A Novel Process of Purifying Soybean Hull Peroxidase

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Original scientific paper
Received: May 5, 2004
Accepted: December 15, 2004

The separation and purification method of peroxidase was studied. A novel and simple purification process was used. RZ value of soybean hull peroxidase (SBP) reached 1. 32 and the recovery of enzyme activity was 65 % after purification. The new process is: the enzyme solution was purified by the ammonium sulphate–acetone cooperation precipitation, the acetone precipitation and zinc sulfate precipitation. The ammonium sulphate–acetone cooperation precipitation, namely, the two-phase system of organic solvent- inorganic salt, was adopted in our process. This is a simple and effective purification method for biomacromolecule.

Keywords:
Soybean hull peroxidase (SBP), purification, cooperation precipitation, acetone fractional precipitation

Introduction

Soybean hull peroxidase (SBP, EC1.11.1.7) is one kind of the family of plant peroxidase. The SBP acting as marker enzyme was widely used in the medical diagnosis, histochemistry of immune cell, and enzyme immunoassay previously.[1] In recent years soybean hull peroxidase absorbed people's attention with its uncommon property.[2] Because of easy source, abundant content in soybean hull, wide range of substrate, high thermostability, and stability within relative wide range of pH, SBP may hopefully substitute horseradish peroxidase.[3] Some new applications of peroxidase suggested include the synthesis of phenolic resin of high quality, the degradation of lignin, and the disposal of the three wastes etc.[4] However, the traditional process of purifying SBP needs to be improved.

So far, a major limitation for the wide uses of the soybean peroxidase is the current high cost of production of the enzyme. One of the effective means to decrease the price of enzyme is the reduction of purification cost. The cost can be decreased either by increasing the recovery and/or by reducing purification steps etc. The common purification methods contain ion-exchange chromatography, hydrophobic and affinity chromatography. A large amount of work has been done in the field of the crystallization of soybean peroxidase. But these studies have nearly all used chromatogram methods. Though the resulted purity is very high, their treatment amount is relatively small without exception, their cost high and time consuming.[5,6] So it is quite important to find a process which can simply purify a mass of soybean peroxidase to get the high purity enzyme. This is significant for reducing the cost. Therefore, in the present study, we have investigated the novel process for the purification of peroxidase from soybean hull.

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Materials and methods

Apparatus and chemicals

Apparatus: Ultraviolet/visible light spectrophotometer (S54 type spectrophotometer, Shanghai Lengguang Technological Co. Ltd., it is a double-beam instrument), high speed refrigerated centrifuge GL-21M (Changsha Xiangyi Centrifuge Instrument Co. Ltd.), FSM-175 separating type soybean milk machine (Shenyang No.3 Machine Tool Factory), high speed tissue triturator DS-1 (Shanghai Sample Model Factory).

All chemicals employed in this research were at least of analytical grade or chemical grade. Dry soybean hull was bought from the free market of agricultural products.

Assay of soybean hull peroxidase activity and purity

According to the documentary[7] method, aniline was used as substrate and ethanol was used as a solvent in which aniline is soluble. The volume fraction of aniline and ethanol (φ) was 1:8. pH of the assay was 5.5 adjusted by hydrochloric acid (0.1 mol L^{-1}).The test process was as follows: firstly, 2 ml of aniline and ethanol mixture was added to 8 ml of soybean peroxidase solution which was diluted to proper concentration. The mixture was

needed to be shaken up. Secondly, 0.2 ml of φ = 0.2 % hydrogen peroxide was added to above solution at 20 °C. The solution was mixed quickly and the absorbance of it at 415 nm was determined by spectrophotometer with the distilled water as a blank. The absorbance was recorded at the intervals of 30s within 4 to 5 min. The mass concentration of enzyme solution was adjusted to obtain the absorbance less than 0.8. One unit of peroxidase activity (U) represents the change of absorbance value in 1 min at room temperature (1 cm quartz cuvette is used in the definition). If there is ammonium sulphate in enzyme solution, enzyme activity should be assayed after dialysis.

Purity of soybean peroxidase was expressed by RZ value. The RZ value was the ratio of absorbance at the wavelength of 403 nm and 275 nm. From the figure of 275 nm of soybean peroxidase solution, the purity of soybean peroxidase can be estimated. Only when the figure is better, the purity of soybean peroxidase was expressed by RZ value as a real value.

The RZ value of pure soybean peroxidase was about 2.0.[8]

The process of separation and purification of SBP

The first step: acetone-ammonium sulphate cooperation precipitation

The soybean hulls were mashed by the high speed tissue triturator after dunking in the 12 fold volumes of deionized water for one hour. Then it was extracted over-night in a refrigerator. Solid ammonium sulphate was added to form 45 % saturation after the extract was filtered to get the original peroxidase solution, and then 0.3 fold acetone of the original volume of enzyme solution were added into it. Acetone was added at 0 °C in all of our tests. The mixture was centrifuged for 10 min at n = 5000 min^{-1}. Then solid ammonium sulphate was added to the supernatant, respectively, to form 50 %, 60 %, 70 % and 80 % saturation of original enzyme solution. The mixture was centrifuged for 10 min at n = 5000 min^{-1}. The activity of resulted precipitants was assayed after dialysis and the recovery was calculated to find the optimum range of the concentration of ammonium sulphate.

Orthogonal design of ammonium sulphate – acetone cooperation precipitation

Volume of adding acetone (0.2, 0.3 fold of the original volume) and saturation of ammonium sulphate (40 %, 45 %, 70 % and 75 %) were optimized by Orthogonal design. The orthogonal table of L8 (2^7) was chosen.

The second step: Acetone precipitation

After ammonium sulphate–acetone cooperation precipitation, the enzyme solution was collected and divided to four equal portions. Firstly, 1.0 fold volumes of acetone (at 0 °C) was added, respectively. And then acetone was, respectively, mixed to the supernatant to form the final volume of 1.6, 1.8, 2.0 and 2.2 fold volumes of acetone after centrifugation. Secondly, the mixed solutions were centrifuged. The precipitations of the second step were respectively dissolved by distilled water, and enzyme activities and RZ value of them were assayed.

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The third step: zinc sulphate precipitation

Effect of the order of adjusting pH on zinc sulphate precipitation

The enzyme solution mixed after acetone fractional precipitation was divided into eight equal portions. The four solutions of them are adjusted, respectively, to be pH as 7.0, 8.0, 9.0 and 10.0 by HCl or NaOH, and then 1.0 mol L^{-1} zinc sulfate solution was mixed to form 0.01 mol L^{-1} Zn^{2+} concentration. The other four solutions are respectively adjusted to be pH as 7.0, 8.0, 9.0 and 10.0 by HCl or NaOH after 1.0 mol L^{-1} zinc sulfate solution was mixed to form 0.01 mol L^{-1} Zn^{2+} concentration. The eight solutions were centrifuged. The RZ value of their supernatant was assayed.

Effect of Zn^{2+} concentration on zinc sulphate precipitation

The enzyme solution mixed after acetone fractional precipitation was adjusted to be as pH 8.0 and divided into four equal portions. The 1.0 mol L^{-1} zinc sulfate solution was mixed to form 0.005 mol L^{-1}, 0.01 mol L^{-1}, 0.02 mol L^{-1}, and 0.04 mol L^{-1} Zn^{2+} concentration. The four solutions were centrifuged. The RZ value of their supernatant was assayed.

Results and discussion

Effect of saturation of ammonium sulphate on purification

The effect of ammonium sulphate saturation on recovery of ammonium sulphate–acetone cooperation precipitation was showed in Fig.1. As it was seen, when the saturation of ammonium sulphate was less than 40 %, the recovery was only 6 %, and when the saturation of ammonium sulphate was more than 80 %, the recovery was not found. So,

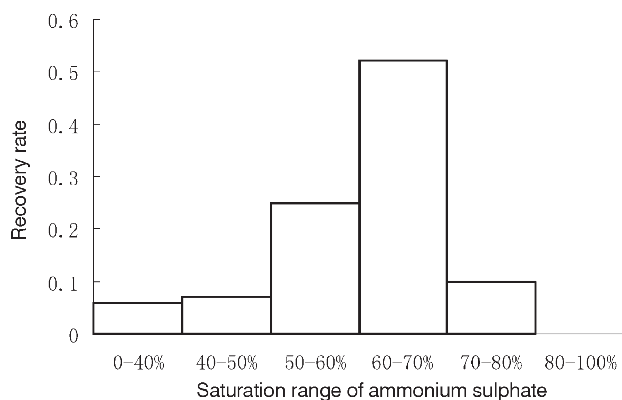


Fig. 1 – *Effect of ammonium sulphate concentration on recovery rate of ammonium sulphate–acetone cooperation precipitation*

the recovery reached 94 % at 40 % – 80 % saturation range of ammonium sulphate and was more than 70 % at 50 % – 70 % saturation range. When ammonium sulphate fractional precipitation was used alone, the recovery was only 62 % at 40 % – 80 % saturation range and was 88 % at 30 % – 80 % saturation range, which were less than the recovery in the ammonium sulphate–acetone cooperation precipitation. The result was led for the volume of solution to become bigger and the saturation of ammonium sulphate was decreased, after the addition of acetone.

Orthogonal design of ammonium sulphate-acetone cooperation precipitation

In order to further optimize the volume of acetone and the initial and final saturation of ammonium sulphate, orthogonal design was used. In the orthogonal design, three variables (initial saturation of ammonium sulphate (C_2), volume of acetone (V), and final saturation of ammonium sulphate (C_2)) were considered. The recovery rate and RZ were assessed parameters. The results were showed in Table 1.

Analysis of variance recovery and RZ were showed in Table 2, Table 3. As was seen from Table 1, Table 2, Table 3, the final saturation of ammonium sulphate had a significant effect on recovery and RZ, and the volume of acetone had a less significant effect on recovery and RZ. Additionally, as was seen from analysis of variance RZ (Table 3), the initial saturation and interaction of initial and final saturation of ammonium sulphate and volume of acetone had significant effect on purification. Considering there was much precipitation when the solution of ammonium sulphate–acetone was first centrifuged, it was advantageous to improve the purity of soybean peroxidase with increasing the initial saturation of ammonium sulphate and volume of acetone, for they had less significant effect on recovery. Therefore, the optimum medium was found

Table 1 – *The results of Orthogonal design of ammonium sulphate-acetone cooperation precipitation*

Run	C_1	V	$C_1 \cdot V$	C_2	$C_1 \cdot C_2$	$V \cdot C_2$	Experimental blank	Recovery	RZ
1	45	0.2	1	70	1	1	1	0.690	0.377
2	45	0.2	1	75	2	2	2	0.816	0.434
3	45	0.3	2	70	1	2	2	0.693	0.437
4	45	0.3	2	75	2	1	1	0.751	0.517
5	50	0.2	2	70	2	1	2	0.721	0.451
6	50	0.2	2	75	1	2	1	0.798	0.463
7	50	0.3	1	70	2	2	1	0.700	0.311
8	50	0.3	1	75	1	1	2	0.760	0.328

Table 2 – *Analysis of variance recovery*

Source of variance	C_1	V	$C_1 \cdot V$	C_2	$C_1 \cdot C_2$	$V \cdot C_2$	e	e+
S_j	0.000	$2 \cdot 10^{-3}$	0.000	$1.3 \cdot 10^{-2}$	0.000	$1 \cdot 10^{-3}$	$3.3 \cdot 10^{-4}$	10^{-3}
f	1	1	1	1	1	1	1	4
S	$1 \cdot 10^{-4}$	$1.8 \cdot 10^{-3}$	0.000	$1.29 \cdot 10^{-2}$	$3 \cdot 10^{-4}$	$9 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	$2 \cdot 10^{-4}$
F value	$5.94 \cdot 10^{-1}$	10.347	$6 \cdot 10^{-3}$	72.82	1.561	5.106		
$F_{1-a}(f_a, f_e)$	10.1	34.1						
significance	*		**					

Table 3 – *Analysis of variance RZ*

Source of variance	C_1	V	$C_1 \cdot V$	C_2	$C_1 \cdot C_2$	$V \cdot C_2$	e	e+
S_j	$6 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	$2.2 \cdot 10^{-3}$	$3 \cdot 10^{-2}$	$1 \cdot 10^{-3}$	0.000	0.000	0.000
f	1	1	1	1	1	1	1	2
S	$6 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	$2.2 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	$1 \cdot 10^{-3}$	0.000	0.000	0.000
F value	81.13	31,45	315.9	49.74	21.05	1.42		
$F_{1-a}(f_a, f_e)$	10.1	34.1						
significance	**	*	**	**	*			

* represent 5% significance levels
** represent 10% significance levels

to be initial saturation of ammonium sulphate 45 %, volume of acetone 0.3 times, and final saturation of ammonium sulphate 75 % from comprehensive consideration of recovery and RZ. The recovery rate was 75.1 % and the RZ value reached 0.517 under the optimum conditions.

In our tests, the two-phase system was ammonium sulphate-acetone two-phase system. The soybean peroxidase was separated out with increasing the concentration of ammonium sulphate and enriched in the low concentration acetone of upper phase. The enzyme became a piece of sheeting after centrifugation. The resulted enzyme is the soybean peroxidase. It was commonly considered that the active macromolecular compound was easy denaturalized in the organic solvent. However, the enzyme was not denaturalized within the range of time and temperature in our process.

The effect of ammonium sulphate–acetone cooperation precipitation was better than that of the ammonium sulphate or acetone fractional precipitation. There were some reasons: firstly, the ammonium sulphate–acetone cooperation precipitation improved the efficiency of purification. The mechanism of ammonium sulphate and acetone precipitation was different, the former neutralizing the surface charge of protein and the latter reducing the dielectric coefficient of protein solution. The effect of them was complementary in the cooperation precipitation, so the efficiency of purification was higher. Secondly, it is propitious to centrifuge. When ammonium sulphate fractional precipitation was used alone, the solution was not propitious to centrifuge with the saturation of ammonium sulphate improving and the density of solution increasing. If acetone of certain volume was added to the ammonium sulphate solution, it was propitious to centrifuge with the density of solution reducing. Thirdly, the ratio of its concentrations was high. Compared with ammonium sulphate fractional precipitation, its deposit of the first step was more and purifying was higher. However, the deposit of the second step was lower, so the impurity in the recycled enzyme was reduced and the purity was improved.

Acetone precipitation

The volume fraction of acetone

The influence of acetone volume fraction on recovery rate and RZ in acetone precipitation is showed in Fig. 2. The RZ value of soybean peroxidase gradually decreased and its recovery shows as a convex curve with increase of volume fraction of acetone (Fig. 2.). Compared with 67 % and 64 % of acetone volume fraction, the recovery rate of enzyme with 69 % of acetone volume fraction decreases. As was seen from Fig. 2, when the final volume fraction of acetone was 64 %, the recovery rate was more than 96 %. Therefore, the 64 % of acetone volume fraction was enough to precipitate the soybean peroxidase solution. Because, the recovery was 72.5 % at the final 62 % volume fraction of acetone, it was not satisfying effect. According to Fig. 2, 64 % of acetone volume fraction was selected as the final volume fraction in acetone precipitation, because the two recoveries and RZ value

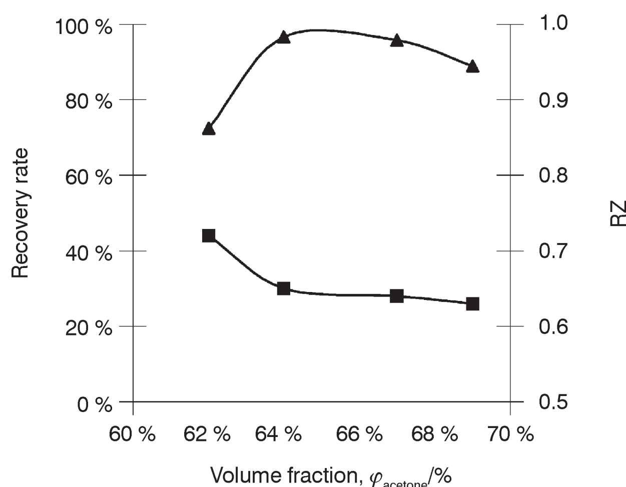


Fig. 2 – *Profiles of recovery rate and RZ. for acetone precipitation ▲ recovery rate ■ RZ*

at 67 % and 64 % volume fraction of acetone were basically the same, it was a satisfying effect and the volume of acetone was lower.

Zinc Sulphate precipitation

Effect of the order of adjusting pH on zinc sulphate precipitation

The effect of pH on zinc sulphate precipitation was showed in Fig. 3. The RZ value of the first adjusting pH was higher for 0.2 than the one of later adjusting pH in zinc sulfate precipitation (Fig. 3). Obviously, it was propitious if the alkaline environment was formed before the zinc sulfate was mixed. In alkaline solution, many proteins had negative electric charge and formed protein-metal compound precipitation with metallic ion. The zinc ion mainly acted on carboxylic acid, amine and heterocyclic compound containing nitrogen etc. The previous research indicated that the zinc ion had formed precipitation not with the horseradish peroxidase but other protein, thus the horseradish peroxidase could be purified.[9] We speculated that the carboxylic acid of protein was easily ionized in the alkaline environment, so protein formed insoluble protein-metal compound with zinc ion easily. But under the later forming alkaline environment, zinc hydroxide precipitation was easily formed because the rate of acid base reaction was very high.

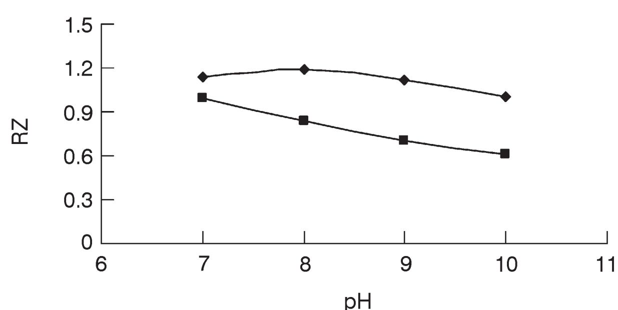


Fig. 3 – *Effect of pH on zinc sulfate precipitation*
◆ *first adjusting pH and then adding zinc sulfate*
■ *first adding zinc sulfate and then adjusting pH*

In addition, Fig. 3 indicated that the low alkaline condition was to the advantage of purification. At the pH range from 7.0 to 9.0, the distinction of purification efficiency was small, but best when pH 8.0. When the pH of solution was more than 10.0, the efficiency of purification was obviously lowered and RZ value was reduced. So we select pH 8 as the optimum pH.

Effect of Zn^{2+} concentration on zinc Sulphate precipitation

The obtained enzyme solution, after acetone fractional precipitation was adjusted to be of pH 8.0, was divided into four equal portions. The effect of Zn^{2+} concentration on zinc sulphate precipitation was showed in Fig. 4. As can be seen from the convex curve (Fig. 4.), the purification efficiency of 0.02 mol L^{-1} concentration was best in the range of Zn^{2+} concentration from 0.005 mol L^{-1} to 0.04 mol L^{-1}. The important property of protein-metal compound was that their solubility was highly sensitive to dielectric constant of medium. Adjusting the dielectric constant of the aqueous solution (adding the organic solvent) could precipitate a lot of protein down by adding Zn^{2+}, and the Zn^{2+} concentration used was about 0.02 mol L^{-1}.

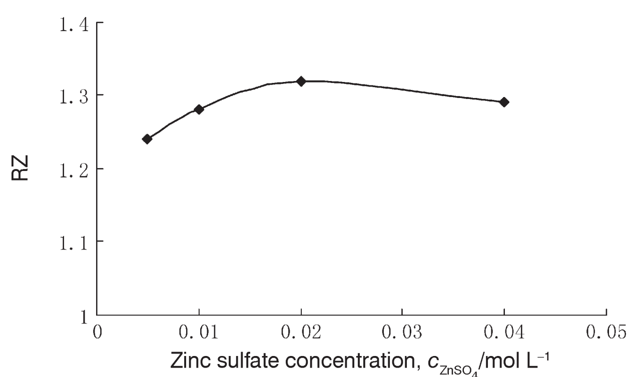
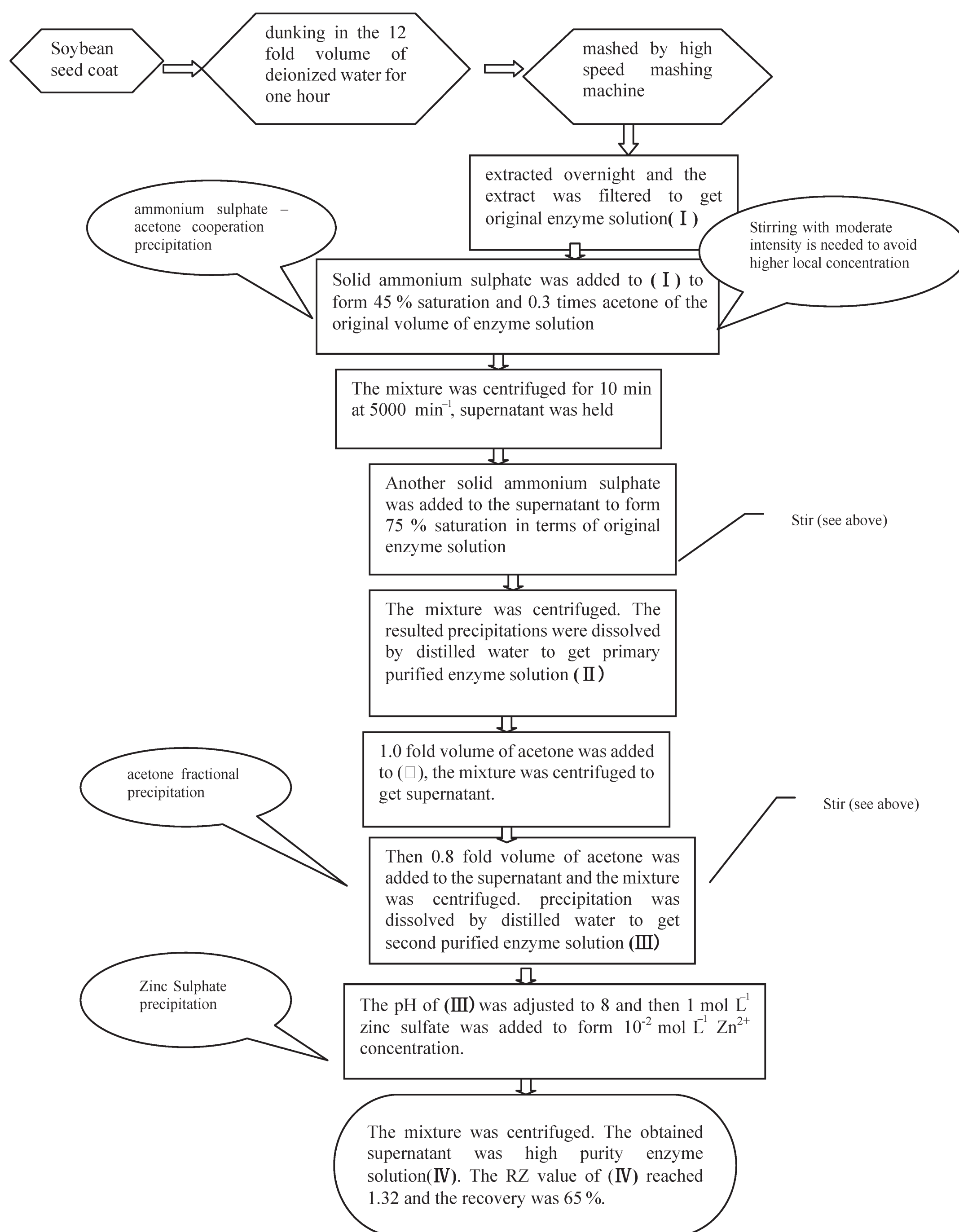


Fig. 4 – *Effect of Zn^{2+} concentration on zinc sulfate precipitation*

Comparison with other purification methods

The soybean peroxidase was a very stable enzyme which was thermo stable with enzyme activity remaining 68 % keeping 65 min in 75 °C, and stable in the pH range from 2.5 to 12.0.[7] Therefore, a major limitation for the wide use of the soybean peroxidase is the current high cost of production of the enzyme. The enzyme cost can be reduced, either, by reducing the purification cost and/or by increasing its recovery. The preferable separation and purification process (see Fig. 5.) obtained from our experiment is very easy. The RZ value of soybean peroxidase reached 1.32 and the recovery was 65 %. Enzyme activities, protein concentrations, enzyme recoveries and RZ for each step were indicated in table 4.

The purification of soybean peroxidase had been already studied in other literatures, such as *Liu* et al.,[10] which obtained pure enzyme that was purified to electrophoretic homogeneity by SDS-PAGE after soybean peroxidase by way of ammonium sulphate fractional precipitation, an anion exchange column (DEAE-Sephadex A50), affinity chromatography on Con A-Sepharose 4B and gel filtration on Bio-Gel P-60; *Zhi* et al.[11] obtained the enzyme of RZ = 1.5 by means of PEG600 aqueous two-phase extraction, anion exchange HPLC (PerSeptive DEAE



F i g . 5 – *Flow diagram of purification of soybean peroxidase in our research*

T a b l e 4 – *Purification table of SBP*

Step	Total volume ml	Total activity U	Recovery %	RZ	Purification times
Original enzyme solution	2300	32200	100		
acetone-ammonium sulphate cooperation precipitation	100	24182	75.1	0.52	
acetone precipitation	10	19449	60.4	0.91	1.75
zinc sulphate precipitation	10	15520	48.2	1.32	2.54

Poros column); *Anette Henriksen* et al.[12] reported that the enzyme was used to crystallization after an anion exchange column(Q-Sepharose HP 26/10), ultrafiltration and ammonium sulphate fractional precipitation; *N. D. Srinivas* et al.[13] purified the peroxidase of leaves of *Ipomoea palmettaenzyme* to 49 times by extraction using a polyethylene glycol (PEG)/ammonium sulphate aqueous two-phase system, followed by gel filtration on a Sephadex G-100 column. These processes had nearly all used chromatogram methods. Though, their efficiency was very high, their treatment amount was relatively small without exception, such as using ml as unit. In addition, the RZ value of soybean peroxidase sold by Sigma company was no less than 0.5.[11] Compared with these purification processes, our process had some advantages of simple procedure, high purification fold, large productive capacity of every batch, and low purification cost. The soybean peroxidase purified by our process can satisfy the demand of purity for sale. With the RZ value more than 1.3 and its large productive capacity, it can also nearly satisfy all laboratory and industry applications, such as medical diagnosis, histochemistry of immune cell, enzyme immunoassay, the disposal of the three wastes, the synthesis of phenolic resin, and the degradation of lignin etc.

Conclusions

In conclusion soybean hull peroxidase has been successfully purified by the ammonium sulphate-acetone cooperation precipitation, the acetone precipitation, and zinc sulfate precipitation in our research. The satisfactory recovery of activity and RZ as well as the simplicity of the procedure make this strategy a useful alternative for the purification of Soybean hull Peroxidase. The scope and limitations of this novel purification method in this field are currently under investigation.

ACKNOWLEDGMENT

The project was supported financially by the National Science Foundation of China (Grant No.20176019)

Nomenclature

c – concentration, mol L^{-1}

n – rotational speed, min^{-1}

φ – volume fraction, %

V – volume, ml

S_j – quadratic sum of variance of any column

f – degree of freedom

S – quadratic sum of variance

F – random variable which compliance with F distribution

$F_{1-a}(f_a, f_e)$ – fractile of random variable (F) with f_a as the first degree of freedom and f_e as the second degree of freedom when degree of confidence is (1-a)

Recovery rate – ratio of total enzyme activity after and before purification, %

RZ – ratio of absorbance of the enzyme solution at the wavelength of 403 nm and 275 nm.

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