Chapter 4

Isolation of Synapse Subdomains by Subcellular Fractionation Using Sucrose Density Gradient Centrifugation

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Abstract

A protocol presents a purification of postsynaptic density (PSD), from rat brain by subcellular fractionation using solubilization of membrane with Triton X-100 and sucrose density centrifugation. The protocol also includes purification of other synapse subdomains such as synaptosome, synaptic plasma membrane, P_1 (nuclei and cell debris), P_2 (crude mitochondria fraction), S_3 (soluble fraction), and P_3 (microsomal fraction). The method presented in this text is the one established by Siekevitz group. The PSDs obtained by this method are mainly excitatory type I PSDs. The method has been widely used and is useful for biochemical analyses such as identification of proteins associated with these subdomains by proteomics methods and western blotting, and morphological analyses at the electron microscopic level.

Key words: Synaptosome, Synaptic plasma membrane, Postsynaptic density, Subcellular fractionation, Detergent-insoluble cytoskeleton, Detergent-insoluble membrane

1. Introduction

Isolation of subcellular compartment is a useful approach to analyze the subcellular complexes at the molecular level. We describe methods to isolate synapse subdomains including P_1 (nuclei and cell debris), P_2 (crude mitochondria fraction), synaptosome, synaptic plasma membrane (SPM), and postsynaptic density (PSD) by subcellular fractionation using density gradient centrifugation. History of development of method for isolation of synaptic complex and PSD is concisely summarized previously (1). The method for PSD purification established by Siekevitz's laboratory (2–4) has been widely used. The methods introduced in this text are basically the same as those used in his laboratory. Both short and long procedures are stated. The method is

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applicable to brains from at least dog, rat, mouse, and human (2, 5, 6), different brain regions (4, 7–10) and brains in various developmental stages (11). Protein yields of synaptosome, SPM, and PSD (short and long procedures, respectively) are approximately 15, 6, and 0.26 and 0.1 mg per 1 g original forebrain of adult rat, respectively, but may fluctuate by unknown factor(s). Protein yields of these fractions change depending on the age of the animals used (11). The short procedure, in which PSD is purified from Triton X-100 (TX-100)-treated synaptosomes, has been widely used and is now a standard method. Protein profiles of the PSD isolated by short and long procedures in one-dimensional gel are similar but not identical (Fig. 1) (2, 12). Contents of neurofilament proteins, at least partly contaminants (12), are higher in the PSD prepared by short procedure (12). Protein yield is also different (12). Major constituent proteins are the same between the two preparations, while the mass spectrometric (MS) analysis revealed that only 70% proteins in these two PSD fractions are common (Suzuki, unpublished data). Purified PSD fraction also contains mRNAs encoding various kinds of proteins (13).

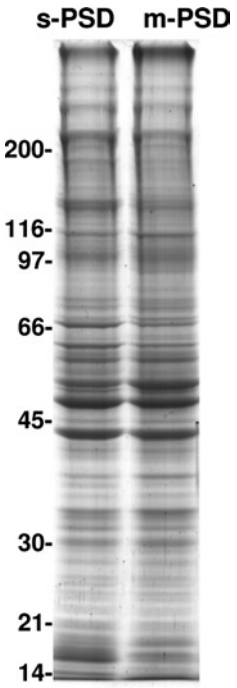

Fig. 1. Protein profiles of postsynaptic density (PSD) fractions purified by short and long procedures. PSD fractions were purified from rat forebrains (Wistar male, 6 weeks old) and separated by 7–17% gradient polyacrylamide gel. s-PSD and m-PSD refer to PSD fractions prepared by short and long procedures via TX-100 treatment of synaptosome and synaptic plasma membrane (SPM), respectively. Molecular weights are shown in kDa on the *left*.

In the early methods to prepare synaptic junctional complex and PSD, *p*-iodonitrotetrazolium violet (INT) was used to separate mitochondria by producing heavy formazan in mitochondria (14–16). However, it was found that INT causes undesirable oxidation of proteins and artificially cross-links synaptic proteins (16–19). Structures of the isolated PSD are tightened by disulfide bonds formed during the PSD isolation using INT. It is suggested that the artificial disulfide bonding of PSD proteins during isolation may occur even in the absence of INT (1, 20, 21). Artificial cross-linking of postsynaptic proteins during isolation gives resistance of the isolated PSD to various treatments including detergent solubilization (1, 20, 21). Blocking of disulfide formation is required for preparing PSD for analyses of its structural and physiological properties.

It is desirable to prepare synaptic subcompartments from freshly dissected brains. PSD fraction can also be prepared from frozen brains (3), which is convenient, in particular, when purifying it from human specimens. However, special attention should be paid when collecting brain tissue, because some proteins, in particular Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), accumulate to PSD in a short duration after decapitation (22). Accumulation of CaMKII is accelerated at room temperature or 37°C. Tubulin also accumulates to PSD fraction in a relatively long time period at 4°C after decapitation (23). Attention should also be paid to "cold-induced exodus of postsynaptic proteins" (24). Exposure of neuron to coldness causes rapid disassembly of unstable microtubules that are present in the spine and associated with PSD. Various proteins also exit from PSD, and spine morphology, at least some, may change by this microtubule disassembly.

The method stated in this text is useful to prepare the fraction enriched in the PSDs of asymmetric type I excitatory synapses, but not of the inhibitory neurons, such as those in the cerebellum. Protein yield of cerebellar PSD fluctuates and sometimes very low by unknown reason. Preparation of type II inhibitory PSD has been reported recently (25). Method using sonication but not detergent has also been reported (26), but up to now the method has been reported only once to the best of author's knowledge.

"One-Triton" PSD and "Two-Triton" PSD are prepared as a pellet after centrifugation of TX-100-treated synaptosomes (27–29). "One-Triton" PSD contains detergent-resistant membrane with light buoyant density (DRM, membrane raft fraction), which is also TX-100 insoluble at 4°C and floats on the 1.0 M sucrose layer (25, 30). Recently, it is demonstrated that "One-Triton PSD" also contains type II GABAergic inhibitory PSD (25).

Nonionic detergent TX-100 is usually used to purify PSD fraction. High quality TX-100 should be used. Other detergent,

such as deoxycholate (31, 32), octyl glucoside (1, 33), and *N*-lauroyl sarcosinate (NLS) (16, 27, 32), has also been used. NLS, a strong ionic detergent, nearly completely solubilizes PSD components when oxidation is prevented with 1 mM *N*-ethylmaleimide (NEM) during isolation of PSD (1). Deoxycholate-insoluble PSD shows clearly a lattice-like core PSD structure (32, 34), which is broken after NLS treatment (32). Octyl glucoside is effective to solubilize rapidly the whole membrane, both raft and nonraft domains (35, 36), and generally does not affect protein–protein interactions.

Presynaptic structure is unstable in alkaline solution, while postsynaptic structures are resistant (37). Therefore, the synaptic junctional structures composing of both pre and postsynaptic cytoskeletal structures can be prepared when synaptosome is solubilized with TX-100 at slightly acidic conditions (37).

2. Materials

Use distilled, double-distilled, distilled-and-deionized, or equivalent grade water. Using ultrapure water sometimes results in low-protein yield of PSD (see Note 1). All chemicals should be of reagent grade. All stocks and working solutions are kept at –20 to –30°C between uses to prevent bacterial and fungal growth. Make sure to mix up the solutions homogeneously after defreezing them, especially those containing dense sucrose solutions. All solutions should be kept at 4°C or on ice during the subfractionation. TX-100 is susceptible to autoxidation upon exposure to air. Store the unused solution sealed and also avoid storage in direct light (see Note 2).

PSD material is extremely sticky to glass and cellulose nitrate and tend to aggregate very easily (2). Therefore, the use of plastic (polyallomer) tubes and pipettes, in particular, after TX-100 treatment, is necessary to avoid undesirable absorption of PSD proteins to glasses.

Add protease inhibitors, phosphatase inhibitors, oxidization inhibitors, or RNase inhibitors as required. Addition of protease inhibitors results in increased yield of PSD proteins. It is desirable to purify PSD in the presence of iodoacetamide or NEM, which prevents harmful oxidation during the purification (1, 20, 21). PSDs prepared in the presence of iodoacetamide are different from those prepared in the absence of iodoacetamide in detergent solubility and aggregation state of PSD. Addition of dithiothreitol interferes with endogenous disulfide bondages necessary for the formation of normal PSD configuration (20, 21).

2.1. Preparation of P_1, P_2, Synaptosome, and PSD Fraction (Short Procedure)

1. 1 M $MgCl_2$ stock. Dissolve 20.33 g of $MgCl_2 \cdot 6H_2O$ (MW, 203.30) in 100 mL H_2O.
2. 1 M $CaCl_2$ stock. Dissolve 14.70 g of $CaCl_2 \cdot 2H_2O$ (MW, 147.02) in 100 mL H_2O.
3. 100 mM $NaHCO_3$ stock. Dissolve 1.68 g of $NaHCO_3$ in H_2O and make up to 200 mL with H_2O.
4. 1 M Tris–HCl (pH 8.1) stock. Dissolve 24.2 g of Tris(hydroxymethyl)aminomethane in H_2O (~150 mL) and adjust pH to 8.1 by HCl, and make up to 200 mL with H_2O.
5. 0.5 M HEPES/KOH (pH 7.4) stock. Dissolve 11.8 g of HEPES, adjust pH to 7.4 with KOH solution, and make up to 100 mL with H_2O.
6. Solution A (0.32 M sucrose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM $NaHCO_3$). Dissolve 109.6 g of sucrose in H_2O. Add 10 mL of 100 mM $NaHCO_3$, 1 mL of 1 M $MgCl_2$, and 0.5 mL of 1 M $CaCl_2$. Make up to 1,000 mL with H_2O.
7. Solution B (0.32 M sucrose, 1 mM $NaHCO_3$). Dissolve 109.6 g of sucrose in H_2O. Add 10 mL of 100 mM $NaHCO_3$. Make up to 1,000 mL with H_2O.
8. 1% TX-100, 0.32 M sucrose, 12 mM Tris–HCl (pH 8.1). Dissolve 109.6 g of sucrose in H_2O. Add 10 g of TX-100 (Sigma) and 12 mL of 1 M Tris–HCl (pH 8.1). Make up to 1,000 mL with H_2O.
9. 1% TX-100, 150 mM KCl. Dissolve 2 g of TX-100 and 2.26 g of KCl in H_2O. Make up to 200 mL with H_2O.
10. 10 mM HEPES/KOH (pH 7.4)-40% glycerol. Dilute 4 mL of 0.5 M HEPES/KOH (pH 7.4) in H_2O (~80 mL). Add 80 g of glycerol and make up to 200 mL with H_2O.
11. Sucrose solution (1.0, 1.4, 1.5, and 2.1 M). Dissolve sucrose (68.5, 95.8, 102.7, and 143.8 g for 1.0, 1.4, 1.5, 2.1 M sucrose solutions, respectively) in H_2O. Add 2 mL of 100 mM $NaHCO_3$ to each solution and make up to 200 mL with H_2O.
12. 1 mM $NaHCO_3$. Dilute 100 mM $NaHCO_3$ into H_2O. 400 mL/20 g of starting brain is required.
13. Plastic disposable pipettes, e.g., Liquipette, polyethylene transfer pipettes of 4 mL capacity, thin stem, 7 mL capacity, with scale, and 6 mL capacity 9″ long (Elkay, Shrewsbury, MA), or other plastic Pasteur pipette such as 3 mL (with scale) etc.

2.2. Preparation of SPM and PSD Fraction (Long Procedure)

The long procedure requires solutions used in Sect. 2.1 and additional solutions listed below.

1. Sucrose solutions (0.85, 1.0, and 1.2 M). Dissolve sucrose (58.2, 68.5, and 82.2 g for 0.85, 1.0, and 1.2 M sucrose solutions, respectively) in H_2O, add 2 mL of 100 mM $NaHCO_3$ and make up to 200 mL with H_2O.
2. 0.5 mM HEPES/KOH (pH 7.4). Dilute 0.5 M stock in H_2O. About 250–500 mL/20–25 g brain is required for SPM preparation.
3. 1 mM $NaHCO_3$. Dilute 100 mM $NaHCO_3$ into H_2O. About 150 mL/20–25 g of starting brain is required.

2.3. Preparation of S_3 and P_3 Fraction

No additional reagent or solution is necessary.

3. Methods

3.1. Preparation of P_1, P_2, Synaptosome, and PSD Fraction (Short Procedure) from Rat Forebrain

The method is based on those developed by Siekevitz's group (2–4). Protocol for PSD purification (short procedure) using 20–25 g forebrain as starting material is described below. The maximum amount of forebrains is about 25 g due to the limitation of capacity of ultracentrifuge. All the processes are carried out at 4°C. The procedure is outlined in Fig. 2.

1. Collect rat forebrains by decapitation and quick dissection (see Note 3). Place forebrains immediately after dissection in a beaker placed on ice. Weigh the pooled brains (weight of the container is better measured before pooling tissues). Proceed for step 2 or freeze and keep the forebrains at −80°C until use.
2. Chop forebrains into small pieces (about less than 2 × 2 × 2 mm) with scissors. When using frozen brains, dip frozen brains into small amount of cooled solution A (approximately a few milliliter) in a beaker, chop or scrape them by scissors. Add solution A to make 80 mL suspension. Keep the suspension on ice for at least 20 min when using frozen brains (see Note 4).
3. Homogenize the suspension at 1,000 rpm with six or seven up-and-down motions with a motor-operated Teflon/glass homogenizer using a loose-fitting pestle (see Note 5) while cooling the container in ice water. Recover suspension into a new beaker and dilute to 200 mL with solution A. (Start preparing sucrose layers necessary at step 9 during centrifugations at steps 3 (or 4) to 8. To make sucrose layers, auto-pipette using 25 mL transfer pipette is convenient.)

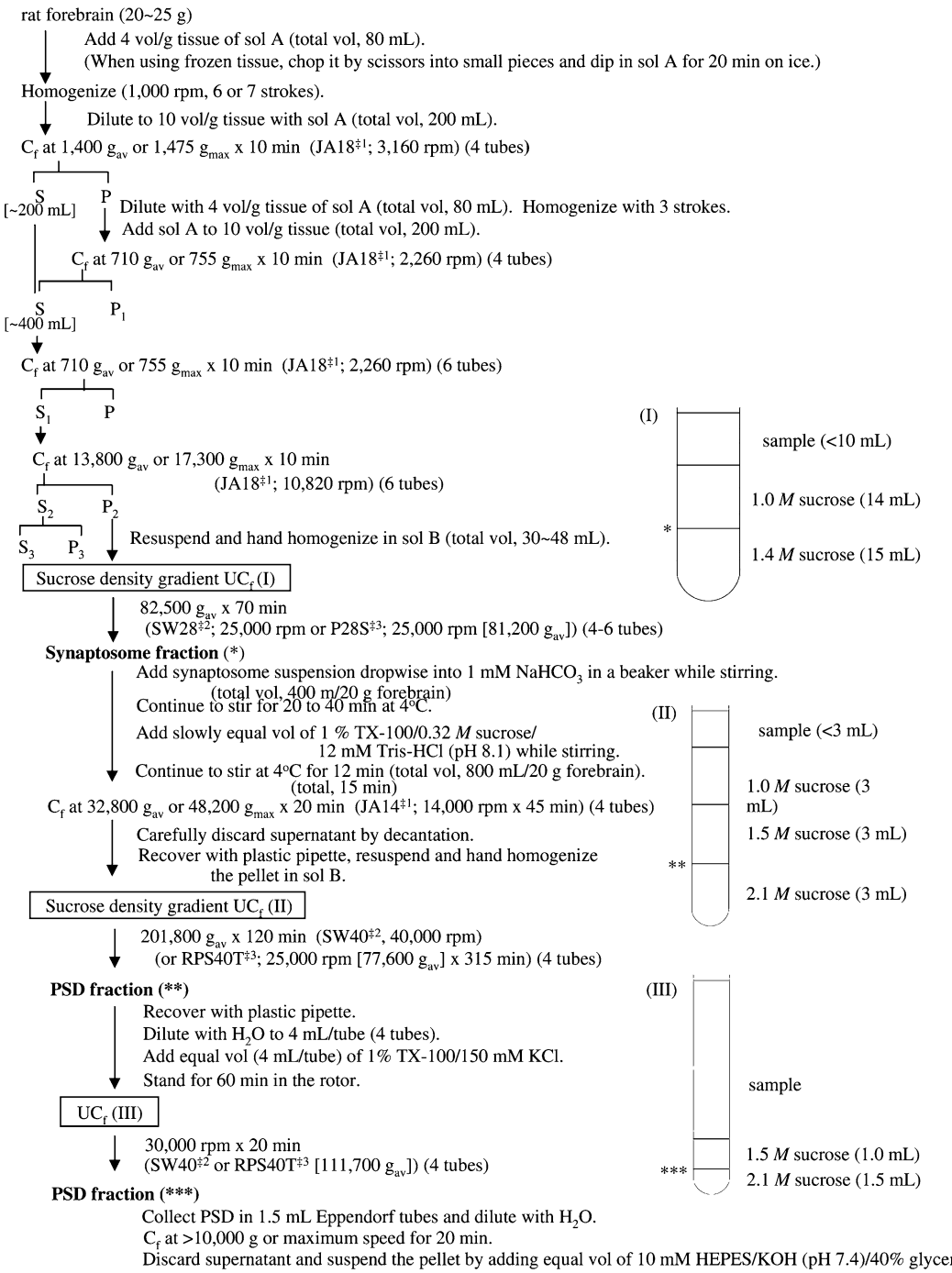

Fig. 2. Purification of synaptosome and PSD by subcellular fractionation using sucrose density gradient centrifugation (PSD purification by short procedure). Examples of centrifugation conditions (rotors and speed) are indicated. Rotors marked with ‡1, ‡2, and ‡3 are those for centrifuges of Avanti J-25 (Beckman), L5-50 (Beckman), and himac CP60E (Hitachi), respectively. Steps using ultracentrifuge are numbered with *roman characters* and surrounded with *parenthesis*. Volumes and number of centrifuge tubes used are those for purifying PSD from 20 to 25 *g* forebrains of rats. *, **, and *** Positions where synaptosome and PSD before and after TX-100/KCl treatment, respectively, are collected. *C_f* Centrifugation; *UC_f* ultracentrifugation; *av* average; *sol* solution; *vol* volume.

4. Centrifuge at $1{,}400 \times g_{av}$ or $1{,}475 \times g_{max}$ for 10 min (JA18; 3,160 rpm, 4 tubes). Save supernatant in a beaker placed on ice or at 4°C.
5. Dilute the pellet with solution A and make 80 mL suspension. Homogenize with three strokes as in step 3. Dilute with solution A to 200 mL.
6. Centrifuge at $710 \times g_{av}$ or $755 \times g_{max}$ for 10 min (JA18; 2,260 rpm, 4 tubes). Collect supernatant. Pellet is P_1.
7. Combine supernatants obtained in steps 4 and 6 and centrifuge at $710 \times g_{av}$ or $755 \times g_{max}$ for 10 min (JA18; 2,260 rpm, 6 tubes) (see Note 6).
8. Collect supernatant (S_1) and centrifuge at $13{,}800 \times g_{av}$ or $17{,}300 \times g_{max}$ for 10 min (JA18; 10,820 rpm, 6 tubes). Supernatant and pellet obtained in this step are S_2 and P_2, respectively.
9. Resuspend the P_2 and gently hand homogenize with a Dounce homogenizer or Teflon-glass homogenizer in solution B (48 mL). Layer the suspension on gradients composed of 1.0 and 1.4 M sucrose, and centrifuge at $82{,}500 \times g_{av}$ for 70 min (SW 28, 25,000 rpm, 4–6 tubes) (see Note 7).
10. Collect the bands in the interface between 1.0 and 1.4 M sucrose layer (synaptosome fraction) (see Note 8) into a small beaker with a plastic pipette of 4 mL capacity with thin stem (see Note 9). Measure the volume of the synaptosome suspension, if necessary. Protein concentration of synaptosome just after recovered from the interface band is approximately 5 mg protein/mL. Save aliquot of synaptosome suspension after dilution to make about 2.5 mg/mL (just an example), if necessary.
11. Pour 1 mM $NaHCO_3$ into a large beaker to make the final volume after mixing of the synaptosome suspension 400 mL/20 g starting forebrains (see Note 10). Place a stirrer bar into the beaker. Add synaptosome suspension dropwise into 1 mM $NaHCO_3$ in a beaker while stirring. Continue to stir for about 20–40 min at 4°C (see Note 11).
12. Add slowly 400 mL/20 g of starting forebrains of 1% TX-100/0.32 M sucrose/12 mM Tris–HCl (pH 8.1) (final 0.5% TX-100, 0.16 M sucrose, 6 mM Tris–HCl) with constant stirring. Take 1 min to add the TX-100 solution. Continue to stir at 4°C. Total time of treatment with TX-100 (from starting addition of TX-100 to starting next centrifugation) should be 15 min. Therefore, transfer the solution to the transparent centrifuge tubes (see Note 12) at about 12 min after starting addition of TX-100 (see Note 13).

13. Centrifuge at 32,800×g_{av} or 48,200×g_{max} for 20 min (JA14; 14,000 rpm×45 min, four 250 mL tubes). (Prepare sucrose layers required at step 14 by using a plastic Pasteur pipette with scale.) Discard upper large portion of supernatant by slow decantation. Discard supernatant using a plastic pipette of 6 mL with 9″-long so that about 2 mL supernatant remains in the tube. Be very careful not to disturb the pellet. Recover pellet with plastic pipette by peeling and aspirating the pellet as a mass. Collect the pellet as small a volume as possible. Resuspend the pellet in solution B. Gently hand homogenize the pellet with a Dounce homogenizer or loose Teflon-glass homogenizer.
14. Layer the solution on gradients composed of 1.0, 1.5, and 2.1 M sucrose, and centrifuge at 201,800×g_{av} for 120 min (SW40, 40,000 rpm or RPS40T; 25,000 rpm 315 min, 4 tubes) (see Notes 14 and 15). (In the latter case, next step begins next morning).
15. Recover PSD fraction (**) with a plastic pipette (4 mL with thin stem) into 15 mL plastic tube. Dilute with cold H_2O to 4 mL/1 tube and mix homogeneously. Add equal volume [4 mL/tube] of 1% TX-100/150 mM KCl (final 0.5% TX-100, 75 mM KCl) and mix homogeneously. Stand for 60 min (see Note 16).
16. Layer the solution on gradients composed of 1.5 and 2.1 M sucrose, and centrifuge at 30,000 rpm (SW40) for 20 min (RPS40T; 30,000 rpm, 2 tube) (see Note 14).
17. Retrieve PSD fraction (***) with a plastic pipette (4 mL with thin stem) into 1.5 mL Eppendorf microfuge tubes. Dilute with more than an equal volume of cold H_2O (see Note 17). Centrifuge at >10,000×g for 20 min. (Swing rotor is favorable.)
18. Discard supernatant and weigh the PSD material. Add equal amount of 10 mM HEPES/KOH (pH 7.4)/40% glycerol and mix homogeneously (see Note 18). Divide into small aliquots and keep them in plastic tubes at −80°C until use.

3.2. Preparation of SPM and PSD Fraction (Long Procedure)

Protocol (long procedure) for PSD purification using 20–25 g forebrain as starting material is described below. All the processes are carried out at 4°C. The procedure is outlined in Fig. 3. Steps 6–11 are the same as steps 12–18 of short procedure except for volumes of the samples and the number of centrifuge tubes used.

1. Prepare synaptosome fraction following the protocol described in Sect. 3.1.
2. Pour 0.5 mM HEPES/KOH (pH 7.4) into a large beaker to make the final volume after mixing the synaptosome suspension 400 mL. Place a stirrer bar into the beaker. Add synaptosome suspension (~50 mL) dropwise into the HEPES/KOH

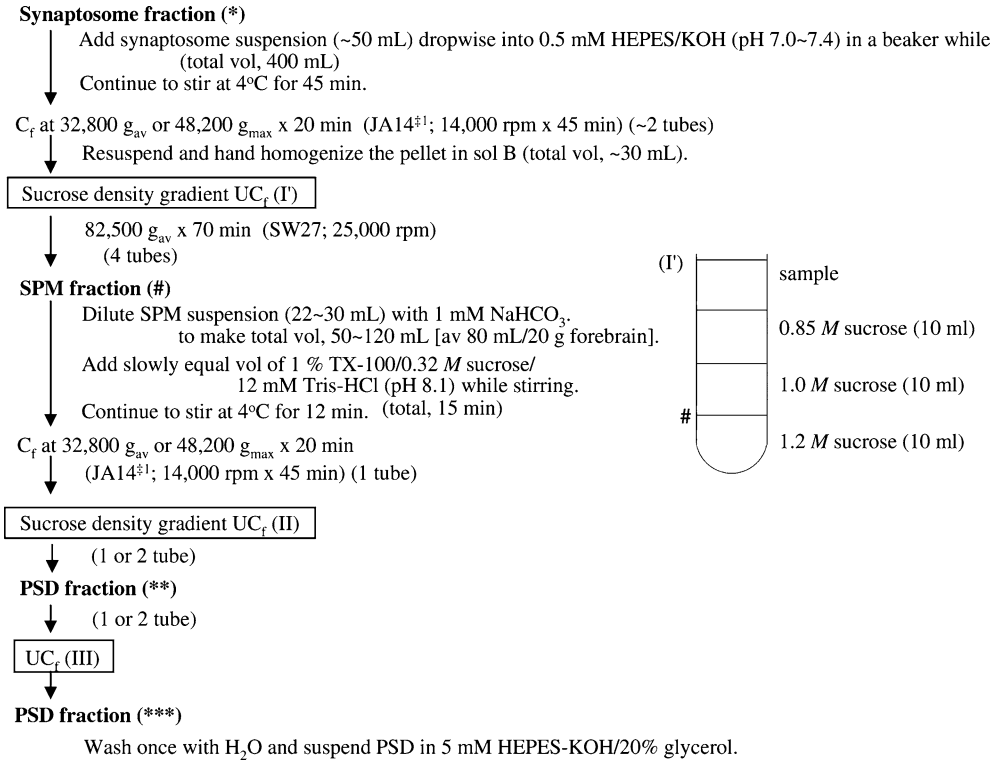

Fig. 3. Purification of SPM and PSD by subcellular fractionation using sucrose density gradient centrifugation (PSD purification by long procedure). Protocol to prepare synaptosome and steps after sucrose gradient ultracentrifugation (II) are the same as those shown in Fig. 2. Comments and abbreviations are the same as in Fig. 2.

buffer in a beaker while stirring. Continue to stir for about 45 min at 4°C.

3. Centrifuge at 32,800 × g_{av} or 48,200 × g_{max} for 20 min (JA14; 14,000 rpm × 45 min). Collect pellet and resuspend in solution B.
4. Layer the suspension on gradients composed of 0.85, 1.0, and 1.2 M sucrose, and centrifuge at 82,500 × g_{av} for 70 min (SW 28, 25,000 rpm). Use 4 tubes.
5. Collect materials in the 1.0–1.2 M sucrose interface (#). Volume of this suspension is usually 20–30 mL (*approximately 3–4 mg protein/mL). Save aliquot if necessary. Dilute SPM suspension with 1 mM $NaHCO_3$ (final volume, 50–120 mL [average 80 mL/20 g forebrain]) (see Note 19).
6. Treat the SPM suspension by adding equal volume of TX-100 as stated in Sect. 3.1, step 12.
7. Centrifuge at 32,800 × g_{av} or 48,200 × g_{max} for 20 min (JA14; 14,000 rpm × 45 min, 1 or 2 tubes). Collect pellet and resuspend in solution B as stated in Sect. 3.1, step 13.

8. Layer the solution on the top of the sucrose gradient and centrifuge at $201{,}800 \times g_{av} \times 120$ min (SW40, 40,000 rpm or RPS40T; 25,000 rpm 315 min, 1 tube with sample).
9. Recover PSD fraction (**) as stated in Sect. 3.1, step 15.
10. Centrifuge at 30,000 rpm (SW40) 20 min (RPS40T; 30,000 rpm, 1 tube) as stated in Sect. 3.1, step 16.
11. Retrieve PSD fraction (***), process, and save as stated in Sect. 3.1, steps 17 and 18.

3.3. Preparation of S_3 and P_3 Fraction

Centrifuge S_2 material at $100{,}000 \times g$ for 1 h. Supernatant and pellet obtained are S_3 and P_3 fractions, respectively.

4. Notes

1. Subtle changes in ionic strength and metal concentration may affect sedimentation of subcellular organelles and protein complexes. It is not necessary to use ultra pure water, such as nanopure or miliQ water, for this subfractionation, and the use of ultra pure water may sometimes result in low yield of synaptosome and PSDs. Some unidentified factor(s) affect on the sedimentation and/or are necessary for stabilization of PSD protein complex.
2. Commercial TX-100 has been found to contain impurity with oxidizing activity (38).
3. If brains are homogenized or rapidly frozen in liquid nitrogen within 30 s to 1 min after decapitation, content of CaMKII, both α and β, is very low in the PSD fraction (22). Neurofilament content is increased in such PSD fraction.
4. Defrozen and chopped brains should be kept in cooled solution A for at least 20 min to depolymerize actin cytoskeleton. Inadequate depolymerization causes unfavorable sedimentation.
5. Literatures (1, 2) recommend loose homogenizer (e.g., Teflon-glass homogenizer with a clearance of 0.25 mm or Dounce homogenizer with a loose-fitting pestle) to preserve morphological integrity of PSD. However, 0.25 mm clearance homogenizer does not appear to be a must.
6. It is very difficult to separate clearly the supernatant and pellet from total brain homogenate by centrifugation at $755 \times g_{max}$. Therefore, the first centrifugation was carried out at $1{,}475 \times g_{max}$. Supernatant obtained in the first centrifugation and the second centrifugation at $755 \times g_{max}$ is combined, centrifuged again at $755 \times g_{max}$, and thus, S_1 fraction was obtained. Removing

$755 \times g_{max}$ pellet is important to minimize contamination of nuclear materials to synaptic fractions (39). Methods omitting this step (e.g., one step purification of synaptosome) cannot avoid large amounts of contamination of nuclear proteins.

7. The first sucrose gradient was originally composed of 0.85, 1.0, and 1.2 M sucrose (2, 4), but can be replaced by those composed of 1.0 and 1.4 M sucrose with equivalent result (3).
8. Use fresh unfrozen brain as starting tissues for functional analysis of synaptosome. It is required to incubate synaptosome suspension in normotonic buffer to bring the terminals to a physiological steady state (40). Synaptosomes recovered from the sucrose gradient and are not incubated in normotonic buffer are shrunken due to high osmotic pressure.
9. Using disposable plastic pipettes to collect synaptosome, SPM, and PSD enriched bands after sucrose gradient centrifugation is convenient. See also 2.1.13. Be careful not to warm the plastic pipette (it means protein sample) by holding it with warm hand or fingers with wide contact areas for long time.
10. Fixed volume (400 mL for 20 g starting tissue) of the synaptosome suspension just before the TX-100 treatment is based on the protein concentration (2) estimated by the A_{260} and A_{280} using nomogram (distributed by California Corporation for Biochemical Research, LA) based on the equation by Warburg and Christian (41). Dilute synaptosome solution by 40-times for measurement of A_{260} and A_{280} (This also applies to SPM solution). The protein concentration estimated by Warburg–Christian method is about fourfold of the value obtained by Lowry method using BSA as standard. (The values were 4.3 ± 1.2 [$n = 7$] and 3.3 ± 1.2 [$n = 12$] folds for synaptosome and SPM fractions, respectively.) Therefore, protein concentration of the synaptosome suspension in 400 mL/20 g original forebrain is approximately 1 mg protein/mL (not 4 mg protein/mL as written in the original paper). Volume should be changed when starting from other parts of the brain, such as cerebellum.
11. This process is required before treatment with TX-100 and important to obtain good yield of PSD proteins, although the reason is unknown. Omitting this process may bring low yields of PSD.
12. Use transparent centrifuge tube to see the pellet clearly with the naked eye. The pellet obtained is very soft and easy to disturb. It is required to collect the pellet in a small volume to load on the top layer of the next sucrose density gradient.
13. The duration of TX-100 treatment affects the recovery of PSD.

14. PSD is extremely sticky to glass and cellulose nitrate tubes (2). Use polyallomer centrifuge tubes (2) to prevent adherence of PSD to the tubes.
15. Keep temperature to be around 4°C during ultracentrifugation. Raise of temperature loses some enzyme activity.
16. Inadequate treatment at this step leaves membrane materials to the final PSD preparation.
17. Repeat wash once or twice if complete removal of TX-100 is required.
18. Glycerol (20–50%) should be added to prevent artificial aggregation of the PSD proteins during storage at −80°C. Again, PSD material is extremely sticky to glass and cellulose nitrate, and tend to aggregate very easily, in particular, after freezing and defreezing.
19. The volume was determined by protein concentration read off with the nomogram, which was described in the original method for the PSD purification (2). The volume (average ± SD) was 80.3 ± 25.7 mL/20 g forebrain ($n = 13$) (see also Note 10).

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