The data showed that the specific activity of GAD and subsequently the purification fold declined from 71.73 to 23.27 with the addition of NaCl, as seen from Table 3, which was in accordance with the reports that addition of NaCl to PEG–salt systems would increase protein solubility as a result of the addition of neutral electrolytes, e.g., NaCl, at a concentration in the order of 0–1.0 M, which drove the target protein into the lower phase [14]. Similar behavior was observed for the recovery efficiency and total enzyme units.

According to the results above, the ATPS, consisting of 20.0% (*w*/*w*) PEG 4,000, 10% (*w*/*w*) sodium sulfates, and no NaCl, was selected for further examinations, in which the percent extraction yield in the ATPS was 90.39%.

Effect of the Sample Concentration on GAD Purification and Comparison with Data of Chromatography

In order to enhance the potential recovery process, the effect of increasing sample concentration on the performance of ATPS was investigated. A stepwise increment in sample concentration (from 0.3 to 0.8 g/mL) after ultrasound sonication was employed to examine its impact on the top phase product recovery using the selected ATPS in the previous session.

Table 3 Effect of salt addition on the purification performance of ATPS for GAD (top phase)

NaCl (mol/L)	Volume ratio	Total enzyme unit (U)	Enzyme activity (U/mL)	Specific enzyme activity (U/mg)	Partition coefficient (k_p)	Recovery (%)	Purification fold
0	2.31	20,329.92	1,016.50	1,075.98	7.61	90.39	71.73
0.05	2.09	16,344.83	817.24	697.47	5.70	71.06	46.49
0.1	1.88	14,590.91	729.55	524.67	5.04	63.44	34.97
0.5	1.43	11,923.73	596.19	368.70	2.55	51.84	24.58
1	1.33	8,483.16	424.16	349.04	1.62	36.88	23.27
p value	*	*	*	*	*	*	*

* represent significant differences between different treatment groups by ANOVA ($p<0.05$)

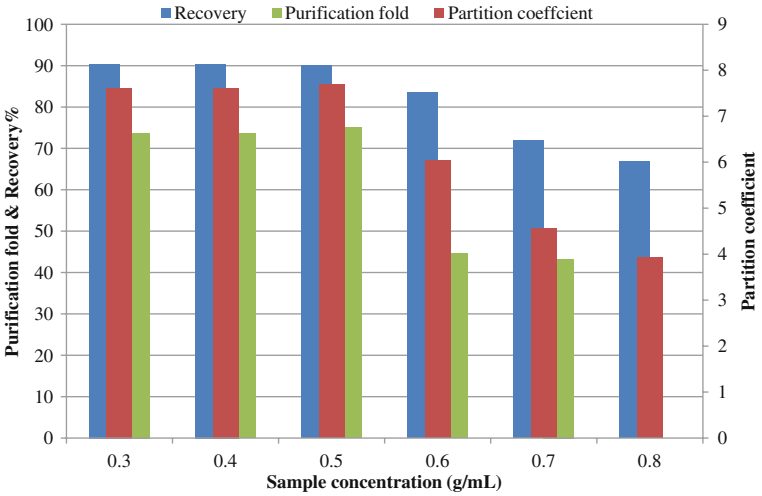

Fig. 1 Effect of sample concentration on purification performance

Figure 1 showed the effect of GAD concentration in the system on top phase product recovery, Kp, and purification fold. Top phase recovery remained relatively constant and over 90%, regardless of the loading concentration of cell disrupts (0.3–0.8 g/mL) to the ATPS. However, it was observed that the top phase recovery of GAD decreased when the concentration of cell disrupts with the product of interest (GAD) loaded higher than 0.4 g/mL. Similar results were obtained for the partition of GAD to the top-PEG-rich phase. These findings can be attributed to the theory that the proteins with a higher affinity for the top phase (because of their size, electrical charge, hydrophobicity, etc.) occupy the free volume available in the top phase [27]. Once the particles with high affinity for the PEG-rich phase have partitioned, molecules with lower affinity can migrate to the remaining free volume. Additionally, the purification fold in ATPS was also not favored by using sample concentration higher than 0.4 g/mL. The reason can be explained in terms of the increase in the contaminant proteins associated with the increase in the concentration of the crude extract loaded to the ATPS. The increment in the amount of contaminants loaded to the systems caused a decrease in the free volume available for the target product [27]. Meanwhile, the loaded concentration of GAD was increased, but the free volume available was limited. Thus, the GAD purification factor in the top phase decreased from 73 to 43

Table 4 Comparison of results between ATPS and chromatography for GAD purification

GAD resources	References	Purification methods	Purification fold	Recovery	Purification step
E. coli	In this study	ATPS	70	90%	One-step
Lactobacillus paracasei	Komatsuzak [31]	$(NH_4)_2SO_4$ saturation and chromatography	27	6.2%	Three-step
Lactobacillus brevis	Ueno et al. [31]	$(NH_4)_2SO_4$ saturation and chromatography	Not listed	9 mg/90.2 g wet cells	Five-step
Rice germ	Zhang [32]	$(NH_4)_2SO_4$ fractionation and chromatography	186	12.6%	Four-step
Aspergillus oryzae	Tsuchiya et al. [33]	$(NH_4)_2SO_4$ saturation and chromatography	40	4.9%	Four-step

(Fig. 1). Based on the discussion above, the selected ATPS comprised a PEG MW of 6,000, TLL of 63.5%, a volume ratio of 2.31, and a sample concentration of 0.4 g/mL that produced a GAD recovery of 90% with the purification fold of 73.73.

Table 4 summarized the methods adopted by past researchers for GAD purification to evaluate the efficiency of different processes in terms of purification and recovery. The ATPS exhibited the highest GAD recovery efficiency (90%) from *E. coli* with the purification fold of 70, which indicated its great potential for being used in scale-up processes. The related purification steps illustrated in literature are mainly salt precipitation and chromatography and are typically developed in the laboratory with milligram quantities of product. For bacteria, Noriko Komatsuzak [31] described a three-step procedure for purification of GAD from *Lactobacillus paracasei* to homogeneity. Ueno et al. [31] purified GAD from a cell-free extract of *Lactobacillus brevis* IFO 12005 by chromatography on Sephades G-100, diethylaminoethyl (DEAE)-sepharose CL-6B, and Mono Q. About 9 mg of purified GAD was obtained from 90.2 g of wet cells. Zhang [32] purified GAD by 186-fold from rice germ using a combination of $(NH_4)_2SO_4$ fractionation, DEAE-sepharose FF ion exchange chromatography, Superdex-200 gel filtration chromatography, and Glu-sepharose CL 4B affinity chromatography. Tsuchiya et al. [33] purified 230 μg GAD from 20 g of the mycelia of *Aspergillus oryzae* by $(NH_4)_2SO_4$ at 30–70% saturation and chromatography on Sephacryl S-300, DEAE-FF, and CM-FF. The purification of GAD from the crude enzyme solution was 40-fold and the recovery rate was 4.9%, indicating that there was still sufficient room for improvement. Based on the results above, the recovery efficiency decreased significantly with increasing operation units even though more purification steps involved could incrementally improve the purity of the target product. In comparison, the ATPS developed in this study could exclusively remove the contaminant protein (high purification fold of 70) while concentrating GAD, omitting the steps of precipitation and chromatography used by the others to remove the contaminants. Therefore, the one-step purification of GAD by ATPS represents a new integrated technique that can be potentially applied to industrial operations for enzyme recovery.

Conclusion

This report describes for the first time the partitioning of GAD produced by *E. coli* after cell disrupts in the aqueous two-phase systems (ATPS). The optimum ATPS system obtained for GAD purification consisted of PEG MW of 4,000, TLL of 63.5%, a volume ratio of 2.31, and a sample concentration of 0.4 g/mL, which produced a 90% GAD recovery with 70 purification fold in only one operation step after cell disruption. Comparing to the current purification processes, the ATPS for GAD partitioning established herein is a novel and integrated technique for GAD purification with a great potential for scale-up applications.

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