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Solubilization and Partial Purification of the Plasma Membrane ATPase of *Rhodotorula glutinis*

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ABSTRACT. The optimal conditions for solubilization of the ATPase activity from the plasma membrane of *Rhodotorula glutinis* by zwitterionic detergent Z-3-14 were found to be: 0.55 mg/ml (5X cmc) of Z-3-14, at room temperature in the presence of 1 mM ATP, 40% (v/v) glycerol, 11.25% ammonium sulfate, 5 mM 2- mercaptoethanol, 1 mM PMSF in 0.1 M Mes-Tris pH 6.5, followed by sonication in a water bath sonicator for 6 min. A 4-fold purification of the solubilized ATPase activity was obtained by centrifugation of the Z-3-14 extract through a glycerol gradient (20-50%, v/v).

Introduction

Membrane proteins embedded inside the lipid bilayer behave as amphiphiles[1, 2] because they possess hydrophilic and hydrophobic domains. It is this sort of amphiphilic character that presents great difficulty in their isolation and purification. Proteins in the intact membrane are associated with the lipid bilayer, and at some stage of their isolation, they must be separated from the lipid. Once separated, they will exhibit the same preferential interactions that cause them to be located in a hydrophobic environment in the first place. Their amphiphilic character will make them unstable in both aqueous or organic solvents. Accordingly, the methods used for isolation of integral membrane proteins do not reach the degree of discrimination of those available for separating soluble proteins. It is wise then to simplify the protein source before starting the solubilization and fractionation procedures. Choosing to work with a population of cells as homo-

geneous as possible for the starting material, will save a lot of work. The membrane fractions obtained from these cells should be carefully fractionated to obtain the highest purity possible[1].

Plasma membrane ATPase from *Schizosaccharomyces pombe*[3-5], *Neurospora crassa*[6-8] and *Saccharomyces cerevisiae*[9,10] have been purified by methods which are quite similar in general outline even though they differ in detail. An important part of the overall purification strategy has been to start with plasma membranes of high specific activity, where contaminating membrane fragments and loosely bound peripheral proteins are removed. Different detergents have been used to solubilize the ATPases. In *Sch. pombe,* lysophosphatidylcholine has been used at a concentration of 1-2.5 mg/ml and at high ratio of 5-8 mg lysophosphatidylcholine/mg protein[3]. In *Neurospora*, deoxycholate has been used at a concentration of 0.6% and at a detergent to protein ratio of 1.2:1, with the obligatory presence of 45% glycerol[8], while other researchers have used lysophosphatidylcholine in the presence of vanadate and chymostatin, a protease inhibitor in the same yeast[5]. In *S. cerevisiae*, the zwitterionic detergent, N-tetradecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate, has been used at a detergent to protein ratio of 0.75:1[9]. Although a variety of purification methods have been attempted, the most successful one has been density gradient centrifugation, either in sucrose (10-30%)[3] or in glycerol (45-80%)[8] or (20-50%)[9].

In this report, a first attempt at solubilization and purification of the ATPase from the plasma membrane of the yeast *Rhodotorula glutinis* is described. Starting with a pure, homogenous preparation of the plasma membrane which is free from any ATPase contamination from intracellular organelles, a successful solubilization of the ATPase in active form was accomplished using the zwittergent detergent, zwittergent-3-14, in the presence of stabilizing agents. Also, attempts at partial purification are described.

Materials and Methods

Materials

Zwittergents, 3-10, 3-12, 3-14, 3-16 and Chaps (3-[3{cholam- idoropyl} dimethylammonio]-1-propane sulfonate) were obtained from CALBIOCHEM, La Jolla, CA. Coomassie Blue R-250, Coomassie Blue G-250, N, N-methylene bis acrylamide, SDS (sodium dodecyl sulfate, electrophoresis purity), glycine (electrophoresis purity) and urea were products of BioRad Laboratories, Richmond, CA. BCA protein assay kit was purchased from Pierce Chemicals Co., Rockford, IL. Acrylamide was a product of Serva, New York, NY. Glycerol, ammonium sulfate, sodium deoxycholate, 2-mercaptoethanol, lysophosphatidyl-

choline (lysolecithin), octyl glucoside, sodium cholate, ammonium persulfate, TEMED (N, N, N', N'- tetramethylethylenediamine), Lubrol PX, and L-α-phosphatidylcholine (from soybeans) were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade.

Detergent Solubilization

To examine the solubilizing ability of various detergents on *Rhodotorula* plasma membrane ATPase, the following protocol was used. The isolated plasma membranes were centrifuged at 140,000 g for 30 min. The supernatant was discarded and the pellet was resuspended in the solubilizing buffer containing detergent. The suspensions were incubated at room temperature for 10-30 min to allow for solubilization, and then centrifuged at 140,000 g for 30 min. The supernatant (which would contain the solubilized ATPase) and the resuspended pellet were then assayed to determine whether ATPase solubilization had occurred. The detergents employed in these studies were Chaps, the Zwittergents series (3-10 through 3-l6), octylglucoside, Triton X-100, sodium cholate, lysophosphatidylcholine and Lubrol PX.

Preparation of Linear Glycerol Gradient

Linear glycerol gradients (25-50%, v/v) were formed mechanically using a linear gradient maker[11]. To prepare 11.5 ml of the above gradient, 6.0 ml of gradient solutions, (0.22 mg/ml Z-3- l4, 1 mM ATP, 5 mM 2-mercaptoethanol, 1 mM PMSF, buffered with 0.1 M Mes-Tris pH 6.5, (containing either 25% or 50% glycerol), were placed in the gradient maker chambers. The gradients were collected in a 5/8 Dome-top tube (capacity was 13.5 ml). After layering the sample on the gradient, the tubes were sealed using the Quick-Seal Tube Sealer.

Cell Growth and Plasma Membrane Isolation

Rhodotorula glutinis cells were grown and maintained and its plasma membrane fractions were isolated as described in[12].

Phospholipid Purification

L-α-phosphatidylcholine (from soybeans) was partially purified by an adaptation of the method of Ragan and Racker[13]. The lipids (20 g) were suspended in 200 ml of acetone in a brown reagent bottle. This bottle was flushed with nitrogen gas, quickly sealed and covered with aluminum foil. This suspension was rapidly stirred for 3-4 days; the lipids were suction-filtered and thoroughly washed with acetone. When dry, the acetone-extracted phospholipids were stored in desiccators at –20ºC.

Liposome Preparation

L-α-phosphatidylcholine (150 mg) was placed in a 25 × 100 mm Pyrex screw-cap culture tube. Two milliliters ATPase buffer was added and the tube was flushed with nitrogen gas to prevent peroxide formation. The phospholipids were sonicated to clarity in a bath-type sonicator. Sonicated unilamellar liposome vesicles were stored at 4ºC for several hours until needed.

Protein Assays

The protein concentrations of plasma membrane suspensions, solubilized enzyme and particulate material left after solubilization were determined by either the Bradford assay[14], the BCA assay[15], the modified Lowry assay[16] or modified Bradford assay[17]. All methods used give same values.

ATPase Assay

The ATPase activities of the plasma membrane suspensions, solubilized enzyme, the particulate material left after solubilization and fractions obtained from purification were determined by either the coupled enzyme assay (by coupling the hydrolysis of ATP with the oxidation of NADH) or the molybdic acid assay (works by measurement of the number of micromoles of inorganic phosphate released by the action of the ATPase as a measure of its activity) as described in[12].

SDS Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed using the Laemmli SDS gels[18].

Result

A variety of detergents were screened to demonstrate the best detergents effective in solubilizing the enzyme (Table 1).

Zwittergent 3-14, extracted about 21% of the ATPase activity into the supernatant. Because of the low total recovery of enzyme activity, different stabilizing agents were tested in the solubilization process. The results showed that glycerol and phospholipids liposome were able to increase the activity of the solubilized enzyme by providing some kind of protection in its new environment. Accordingly, a new series of experiments were run aimed at increasing the solubilization power of the detergent, either by allowing the detergent to function more effectively or to provide more protection to the solubilized enzyme. Using different concentrations of the Zwittergent 3-14 to determine the

optimum concentration needed to solubilize the ATPase, a concentration of 0.11% (w/v) which is equivalent to 10 × the cmc (critical micelle concentration) of Z-3-14 yielded the highest activity in the supernatant (Fig. 1).

TABLE 1. Ability of different detergents to solubilize the plasma membrane ATPase.

The plasma membrane suspension were treated with different detergents at different concentrations as shown below. The suspensions were incubated 10 min at room temperature and centifuged at 140,000 × g for 30 min. Both the supernatant (S) and the pellet (P) were assayed for ATPase activity using the coupled enzyme assay.

Detergent	CMC % (w/v)	Conc. used % (w/v)	Activity in S %	Activity in P %	Total activity %
Lysophosphatidylcholine	0.001	0.013	6	68	74
		0.026	9	83	92
		0.046	7	12	19
Octylglucoside	0.73	6.4	8	62	70
		7.3	7	10	17
Sodium cholate	0.36	0.5	7	16	23
		2.0	0	16	16
		5.0	0	1	1
Chaps	0.49	0.4	0	12	12
		4.0	0	12	12
Lubrol PX	0.006	0.0055	6	43	49
		0.014	11	38	49
		0.06	7	44	51
Triton X-100	0.02	0.17	10	36	46
Z-3-12	0.11	0.11	8	36	44
Z-3-14	0.11	0.11	21	10	31
Z-3-16	0.001	0.011		66	68
		1.2	2	11	13

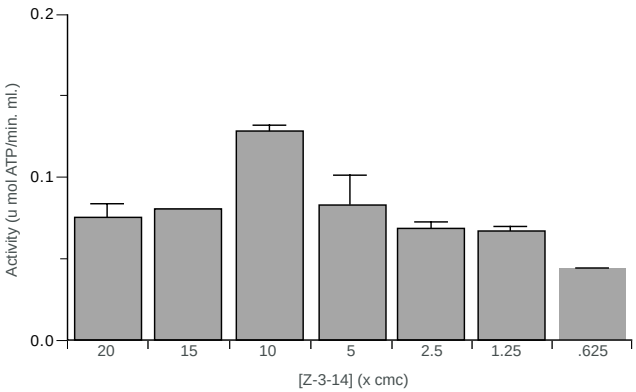


FIG. 1. Determination of the optimum concentration of Z-3-14 needed to solubilize the plasma membrane ATPase.

As shown in Fig. 2, the detergent Z-3-14 showed more solubilizing power with increasing incubation time at room temperature.

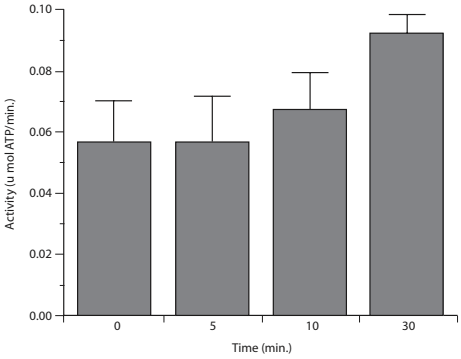


FIG. 2. Determination of incubation time of Z-3-14 with plasma membrane to have maximum solubilization.

It seems that the detergent solubilizes more ATPase if it reacts with the plasma membrane suspensions for a longer time. Solubilized membranes were sonicated using bath-type sonicator for various times. The result showed that more ATPase was solubilized with increasing time of sonication (Fig. 3).

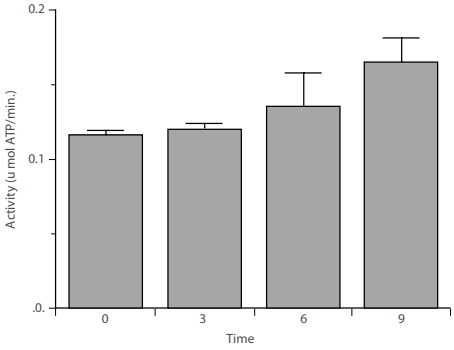


FIG. 3. Determination of sonication time to have maximum solubilization of the plasma membrane ATPase.

Addition of phospholipids in liposome form increases the activity of the solubilized ATPase. Addition of 7.5 phospholipids % (w/v) doubled the activity of the enzyme as shown in Fig. 4.

In Fig. 5, different concentrations of glycerol were included in the solubilization buffer and solubilization was carried out as usual. The resulting solubilized preparations were centrifuged further at 140,000 g for 5 hr to determine if the enzyme was really solubilized or whether the result was an effect of increased density and viscosity.

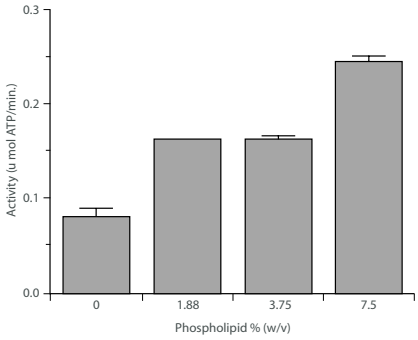


FIG. 4. Determination of phospholipids concentration in liposome form needed to increase the activity of the solubilized plasma membrane ATPase.

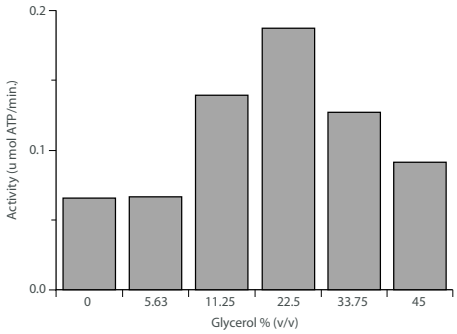


FIG. 5. Determination of glycerol concentration needed to protect the solubilized enzyme.

The results showed that a concentration of 22.5% (v/v) glycerol was the most effective concentration in maintaining the ATPase activity. To increase the yield of the solubilized enzyme, the resulting pellet after the first solubilization was treated once more with solubilization buffer, incubated at room temperature for 10 min, and the resulting solubilized ATPase was assayed for ATPase activity as shown in Table 2.

The complete procedure for solubilization of the ATPase is shown in Scheme 1.

The procedure is very efficient since solubilization of around 70% of the total activity of plasma membrane ATPase in an active form was obtained. The activity of the enzyme was stable up to 6 months if stored in dry ice (data not shown) but it had a tendency to develop turbidity if the enzyme was solubilized in the presence of liposome.

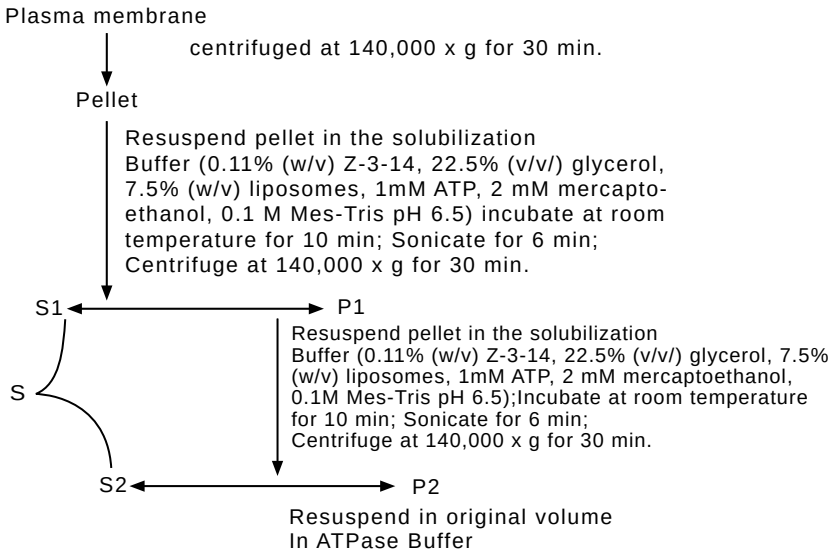
The use of density gradient centrifugation was used as a purification method. A medium containing 0.22 mg/ml Z-3-14, 1 mM ATP, 5 mM 2-mercaptoethanol, 1 mM PMSF, buffered with 0.1 M Mes-Tris pH 6.5 was used through-

TABLE 2. Reextraction of the pellet from the first solubilization.

The solubilization buffer which includes 10 × cmc Z-3-14, 22.5% (v/v) glycerol, 7.5% (w/v) liposomes in ATPase buffer was used to resuspend the pelleted plasma membrane suspensions, incubated at room temperature for 10 min, sonicated for 6 min and centifuged at 140,000 × g for 30 min. The solubilized enzyme (S1) was removed and the resulting pellet was treated with additional solubilization buffer, incubated at room temperature for 10 min, sonicated for 3 min and centifuged one more time at 140,000 × g for 30 min. The resulting solubilized enzyme (S2) together with the pellet and (S1) were assayed for ATPase activity using the coupled enzyme assay system.

Components	% Activity
S1	49.7 ± 14.6
S2	29.7 ± 9.6
P2	12.2 ± 0.5

The plasma membrane suspension had an activity of 1.23 umol ATP/min/mg. Each point is the average of 4 determinations.



SCHEME 1. Complete procedure for solubilization of the plasma membrane ATPase.

out in the gradient, only the glycerol concentration was changed. Centrifugation using slow mode of acceleration and deceleration helped to sharpen the fractions collected. This high specific activity from the first linear glycerol gradients was associated with fraction 7 which sediment to the region of 25-30% glycerol in the gradient, on the other hand, the rest of the proteins moved much further. However, some of the ATPase activity was recovered as a pellet, which

might indicate large aggregates of incompletely solubilized enzyme. Fraction 7 from the gradient was diluted 2 fold with a solution of 0.1 M Mes-Tris pH 6.8 to reduce the concentration of glycerol. About 1.3 ml of this diluted solution was applied to a second linear glycerol gradient (20-50%) which differed from the first only in that the Z-3-14 concentration was 0.15 mg/ml, because higher concentrations in the second gradient were found to inactivate the ATPase. The activity in the second gradient sediment to the region of 20-25% glycerol in the gradient. The results of a typical purification are summarized in Table 3. The ATPase was purified about 4-fold compared to the solubilized enzyme. The purified enzyme was stable at 4ºC for several days and at –70ºC for several weeks.

TABLE 3. Purification of the plasma membrane ATPase from the yeast *R. glutinis.*

Fraction	Total activity (umole/min)	Total protein (mg)	Specific activity (umole/min/mg)
Plasma membrane[a]	3.15	12.58	0.25
Solubilized enzyme	3.28	12.13	0.27
First glycerol gradient	0.42	2.21	0.19
Second glycerol gradient	0.02	0.026	0.77
	0.03	0.026	1.15[b]

[a]protein concentration was estimated as the sum of the protein concentration of the solubilized enzyme + the protein concentration of the particulate materials left after solubilization.
[b]assayed in the presence of 500 ug sonicated soybean phospholipids.

Discussion

The study of biological membrane components, either lipid or protein, although interesting in itself, should ultimately be aimed towards a full understanding of the role that they play in the functioning and survival of the living organism.

The aim of this study was to isolate one of the plasma membrane components (the ATPase) in order to understand its role in the active transport process of *Rhodotorula glutinis.*

The plasma membrane of *Rhodotorula* was isolated through a process of differential centrifugation and pH precipitation. The purity of the resulting plasma membrane fractions was high, as judged by the absence of any ATPase contamination either from mitochondria or vacuoles as shown in[12].

In general, the isolation of a membrane in itself provides a reasonable degree of purification of the protein associated with it. However, a very high degree of purification may be needed to achieve high degree of purity of soluble protein; much less purification may be needed for membrane proteins[19].

A variety of detergents were employed in attempts to solubilize the ATPase in an active form. These detergents included: Chaps, Triton, deoxycholate, sodium cholate, lysophosphatidyl choline, octylglycoside, Lubrol PX and the Zwittergent series (Z-3-10 to Z-3-16).

None of the above mentioned detergents proved successful in solubilizing the ATPase in an active form except for the synthetic zwitterionic detergent, N-tetradecyl-N, N-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-14), which showed some satisfactory results. Although, it was reported that proteins were not denatured by this detergent[19], we have found that at > 1.5 mg/ml the *Rhodotorula* ATPase is inactivated. Nevertheless, this detergent at 1.1 mg/ml, solubilized about 21% of the ATPase activity and it has been the only detergent capable of solubilizing the plasma membrane ATPase from *Rhodotorula* in an active form.

The solubilized enzyme is very stable if stored in dry ice up to 6 months. This duration of stability is very helpful since a large quantity of the solubilized enzyme can be prepared and stored for studying different purification methods.

Purification procedures starting with chromatographic methods including gel filtration, ion exchange, and affinity chromatography were proven ineffective (data are not shown). In each case, the enzyme has been eluted in or near the void volume. It seems that the ATPase forms an aggregated complex during purification similar to that reported for L-lactate dehydrogenase which forms oligomeric complexes consisting of more than ten molecules of single polypeptide in an aqueous buffer[20].

Linear glycerol gradient centrifugation was success. The first glycerol gradient did not increase the specific activity of the enzyme as expected, since some inactivation did occur. Nevertheless, as is shown in the gel (data is not shown), some proteins were removed. The second linear glycerol gradient showed some increase in the specific activity, but it was diluted and contained too low a protein concentration to be observed in the gel. The ATPase was purified 4-fold using this procedure compared to the solubilized enzyme.

Isolation of the plasma membrane fractions from crude cell homogenate resulted in tremendous purification. Solubilization of the enzyme was another improvement towards the enzyme purification. And finally, removal of some of the solubilized proteins was achieved using a linear glycerol gradient centrifugation.

One of the problems encountered in evaluating the degree of purification of the enzyme was the difficulty of measuring the protein concentration of the plasma membrane. Plasma membrane protein could not be determined exactly

until totally solubilized. However, another difficulty was encountered to determine the protein concentration of the solubilized ATPase. The presence of phospholipids in the solubilization buffer interfered with the protein determination. Treatment of the solubilized enzyme with different concentrations of TCA (trichloroacetic acid) in the presence of 95% ethanol, ethyl ether, or petroleum ether did not help much, since in each case the phospholipids precipitated, too. Treatment of the solubilized enzyme with 10% TCA in the presence of a mixture of chloroform: methanol (1:1) separates the protein between the two layers, but it was difficult to remove the protein without disrupting the phospholipids layer.

Another problem encountered during purification of the ATPase was its instability after extraction from the plasma membrane. The same problem has been reported by other researchers working in this field[3, 8,9,10,21,22]. However, the presence of protease inhibitors such as phenylmethylsulfonylfluorid[10,19] and more importantly, a high concentration of glycerol (l0- 40%)[8, l0,11,20,22, 23] have improved the stability to a certain extent.

The fold-purification of the ATPases from different sources has ranged from 5-15[3,8,10,19,22]. It is difficult to evaluate the different procedures of purification due to the phenomena of activation and inactivation caused by detergent. It seems that solubilization by the detergent combined with gradient centrifugation may produce between 30-70% inactivation of the plasma membrane ATPases[3,10,19]. This inactivation is possibly due to loss of some essential lipids during solubilization and purification. However, lysolecithin[3,19,20], soybean phospholipids[8,10,21] or bovine brain phospholipids[22] when included in the assay mixture could not restore activity. Other enzymes solubilized and purified (phenylalanine ammonia lyase and cephalosporin-C deacetylase) from *Rhodotorula* used almost same procedures[23,24] and same stabilization reagents[25].

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الإذابة والتنقية الجزئية لإنزيم الأتيبيز من الغشاء البلازمي لخميرة الرودوتيلا جليتينس

طه عبدالله قمصاني* وشارلي جرفين**

**قسم الكيمياء الحيوية ، كلية العلوم ، جامعة الملك عبدالعزيز*

جـــدة – المملكة العربية السعودية

***معامل هيوج ، قسم الكيمياء ، جامعة ميامي*

أكسفورد – اوهايو – الولايات المتحدة الأمريكية

المستخلص. الظروف القصوى لإذابة إنزيم الأتيبيز من الغشاء البلازمي لخميرة الرودوتيلا جليتينس بالمذيب (Z-3-14) ذي الأيونات المزدوجة كانت: ٥٥ , ٠ مليجرام/ مل (٥ × التركيز الحرج لتكوين المايسيل) للمذيب (Z-3-14) ، عند درجة حرارة الغرفة في وجود ١ ملي مولار من الأدينوسين ثلاثي الفوسفات ، و٤٠٪ (حجم/ حجم) من الجليسرول ، و٢٥ , ١١٪ من كبريتات الأمونيوم ، و٥ ملي مولار من ٢- ميركابتو ايثانول، و١ ملي مولار من مثبط البروتيزات (PMSF) في ١ , ٠ مولار من المحلول المنظم Mes-Tris عند الأس الهيدروجيني ٥ , ٦ . تم بعد ذلك تعريض المحلول لموجات صوتية في حمام مائي صوتي لمدة ٦ دقائق. تمت تنقية الأنزيم المذاب ٤ درجات باستخدام عملية الطرد المركزي المستمر للمستخلص المذاب في (Z-3-14) من خلال التراكيز المختلفة للجليسرول (٢٠-٥٠٪، حجم/ حجم).