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Separation of Soybean Trypsin Inhibitor Using Reverse Micellar System

Sang-Woo Jo, Choon-Soon Choi* and Joon-Shick Rhee
Department of Biological Science & Technology, KAIST, Seoul
**Department of Food Science & Technology, Gwangju Health Junior College, Gwangju*

역미셀계를 이용한 대두 단백질로부터 트립신 저해제의 분리에 관한 연구

조 상 우 · *최 춘 순 · 이 준 식
한국과학기술원 생물공학과, *광주보건전문대학 식품가공과

ABSTRACT

The separation of soybean trypsin inhibitor using reverse micellar system was investigated. Among the buffer systems tested, 1.0M $CaCl_2$ soltion (pH 3.0) and 1. OM NaCl solution (pH 11.5) were most effective for solubilization and de-solubilization of protein, respectively. When these conditions were applied to two model samples, one of which was composed of the same amounts of 7S protein and trypsion inhibitor, and the other of which was composed of the same amount of soluble soybean protein isolates and trypsin inhibitor, highly pure trypsin inhibitor was obtained. And in the real soybean, *Kwang Gyo*, pure trypsin inhibitor was also obtained.

INTRODUCTION

Though upstream processing of biotechnology are well developed, many biochemicals are still separated and purified by intrinsically batch-mode processes, such as column chromatography, that are at best only scaled up to a preparative scale. Thus, there is a clear need for efficient and scalable bioseparating processes that can be operated on a continuous basis. Liquid-liquid extraction processes are attractive from this standpoint and several methods are investigated by a number of research groups (1-5). The most frequently encountered two-phase systems are composed of low concentrations of polyethylene glycol (PEG), dextran and water.

Recently, process using reverse micelle is being widely studied. A reverse micelle is composed of amphiphilic molecules in organic solvent with a nanometer-scale droplet which has the ability to solubilize hydrophilic macromolecules, such as proteins, in an organic environment without denaturation (6, 7). As different proteins possess different propensities to be solubilized in a micellar phase it would be anticipated that this would allow the separation of proteins by selective solubilization in the micellar phase (8).

However, extraction of enzyme by organic solvents using reverse micelles is a relatively new concept. While many earlier workers have demonstrated the importance of this procedure and investigated the phenomenon of solubilization, there have been a few examples and systematic investigation of this system for protein separation (9, 10). Therefore, we applied this reverse micellar system to the separation of trypsin inhibitor (TI) from the whole soybean protein. The objective of our work was to develop the most suitable condition for the separation of TI using the reverse micellar system.

MATERIALS AND METHODS

The anionic surfactant, Aerosol-OT (AOT), was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.) and purified according to the method of Tamamushi and Watanabe (11). Trypsin, type IX and trypsin inhibitor (TI), type II-S (crude form) and type I-S (highly purified form) were also obtained from Sigma. A soybean gift of Crop Experimental Station, Rural Development Adminis-

tration was *Glycin max*, cultivar *Kwang Gyo*. Storage protein of soybean, glycinin and conglycinin, were prepared by the method of isoelectric precipitation (12) and DEAE-Sephadex A-50 column chromatography (13-14).

As for standard conditions, forward transfer experiments were performed by contacting 5 ml samples of a protein solution at the desired pH and ionic strength with an equal volume of AOT-isooctane solution in a 3 × 8.5cm vial(30ml) and agitated with a magnetic stirring bar for 5 min at about 750 rpm. Then the resulting dispersion was centrifuged at 2000rpm for 15 min to obtain a distinct phase boundary. The phases were then separated and their UV absorption was measured. For the backward transfer experiments, 4 ml of the upper phase of the forward transfer experiments was taken and contacted with a new aqueous phase (4ml) of the desired pH and ionic strength.

The protein concentration was measured by UV absorption at 280 nm minus at 310 nm (△Abs) in order to minimize the effect of turbidity on a Beckman DU-6 spectrophotometer. TI activity was measured by the degree of trypsin activity decreased according to Hummels method (15), and defined as the number of trypsin units inhibited(TUI).

RESULTS AND DISCUSSION

Forward transport of TI

Forward transport experiments were performed with solutions of TI to determine the most suitable conditions of the aqueous phase for the separation of TI. Using the standard conditions described earlier, TI was transfered from aqueous to organic phase at various pH of aqueous phase. Fig. 1 shows the effect of pH on the solubilization of TI. No solubilization was observed at pH above isoelectric point (pI) of TI (pH 4.5). At this pH, net charge of TI was negative and the repulsion between protein and AOT head group might have resulted in the insolubilization. As the pH of the system was lowered below the pI, an increase in solubilization was observed with 90% solubilized at pH valuse slightly less than pI value. However, a downturn in solubiliztion was observed at extremely low pH, which cannot be explained in terms of electrostatic interaction. It is possible, however, that TI was more apt to denature at these pH, which resulted in their transition to a random coil. A solid precipitate was observed at the interface indicating

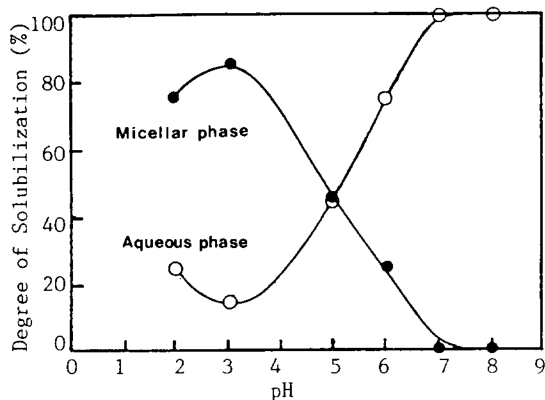

Fig. 1. Effect of pH on trypsin inhibitor solubilization.

that the protein might have formed an insoluble complex with AOT at low pH.

The effect of salt type and the electrolyte concentration was also studied. Four types of salt were investigated at their various concentrations. Table 1 shows that 1.0 M $CaCl_2$ solution was most suitable for aqueous phase. Goklen speculated that at low ionic strength of the feed solution, the micellar size became larger than that at high ionic strength and that there was greater probability to solubilization of proteins thermodynamically at low ionic strength(8). Our result corresponds well with that of Goklen in the case of KCl solution. However, in the case of NaCl and $MgCl_2$ solutions at low ionic strength, both phases became turbid when aqueous phase

Table 1. The effects of salt type and ionic strength on solubilization of trypsin inhibitor*

Salt solution	Concentration in organic phase	Concentration in aqueous phase
0.1 M NaCl	12.0%	13.0%
1.0 M NaCl	85.7%	14.3%
0.1 M KCl	84.0%	16.0%
1.0 M KCl	66.7%	29.3%
0.1 M $CaCl_2$	80.7%	19.3%
1.0 M $CaCl_2$	90.7%	9.2%
0.1 M $MgCl_2$	7.1%	10.7%
1.0 M $MgCl_2$	71.8%	7.4%

*) The pH of all feeding aqueous phase was 3.0.

of protein came into contact with organic solvent phase. And particularly in the case of $MgCl_2$ solution, gel-like precipitates were observed just below the interface even in the absence of proteins. It may be due to Mg^{++} interacting with AOT which resulted in insoluble precipitates to occur. In both cases, it is speculated that the salt and surfactant might have caused aggregation of TIs which resulted in the denaturation of proteins. But in the case of $CaCl_2$ solution, a contradictory result was shown. Wells (16) reported that there exists a specific cation-protein interaction between the phospholipase A_2 and Ca^{++} when the enzyme was solubilized. Our result also indicates that there exists interaction between protein and Ca^{++} ion for favorable solubilization of TI. However, further research is warrented for the detailed study of the mechanism.

In summary, we chose the following conditions for solubilizing the TI : pH of feeding aqueous solution, 3.0 ; type of salt and concentration, $CaCl_2$ at 1.0 M, respectively.

Backward transport of TI

To extract the TI in micellar phase to a new aqueous phase, the net charge of the protein must be negative so that repulsive force against the head group of AOT makes it possible to be excluded from micellar phase. As shown in Fig. 1, no solubilization was observed above pH 6.0 of the aqueous phase. We chose pH 11.5 for the pH of the recovering aqueous phase because of slight buffering capacity of salt solution and tolerance of TI in this pH. Table 2 represents the effect of types of salts and ionic strength on de-solubilization of TI from micellar phase to aqueous phase. From this result, 1.0 M NaCl solution was found to be most suitable for de-solubilizing the TI. In the case of $CaCl_2$ solution, only a small amounts of TI was recovered. It was thought that the divalent, Ca^{++}, was bound to the negative charge of the TI, masking the net negative charge. So the repulsive force resulted in the decrease. Another possible explanation for this behavior might be a random distribution of Ca^{++} to the protein surface. At any rate it was thought that the Ca^{++} ions render the TI more attractive AOT-reverse micelles.

Table 2. The effects of salt type and ionic strength on de-solubilization of trypsin inhibitor*

Salt solution	Concentration in organic phase	Concentration in aqueous phase
0.1 M NaCl	2.5%	14.0%
1.0 M NaCl	30.0%	48.0%
0.1 M KCl	55.9%	21.6%
1.0 M KCl	54.5%	10.7%
0.1 M $CaCl_2$	82.0%	11.7%
1.0 M $CaCl_2$	83.4%	11.2%

*) The pH of all contacting aqueous phase was 11.5.

Separation of TI from complex system

The ultimate test of this process as a selective extraction operation for protein recovery is its ability to resolve protein mixtures. In order to test the feasibility of applying reverse micellar system in actual system, several model systems consisting of TI and other components of soybean were composed and tested. The details of this process is illustrated in Fig. 2.

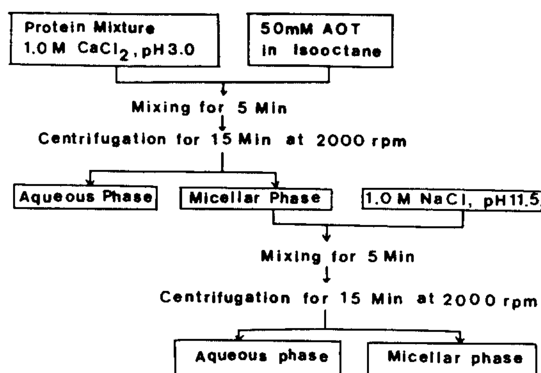

Fig. 2. Experimental procedure of protein separation.

First, separation of TI from 11S protein was tested. 11S globulin and TI, each at 1 mg / ml in 1.0M $CaCl_2$, were employed. However, since 11S globulin coagulated and precipitated in the presence of $CaCl_2$(17-19) the separation of TI from the 11S globulin could be done without applying the reverse micellar system.

Second, 7S globulin was mixed with TI and the mixture was contacted with 50 mM AOT in isooctane solution, using standard method as mentioned above. Table 3 presents the protein content and its activity at the initial

Table 3. Result of the separation of trypsin inhibitor for two model samples and a real sample using reverse micellar system.

Composition	Total volume	Total protein	Total activity[1)]	Specific activity[2)]	fold
7S + TI[3)]	5 ml	10.0 mg	229.80	22.98	1
	4 ml	2.5 mg	135.64	54.26	2.36
SBP + TI[4)]	5 ml	10.0 mg	339.70	33.97	1
	4 ml	2.7 mg	155.96	56.13	1.68
Kwang Gyo	5 ml	5.0 mg	52.05	10.41	1
	4 ml	0.3 mg	5.44	16.48	1.56

The compositions of upper two model samples are composed of the same amount of trypsin inhibitor and other proteins in 1 ml/mg.
1) Activity was defined as the number of trypsin units in hibited,
2) The unit of specific activity was TUI/mg protein,
3) TI = trypsin inhibitor,
4) SBP = soluble soybean proteins.

and final aqueous phase. The separaion of TI from 7S globulin was very successful. However, considering the pI of the 7S globulin(pI=4.9), electrostatic interaction could not be selective force for the separation of TI. The 7S globulin is very large protein(MW=330,000) and its incorporation into the reverse micelles whose radius is in several nm would not by easy(20). Therefore, the 7S globulin needs more larger reverse micelle to be solubilized which was formed by the fusion of several small reverse micelles. But it was thermodynamically unfavorable, resulting in exclusion of 7S in the solubilization process.

Finally, separation of TI from whole soluble soyprotein of defatted soybean meal and TI was tested. In this case, other than 11S and 7S globulin were presented in the proteins solution, especially 2S fraction which is similar in molecular weight with TI. As shown in Table 3, purification of TI from this model system was also successful. The specific activity was increased about 2 folds.

This results were applied to the soybean *Glycin max*, cultivar *Kwang Gyo* for purification of TI. Table 3 shows that only 10% of initial activity was recovered, but the specific activity was increased about 1.6 folds.

All purified protein samples were applied to SDS-PAGE and the results were shown in Fig. 3. Lane 5 is pure TI. Lane 2 is soluble soybean protein from defatted soybean meal and lane 6 is purified TI using reverse

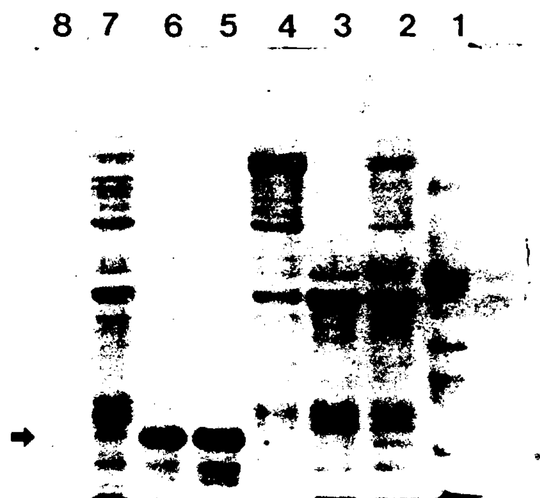

Fig. 3. SDS-PAGE of various proteins.
1. Standards,
2. Soluble soybean proteins from defatted soybean meals,
3. 11S protein,
4. 7S protein,
5. Pure trypsin inhibitor,
6. Purified trypsin inhibitor from soluble soybean protein and pure trypsin inhibitor by reverse micelles,
7. Soluble soybean proteins from *Kwang Gyo*,
8. Purified trypsin inhibitor from *Kwang Gyo* by reverse micelles.

micellar system from the same amount of soluble soy proteins and TI. Lane 7 is soluble soybean proteins from *Kwang Gyo* and lane 8 is the recovered aqueous phase which shows highly purified form. This result shows that all TI recovered by reverse micellar system were highly pure.

요 약

역미셀계를 이용하여 대두 트립신 저해제의 분리에 대하여 고찰한 바, 여러 완충 수용액 중에서 1.0M $CaCl_2$ 용액(pH 3.0)과 1.0M NaCl용액(pH 11.5)이 단백질의 용해와 회수에 가장 적당하였다. 이 조건을 두 모델계와 하나의 실제시료에 적용해 본 결과, 7S 단백질과 트립신 저해제로 구성된 모델계에서는 비활성이 두배이상 증가되었고, 전수용성 단백질과 트립신 저해제로 구성된 모델계에서는 1.6배의 비활성 증가를 볼 수 있었다. 광교대두로부터 추출한 전유출물의 경우, 1.56배의 비활성 증가가 있었다. 이들을 SDS-PAGE로 확인한 결과 모두 매우 순수한 단백질임을 알 수 있었다.

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