8
Proteome Analysis of Synaptic Vesicles

Mads Grønborg, Matthew Holt, Florian Richter, Shigeo Takamori, Dietmar Riedel, Reinhard Jahn and Henning Urlaub

8.1
Introduction

8.1.1
The Synaptic Vesicle

Chemical synapses can be regarded as the elementary structures at which information transfer between neurons occurs. Neurons transmit information through the nervous system by releasing neurotransmitters from the presynaptic terminal. In a resting state, transmitters are stored in small organelles of uniform size and shape called synaptic vesicles. When an action potential (stimulus) arrives in the nerve terminal, the membrane depolarizes and voltage-gated Ca^{2+} channels open. The resulting Ca^{2+} influx triggers fusion (exocytosis) of synaptic vesicles at specialized release sites on the membrane, resulting in the release of neurotransmitter. This transmitter then diffuses to and binds appropriate receptors on the postsynaptic membrane, eliciting a response. At the same, the synaptic vesicle membrane is rapidly retrieved by endocytosis and reutilized for the reformation of synaptic vesicles.

Although a highly specialized process, the synaptic vesicle cycle shares basic properties with other intracellular membrane pathways: these include directed transport to the release site (along cytoskeletal tracks with the aid of a motor protein) (Soldati and Schliwa, 2006), recognition of the target membrane and docking (via rab GTPases) and fusion (executed by the SNARE proteins (Jahn and Scheller, 2006)) followed by several specialized steps including retrieval (interaction of synaptotagmin I with cytosolic proteins) and refilling with neurotransmitter (by specific transporters (Ryan, 2006)). Hence, not only can synaptic vesicles be considered as the basic minimal units of synaptic transmission but they can also be regarded as the basic minimal units of membrane transport, whose integral protein composition serves as the basis for all the functions that a trafficking vesicle

must perform (including the recruitment of protein complexes from the cytoplasm).

In fact, the composition of synaptic vesicles is, at present, better understood than any other trafficking organelle (for a detailed characterization of their composition see Takamori *et al.*, 2006) and several proteins first identified in synaptic vesicles have turned out to be members of conserved protein families which operate in all trafficking steps (Jahn *et al.*, 2003; Jahn and Scheller, 2006). This is because synaptic vesicles possess several unique properties that make them amenable to biochemical studies. Synaptic vesicles represent the most abundant class of trafficking organelles known (e.g. the human central nervous system (CNS) alone contains approximately 10^{17} vesicles), and large amounts of nervous tissue can easily be obtained in the laboratory. They are also the most uniform class of organelle in the nervous system, comprising a relatively homogeneous population with diameters between approximately 40 and 50 nm, allowing the application of standard size fractionation techniques. Finally, many of the major integral membrane proteins are already known and provide a further basis for manipulation. In summary, there is no other trafficking organelle of comparable simplicity and abundance that offers biochemical access to the membrane proteins involved in its function. This provides the opportunity of identifying novel trafficking proteins by mass spectrometry (MS), while also providing a platform for the optimization of existing MS protocols, using previously identified membrane proteins.

8.1.2
Purification of Synaptic Vesicles

8.1.2.1 General Remarks

The purity of the sample is the single most important factor for a successful proteome analysis of any isolated subcellular compartment and/or protein complex. Therefore, much attention is currently devoted to techniques of sample preparation. In particular, purification of synaptic vesicles requires specialized protocols to avoid co-purification of plasma membrane fractions and other trafficking vesicles of similar size, density or composition such as clathrin-coated vesicles (CCVs). Therefore, it is necessary to monitor both the enrichment and the purity of the material at various steps of the purification procedure by the detection of highly specific marker proteins (e.g. synaptobrevin-2, which is an integral synaptic vesicle membrane protein critical for fusion with the plasma membrane; see below).

Synaptic vesicles can be purified in sufficient amounts from rat brain. Purification protocols for synaptic vesicles can be divided into two major groups. The first group involves those that separate exclusively on physical parameters, such as shape and density (Hu *et al.*, 2002). Synaptic vesicles prepared using these methods involve the purification of isolated nerve terminals (synaptosomes)

followed by osmotic lysis to release synaptic vesicles (Huttner *et al.*, 1983). Purification of synaptic vesicles from synaptosomes has the advantage that small membrane fragments generated during homogenization are removed before vesicle extraction. Although these purifications are time-consuming and result in comparatively low yields, the vesicles obtained are of exceptionally high purity. The second group involves immunoaffinity purification using known vesicle proteins, for example, synaptophysin (Burger *et al.*, 1989). Using this method, synaptic vesicles can even be isolated directly from rat brain homogenate (without the prior isolation of synaptosomes). The crucial step in this purification method is the homogenization phase, during which relatively harsh conditions have to be used in order to release a sufficient amount of synaptic vesicles from the brain.

An important general point to consider during the sample purification prior to any proteome analysis is whether a particular purification method will select for a biochemically distinct pool of (sub)compartments and/or protein complexes, producing a bias in the final analysis. In neurons, the major sources of variation will arise from (i) differential protein expression within a population of neurons and (ii) the activity status of the cell. This variation may be found in the integral membrane proteins, or in cytosolic factors recruited to the synaptic vesicle at various stages of its life cycle, and is discussed in the following paragraphs in more detail:

(i) Neurons in the brain show differential expression profiles across a range of proteins. Nowhere is this more apparent than in the case of the neurotransmitter transporter proteins carried on the synaptic vesicle. To date, seven transmitter-specific transporters have been identified, namely VMATs 1 and 2 (monoamines), VAChT (acetylcholine), VGLUTs 1, 2, and 3 (l-glutamate), and VGAT (GABA and glycine) (Ahnert-Hilger *et al.*, 2003), and these show non-overlapping but complementary expression profiles in the adult brain. Interestingly, glutamatergic and GABAergic neurons predominate in the CNS and this may reflect why these transporters have been previously identified by mass spectrometry of synaptic vesicles purified from whole brain, while those expressed at low(er) levels (VMAT and VGAT) have remained elusive (Takamori *et al.*, 2006). In addition, other key vesicular proteins, which appear to be present on every vesicle, such as synaptobrevin, synaptotagmin, and synaptophysin, also occur in several isoforms displaying differential localization in the CNS. At present it is impossible to say whether the copy number of these individual vesicle proteins may vary between different neurons, or even between individual vesicles. In addition to the different proteomic expression pattern, some synaptic terminals release neuropeptides from large-dense core vesicles (LDVs), alongside the classical neurotransmitters released by synaptic vesicles (see Chapter 10). Fusion and recycling of these LDVs utilizes many of

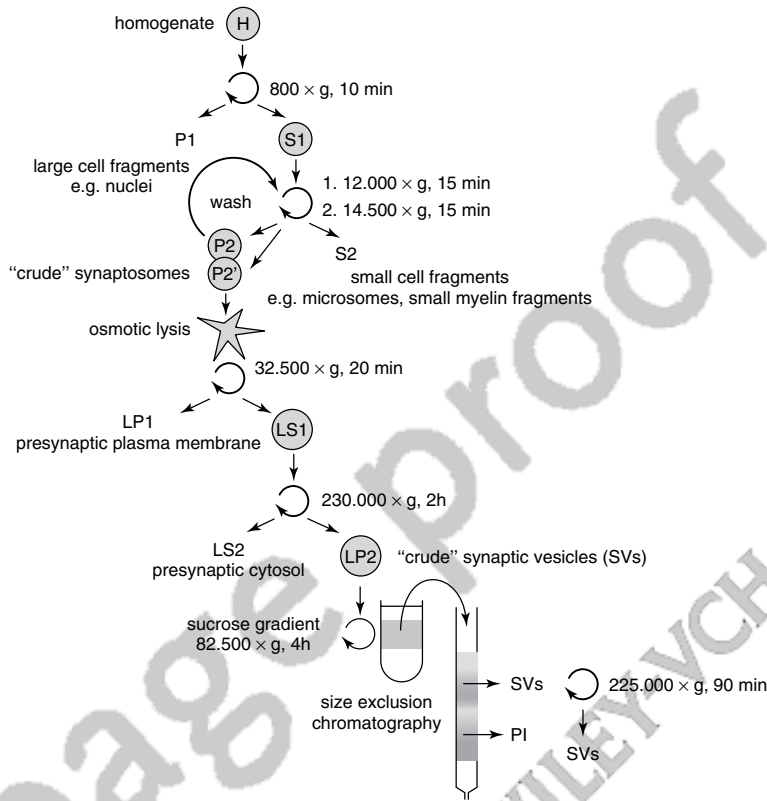the same proteins as synaptic vesicles so that differences between these two species are hardly detectable at the proteomic level.

(ii) Synaptic vesicle composition may also be affected by the activity status of the neuron. For instance, some synaptic vesicles seem to be preferentially recycled and released—although it is unclear whether this is related to the molecular composition of the vesicle or a posttranslational modification such as phosphorylation (von Schwarzenfeld, 1979). Recent evidence also suggests that the protein stoichiometry of a vesicle may not be fixed and that synaptic vesicles may exchange proteins with the plasma membrane upon fusion, influencing its composition (Burre *et al.*, 2006a; Wienisch and Klingauf, 2006). Furthermore, recycling of synaptic vesicles in neurons occurs in a clathrin-dependent manner, explaining why the vesicle preparation contains many clathrin-related proteins, in particular the components of the AP-2 adapter complex, responsible for coat recruitment to the vesicle, and why partially decoated vesicles derived from these recycling synaptic vesicles have been shown to cofractionate with synaptic vesicles. It is still unclear whether any, or all, of these recycling synaptic vesicles utilize an early endosomal intermediate that would allow a further opportunity to modify the protein composition of the vesicle. Furthermore, it should also be expected, that there will be some variability in the recovery of soluble proteins that are recruited from the cytosol to the vesicle at various points in the trafficking life cycle, thus resulting in hetereogeneous vesicle populations. Indeed, negatively stained electron micrographs of synaptic vesicles purified from synaptosomes (although perfectly aligning in size and overall shape) show clearly distinct surface staining suggesting different proteins and/or quantities of proteins on the corresponding synaptic vesicles (see Section 8.1.2.5 and Figure 8.3). This individual variation among synaptic vesicles perhaps explains why a differential distribution of synaptic vesicles derived from synaptosomes can be seen in sucrose-density centrifugation (Maycox *et al.*, 1992).

8.1.2.2 Purification of Synaptic Vesicles from Synaptosomes

The standard purification procedure for purification of synaptic vesicles from synaptosomes is based on a modified version of a classical fractionation protocol originally developed by Whittaker and co-workers (Nagy *et al.*, 1976; Huttner *et al.*, 1983). It can be divided into six major steps: (i) homogenization of whole rat brain, (ii) differential centrifugation of the homogenate to obtain a crude synaptosomal pellet, (iii) hypo-osmotic lysis of the syaptosomes to release synaptic vesicles, (iv) differential centrifugation of the crude synaptosomal lysate to obtain a crude synaptic vesicle fraction, (v) purification of the synaptic vesicles by continuous sucrose-density gradient centrifugation and (vi) size-exclusion chromatography on controlled-pore glass. The purification procedure is outlined in Figure 8.1 and in Section 8.2.1.

Figure 8.1 Purification of synaptic vesicles from rat brain. Diagram for the purification of synaptic vesicles according to (Huttner *et al.*, 1983). After non-equilibrium sucrose density gradient centrifugation, the zone between approximately 0.04 and 0.4 M sucrose (gray) was collected and separated by chromatography on controlled-pore glass beads (CPG). See text and Section 8.4.1 for further details.

8.1.2.3 Purification of Synaptic Vesicles by Immunoisolation

Analytical amounts of synaptic vesicles may also be obtained by using immunoaffinity purification, which takes advantage of the high binding affinity and specificity of an antibody for its antigen to allow large quantities of synaptic vesicles to be isolated rapidly. Immunoisolation has been performed both from crude brain homogenate and from isolated synaptosomes. Obviously, using a crude brain homogenate largely avoids the damaging steps associated with purification from synaptosomes (such as osmotic stress), although extra care has to be taken to maintain sample purity. In both cases, the time-consuming size-exclusion chromatography employed in other methods is avoided (Huttner *et al.*, 1983). Moreover, immunoisolation can be used to isolate a distinct synaptic vesicle population, for instance those containing a specific neurotransmitter transporter (see above).

Immunoisolation of synaptic vesicles has recently been carried out using magnetic Dynabeads (Morciano *et al.*, 2005) and the reader is referred to this resource for more detailed protocols. The general requirements for any bead-based system used for immunoisolation however can be summarized as follows: the beads must be small (1–2 μm average diameter) to maximize surface-to-volume ratio; they should be non-porous with a hydrophilic surface and a hydrophobic core to restrict the binding of antibody to the bead surface and to allow easy washing; and they should have almost no unspecific binding when assayed with ^{35}S-labeled proteins from cell-homogenates or ^{3}H-labeled glutamate, GABA or acetylcholine. The use of protein A/G sepharose beads coupled with antibodies is not recommended as these beads are porous, resulting in a large amount of ''internal'' antibody coupling which is not accessible for binding owing to the size of the synaptic vesicles.

In general, any antibody—either affinity-purified polyclonal or mono-clonal—can be successfully used for immunoisolation against proteins on synaptic vesicles. Specific polyclonal antibodies have already been used for the successful isolation of distinct synaptic vesicle populations (Takamori *et al.*, 2000a, 2000b). Antibodies also have the advantage that synaptic vesicles can be eluted under less harsh condition from the beads, that is, by an excess of the antigenic peptide. However, because finding the best antibody/coupling conditions for immunoaffinity purification is an empirical task, and potentially time- and resource-consuming, commercial sources of antibody are rarely an economical option, and, at the least, researchers should consider producing their own polyclonal antibodies.

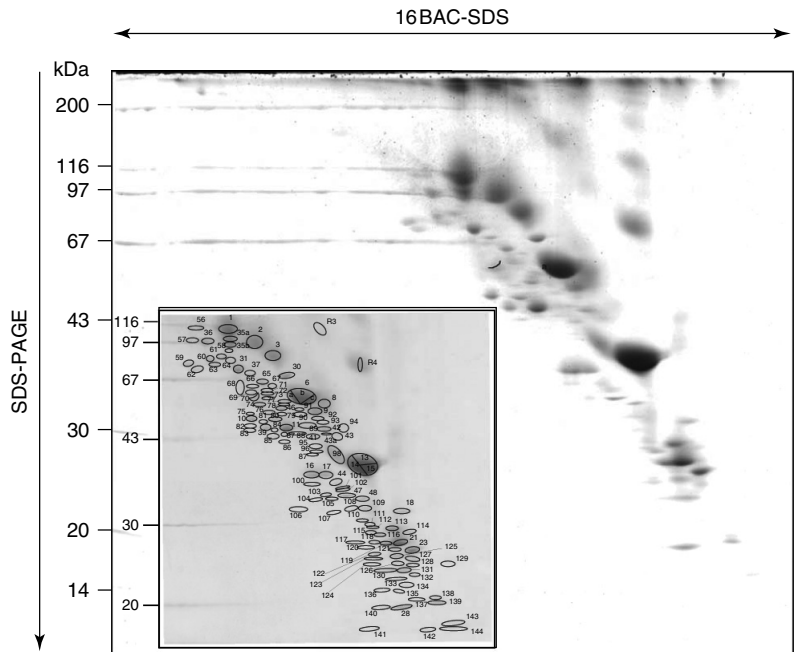### 8.1.2.4 Reducing Contaminating Peripheral Proteins

As mentioned above, the purity of a complex biological sample is an essential prerequisite for a comprehensive analysis of its proteome. In particular, the resolving power and dynamic range of liquid chromatography coupled to mass spectrometry (LC-MS) are drastically decreased by highly abundant protein components that either are naturally present or are contaminants because of their high abundance in the cell. Either such components should be specifically depleted (in case of a highly abundant intrinsic protein component) or the purification protocols should include an additional step to remove contaminating proteins that interact non-specifically with the biological sample.

Synaptic vesicles obtained using either isolation technique contain various amounts of soluble proteins with affinity for membranes such as glyceraldehydes phosphate dehydrogenase, aldolase, actin, and tubulin. To remove these peripheral proteins from the synaptic vesicles, the purified vesicle fraction can be washed with sodium carbonate (see Figure 8.2 and Section 8.2.2).
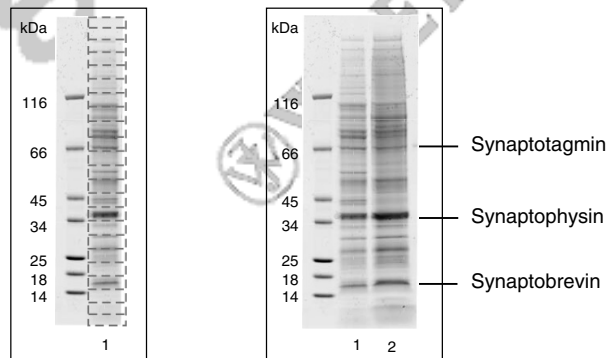
8.1.2.5 Assaying the Purity of the Synaptic Vesicle Preparation

Monitoring the purity of the sample during the various steps of purification is another important issue throughout the preparation. The most practical

(a)

(b)

Figure 8.2 Fractionation of synaptic vesicles by 16-BAC/SDS-PAGE and/or 1D SDS-PAGE. Purified synaptic vesicles can be resolved by either 2D (16-BAC/SDS-PAGE) or 1D (SDS-PAGE) gel electrophoresis. (a) In 16-BAC/SDS-PAGE gel electrophoresis the synaptic vesicle proteins are first fractionated by a cationic detergent benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) and secondly by SDS-PAGE. Protein spots of interest are labeled, excised, and digested by trypsin prior MS analysis. (b) Alternatively, synaptic vesicle proteins can be resolved by 1D SDS-PAGE. To minimize the number of (contaminating) peripheral proteins, synaptic vesicles can be treated with sodium carbonate prior SDS-PAGE (right panel, lane 1) or left untreated (right panel, lane 2). To maximize the total number of identified proteins in the MS analysis the gel can be divided into 20–25 lanes and subsequently digested by trypsin (left panel).

means of assessing both the degree of enrichment and the purity of the synaptic vesicle preparation is immunoblotting, for which excellent antibodies are available (e.g. Synaptic Systems, Göttingen, Germany, www.sysy.com). During purification, proteins associated with the postsynaptic density (e.g. the NMDA receptor subunit 1 and PSD-95) should be lost, while the integral membrane proteins of synaptic vesicles (e.g. synaptophysin) should be enriched by about 20- to 25-fold in comparison to the homogenate (Jahn *et al.*, 1985). Contamination by other subcellular compartments can also be monitored by assaying for marker enzymes of the plasma membrane, mitochondria or endoplasmic reticulum (Hell *et al.*, 1988). Alternatively, although less sensitive and specific, synaptic vesicles have a well-documented protein profile, as can be observed by sodium dodecyl sulfate polyacrylamide-based gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue (CBB). Protein bands of the major membrane proteins of synaptobrevin (18 kDa), synaptophysin (38 kDa), and synaptotagmin (65 kDa) are clearly visible (see Figure 8.2b).

Besides the detection of marker proteins, the morphology of synaptic vesicles also adds very valuable information about their purity which can be checked by electron microscopy with negative staining (Takamori *et al.*, 2006). In the final purification step, synaptic vesicles are identified by their small, uniform appearance, with diameters in the range of 40–50 nm (Figure 8.3a). Final confirmation can be obtained by immunogold labeling for the membrane protein synaptophysin (Figure 8.3b). In a highly pure preparation more than 95% of all vesicles must be immunogold stained.

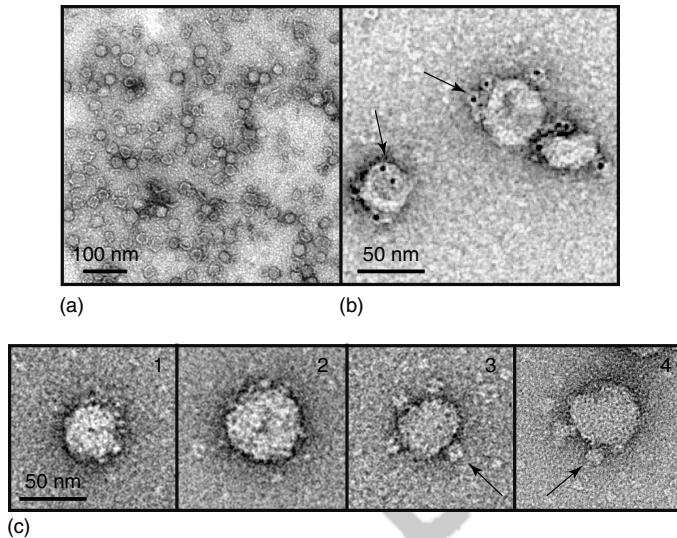### 8.1.3
Proteomic Analysis of Synaptic Vesicles

To date, the identification of synaptic vesicle proteins has been limited to biochemical and immunological techniques. The introduction of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and matrix-assisted laser desorption/ionization coupled to tandem time-of-flight mass spectrometry (MALDI-TOF/TOF) either on-line or off-line has provided a very powerful tool for identification of novel synaptic vesicle proteins (Coughenour *et al.*, 2004; Burre *et al.*, 2006a, 2006b; Takamori *et al.*, 2006). Even though synaptic vesicles have been intensively studied as a trafficking organelle, only limited information is available about the overall protein complexity of the synaptic vesicle.

8.1.3.1 **Fractionation by 16-BAC/SDS-PAGE or 1D SDS-PAGE**
Some issues have to be taken into consideration when a proteomic analysis of synaptic vesicles is to be conducted. First, it has been indicated from previous studies that the synaptic vesicles proteome is rather complex (Takamori *et al.*, 2006). It is therefore recommended that the sample is fractioned prior to MS analysis. Second, synaptic vesicles proteins are mainly membrane-bound proteins or integral membrane proteins and have to be separated by 16-

(a)   (b)

(c)

Figure 8.3 Electron microscopy of synaptic vesicles. The purity and morphology of synaptic vesicles can be checked by electron microscopy with negative staining. Highly purified synaptic vesicles can be obtained from rat brain homogenate by several differential centrifugation steps combined size-exclusion chromatography.
(a) Negatively stained electron micrographs of purified synaptic vesicles.
(b) Immunogold labeling of synaptic vesicles for the known membrane protein synaptophysin (arrows). (c) Purified synaptic vesicles show distinct surface staining indicating a certain degree of heterogeneity. Panels 1 and 2 show synaptic vesicles with a rough surface while panels 3 and 4 show more smooth synaptic vesicles with ATPases present (arrows). Negative staining: A solution containing synaptic vesicles was applied to glow-discharged carbon-coated grids and stained with 1% uranyl acetate. Images were taken in a Philips CM120 electron microscope (Philips Inc.) at a defocus of 2.3 μm using a TemCam 224A slow scan CCD camera (TVIPS, Gauting, Germany). Immunogold-labeling: Purified synaptic vesicles were adsorbed to glow-discharged formwar-coated grids and fixed with a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M potassium–sodium phosphate buffer, pH 7.4. Thereafter unspecific binding sides were blocked with 0.02 M glycin, and 0.5% BSA in phosphate buffer. Labeling with 1 : 500 diluted anti-synaptophysin antibody and 10 nm protein A gold conjugates diluted at 1 : 1000 in 1% BSA in phosphate buffer were performed. The samples were post-fixed for 10 min with 2% glutaraldehyde in phosphate buffer, washed with H_2O, rinsed with three drops of 1% uranyl acetate, and immediately dried with filter paper.

BAC/SDS-PAGE gel electrophoresis (Macfarlane, 1989; Hartinger et al., 1996). This technique has previously been demonstrated to work very well for the fractionation of membrane proteins from synaptic vesicles (Morciano et al., 2005; Burre et al., 2006a, 2006b; Takamori et al., 2006) (Figure 8.2a and Section 8.4.3).

However, when analyzing membrane proteins, one cannot rule out the possibility that some proteins might escape detection when only one fractionation approach is applied so that the proteome is "undersampled". Therefore, it is generally advisable to apply different fractionation approaches in order to

increase the total number of proteins identified in the MS analysis (Burre *et al.*, 2006b; Reinders *et al.*, 2006; Takamori *et al.*, 2006). Accordingly, the fractionation of synaptic vesicle proteins can also be performed by traditional one-dimensional (1D) SDS-PAGE combined with nanoLC-MS/MS or off-line MALDI-MS/MS (Laemmli, 1970; Takamori *et al.*, 2006). After the electrophoresis, gel lanes can be cut into pieces of equal size instead of cutting single protein bands (Figure 8.2b).

Since gel electrophoresis can be considered the first step in the separation of complex protein samples and because proteins can be differentially stained (or not at all) it is advisable to excise not only bands or single protein spots but rather entire lanes on 1D SDS-PAGEs (Figure 8.2b, left panel). This strategy ensures that proteins that are not detectable by a staining procedure still have a chance of being identified in the subsequent MS analysis. In addition, the total number of proteins identified is usually drastically improved when gel lanes rather than gel spots are analyzed.

Depending on the type and size of the gel, approximately 50–200 µg of purified synaptic vesicles can be loaded as starting material for separation by 1D SDS-PAGE and 16-BAC/SDS-PAGE. The amount of starting material can of course be modified as needed. Figure 8.2 shows the separation of the proteins derived from synaptic vesicles by 16-BAC/SDS-PAGE and 1D SDS-PAGE, respectively.

8.2
Protocols

8.2.1
Purification of Synaptic Vesicles from Synaptosomes

The following method is based on the standard protocol that has been used successfully for many years and is considered to be the "gold-standard" in synaptic vesicle preparation (Hell and Jahn, 1994). Slight variations in centrifugal force and spin time have also been successfully used (Takamori *et al.*, 2006). Note that this method preferentially purifies synaptic vesicles that remain membrane associated after synaptosomal lysis. At present it is unclear whether these synaptic vesicles form a functionally distinct subset in the synaptic terminal.

Requirements

Solutions: Homogenization buffer (320 mM sucrose, 4 mM HEPES (pH 7.40 NaOH)); 1 M HEPES (pH 7.40 NaOH); 40 mM sucrose; 50 mM sucrose; 800 mM sucrose; chromatography buffer (300 mM glycine, 5 mM HEPES (pH 7.40, use KOH)), degassed and filtered. Protease inhibitors: 1 mg mL^{-1} pepstatin A in DMSO; 200 mM PMSF in 100% EtOH. Both should be stored at room temperature until use.

Instrumentation: Loose-fitting motor-driven glass–Teflon homogenizer, cooled centrifuge (Sorvall RC5 or comparable with SS34 rotor), ultracentrifuge with fixed-angle and swing-out rotors (Beckman with 50.2Ti and SW28 rotors with corresponding tubes), equipment for column chromatography (peristaltic pump, UV monitor, fraction collector), Corning filter system (0.22 μm polyethersulfone membrane), gradient mixer for forming continuous sucrose gradients.

After collecting the brains, all steps are carried out on ice or at 4 °C.

1. Decapitate 20 rats (180–200 g) and remove the brains into ice-cold homogenization buffer. Wash the brains once with homgenization buffer to remove residual blood. Homogenize the brains in 240 mL homgenization buffer (supplemented with 240 μL PMSF and 240 μL pepstatin A).

2. Centrifuge the homogenate for 10 min at $850 \times g$. Discard the resulting pellet P1 (containing large cell fragments and nuclei) and collect the supernatant (S1).

3. Centrifuge the S1 for 15 min at $12\,000 \times g$. Remove the resulting supernatant (S2), which consists of small cell fragments such as microsomes, small myelin fragments and soluble proteins. The resulting pellet, P2, should be resuspended in homogenization buffer. At this stage care should be taken to avoid the brown bottom part of the pellet that consists of mitochodria. Centrifuge the resuspended pellet at $14\,500 \times g$ for 15 min.

4. The pellet obtained, P2′, represents a crude synaptosomal fraction. To release synaptic vesicles from the synaptosomes, the P2′ is resuspended (again avoiding the brown mitochondrial pellet) to yield a final volume of 24 mL. Transfer 12 mL into a glass–Teflon homogenizer, add 108 mL ice-cold distilled water, and perform three strokes at approximately 2000 rpm. Immediately add 600 μL 1 M HEPES-NaOH and 120 μL PMSF. Repeat for the remaining 12 mL of tissue. Combine both fractions and add 240 μL pepstatin A.

5. Centrifuge the suspension for 20 min at $32\,500 \times g$ to yield a lysate pellet, consisting mainly of presynaptic plama membrane (LP1) and the lysate supernatant LS1. The LS1 should be removed immediately and without disturbing the LP1, which would otherwise significantly reduce the final purity of the synaptic vesicles.

6. The LS1 is centrifuged for 2 h at $230\,000 \times g$ in a 50.2 Ti rotor. The resulting supernatant (presynaptic cytosol; LS2) is discarded and the pellet (LP2; crude synaptic vesicles) is resuspended in 6 mL of 40 mM sucrose, using a small, tight-fitting glass–Teflon homogenizer running at 900 rpm, followed by drawing it through a 20-gauge needle and subsequently out through a 27-gauge needle.

7. During the centrifuge run prepare two linear sucrose gradients from 18 mL of 800 mM sucrose and 18 mL 50 mM sucrose. Layer 3 mL of LP2 onto the top of each gradient and centrifuge for 4 h at $82\,500 \times g$ in

a SW28 rotor. After centrifugation, a turbid (opaque-white) zone is visible in the middle of the gradient (corresponding to 200–400 mM sucrose). This fraction (25–30 mL) is collected with the aid of a peristaltic pump and glass pipette. At this stage synaptic vesicles are enriched approximately 10-fold over the homogenate.

8. The sample is carefully layered onto the top of a controlled-pore glass bead (CPG-3000, see comment below) column pre-equilibrated overnight with glycine buffer. The sample is overlaid and eluted with glycine buffer at a flow rate of 40 mL h^{-1}, with fractions collected every 15 min. The protein content of the eluate is monitored by absorption at 280 nm. Two peaks are obtained from the column. The first contains small amounts of plasma membrane and/or microsomes. The second peak contains the highly purified synaptic vesicles. Fractions comprising the second peak are pooled and centrifuged at 225 000 × g for 90 min. The pellet is resuspended in an appropriate buffer for example, PBS, as in step 7. The sample can then be aliquoted and snap-frozen, before storing at −80 °C. Vesicles handled show no obvious deterioration in quality over many months.

The process of size-exclusion chromatography is omitted in many procedures, although this final step has been shown to be important in separating synaptic vesicles from residual amounts of contamination by larger membrane fragments and soluble protein. Unfortunately the controlled-pore glass beads used by our lab are no longer commercially available. An alternative is Sephacryl S-1000 (GE Healthcare) although these columns have a relatively low capacity, do not tolerate overloading and require some experience in their use. In addition, Sephacryl columns have low flow rates, run at low pressure and have a tendency to adsorb proteins and membrane particles, in particular during the first few separation runs in the life of the column. Alternative methods of synaptic vesicle preparation following synaptosomal lysis have also been reported, including the use of flotation on an Optiprep step gradient (Hu *et al.*, 2002).

8.2.2
Reducing Contaminating Peripheral Proteins

Requirements

Solutions: 0.1 M Na_2CO_3 (pH 11), PBS.

Equipment: Ultracentrifuge with fixed-angle and swing-out rotors (Beckman with 50.2Ti rotor).

1. After purification, the synaptic vesicles are incubated with 0.1 M Na_2CO_3 (pH 11) on ice for 15 min.
2. The synaptic vesicles are subsequently collected by ultracentrifugation for 20 min at 50 000 rpm.

3. Discard the supernatant after the ultracentrifugation and resuspend the synaptic vesicles in an appropriate buffer (e.g. PBS or SDS/LDS sample buffer).

8.2.3
Fractionation of Synaptic Vesicles by 16-BAC/SDS-PAGE Gel Electrophoresis

Requirements

Chemicals: Benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC), urea, glycerol, β-mercaptoethanol, glycine, phosphoric acid, Tris, acrylamide, bisacryladmide, TEMED, SDS, $FeSO_4$, ascorbic acid, H_2O_2, pyronine Y, EDTA, glycerol, DTT.

Solutions: First dimension:
1. $2 \times$ Sample buffer: 1 g 16-BAC, 4.5 g urea, 1 mL glycerol, 0.5 mL of 1.5 M DTT, 100 µL 5% pyronine Y (w/v) solution (in water), ddH$_2$O to 10 mL. Solubilize detergent and urea in glycerol and 4 mL ddH$_2$O by heating in a microwave, then add DDT and pyronine Y. The solution is finally brought to 10 mL with H$_2$O.
2. Running buffer (pH 3): 2.5 mM 16-BAC, 150 mM glycine and 50 mM phosphoric acid (a $10\times$ solution can be prepared if needed).
3. Lower gel (7.5%): 10 mL H$_2$O, 10 mL 300 mM potassium phosphate buffer (pH 2.1), 10 mL AMBA (30% acrylamide, 0.8% bisacrylamide), 1.4 mL 1.7% bisacrylamide, 7.2 g urea, 400 µL 10% 16-BAC (heat to 65 °C to solubilize), 64 µL 0.14% FeSO$_4$ (fresh), 2 mL 80 mM ascorbic acid (fresh), start with 1.6 mL H$_2$O$_2$ (1:1200, diluted from 30% stock solution, make fresh).
4. Upper gel (4%): 2.5 mL 0.5 M phosphate buffer (pH 4.1), 3.0 mL H$_2$O, 1.33 mL AMBA, 1.38 mL 1.7% bisacrylamide, 1 g urea, 70 µL 250 mM 16-BAC (heat to 65 °C to solubilize), 8.5 µL 5 mM FeSO$_4$ (fresh), 520 µL 80 mM ascorbic acid (fresh), start with 500 µL H$_2$O$_2$ (1:750, diluted from 30% stock solution, make fresh).
5. 1:750 H$_2$O$_2$ (fresh).
6. Fixing solution: isopropanol:acetic acid:water (3.5:1:5.5).
7. Staining solution: 0.15% (w/v) Coomassie Brilliant Blue R, 25% (v/v) propanol, 10% (v/v) acetic acid.
8. Re-equilibration solutions: (1) 100 mM Tris-Cl, pH 6.8; (2) 15% (v/v) EtOH, 100 mM Tris-Cl, pH 6.8.

Second dimension:
1. $10 \times$ Running buffer: 150 g Tris, 720 g glycine, 50 g SDS, add ddH$_2$O to 5 L.

2. Upper Tris buffer: 0.5 M Tris, 0.4% (w/v) SDS, pH 6.8.
3. Lower Tris buffer: 1.5 M Tris, 0.4% SDS (w/v), pH 8.8.
4. AMBA (acrylamide stock): 300 g acrylamide, 8 g bisacrylamide, fill up to 1 L with ddH_2O.
5. 3× Sample buffer: 45 g SDS, 124.8 mL upper Tris (pH 6.8), 15.0 mL 0.1 M EDTA, 150 g sucrose or 150 mL glycerol, ddH_2O to 450 mL. Remember to add β-mercaptoethanol (10% final concentration).
6. TEMED and 10% APS.
7. Coomassie stain: 0.15% Coomassie Brilliant Blue R, 25% isopropanol, 10% acetic acid.
8. Destain: 25% isopropanol, 10% acetic acid.

16-BAC discontinuous gel (first dimension):
1. The glass plates used for the gel apparatus are first washed with ethanol and assembled according to the manufactures instructions. The 16-BAC gel is prepared as a slab gel using a stacking gel casted on top of a resolving gel.
2. The 7.5% resolving gel (40 mL final volume) is made accordingly to the above method (lower gel). The polymerization is initiated by adding the H_2O_2 and should be completed in approximately 30 min at room temperature. For optimal results the gel should be allowed to polymerize over night.
3. The 4% stacking gel prepared accordingly to the above method (upper gel) and is poured on top on the resolving gel. The polymerization is initiated by adding H_2O_2 and should be completed in approx 20–30 min. Appropriate gel combs are inserted into the stacking gel before it polymerizes.

First-dimension electrophoresis:
1. After the comb has been removed from the gel, the wells are washed with 1× running buffer.
2. The samples are diluted in the prepared 2× sample buffer (to 1×) and heated for 5 min at 65 °C. Do not boil the samples. The samples are then loaded on the gel.
3. Remember that the electrophoresis is carried out such that the proteins move towards the cathode (opposite of SDS gels!).
4. For mini-gels (0.75 mm) run the gel at 10 mA/gel until the dye front enters the separation gel, then 20 mA/gel until the dye front has completely run out of the gel (approx 1.5 h). For larger gels (14 × 16 cm) the current is initially 25 mA/gel and subsequent at 80 mA/gel (approx 7.5 h).

Note: Never store samples in sample buffer. Due to unfolding of the proteins in the presence of urea they are more susceptible to proteases and can degrade overnight. If dealing with membrane proteins which are hard to dissolve one may tip-sonicate samples before adding sample buffer and centrifuge them before loading.

Staining, destaining, and re-equilibration:

1. After first-dimension electrophoresis the gel is fixed in fixing solution for at least 1 h. Several changes should be made during fixation.
2. Stain the gel with Coomassie Blue (15–30 min).
3. Destain the gel with fixing solution (several changes).
4. For re-equilibration, the gel is first incubated for 3×10 min with re-equilibration solution 1 and subsequently for 5–10 min with re-equilibration solution 2.
5. The strip of interest is carefully excised by cutting with the edge of a glass plate (0.3 and 0.6 cm for mini-gels and larger gels, respectively) and stored at $4\,^{\circ}$C.

Second-dimension electrophoresis:

1. The SDS-PAGE is done under standard conditions using the lower Tris buffer for the separations gel and the upper Tris buffer for the stacking gel. Either a gradient (9–15%) or a 10% gel can be used depending on the complexity of the sample to be analyzed. Remember to prepare the stacking gel with a large well that can accommodate the strip generated from the first dimension. There should also be an extra lane for molecular mass markers.
2. After polymerization, the gel is placed in the appropriate gel apparatus and the well is filled with $1\times$ running buffer.
3. Place the strip at the bottom of the well (stacking gel) with the aid of a spatula.
4. Fill the well with 100–300 µL $3\times$ sample buffer and incubate for 5 min.
5. The gel is first run at 5 mA for mini-gels and 12 mA for larger gels (16×16 cm, 1 mm thick) until the dye front enters the separation gel. Separation is carried out at 20 mA for mini-gels and 80 mA for larger gels. The electrophoresis will run for approx 1 h for mini-gels and 3–3.5 h for larger gels (10%).
6. The gel apparatus is dismantled carefully and the gel stained with colloidal Coomassie Blue stain.

8.2.4
Fractionation by 1D SDS-PAGE

This protocol is based on the use of pre-cast gels which can be bought from several vendors. This paragraph is based on the NuPAGE system (Invitrogen).

Requirements

Equipment: Novex Bis-Tris pre-cast gels (10% or 4–12% gradient gels), gel chamber, running buffer (MES or MOPS), molecular weight markers, LDS sample buffer, reducing agent and power supply. The Novex Bis-Tris gels

come in thickness of 1 mm (approx 30 µL loading capacity) and 1.5 mm (approx 40 µL loading capacity).

Sample preparation:
1. The sample is dissolved in LDS sample buffer (1×) containing reducing agent.
2. Heat (do not boil) the samples for 5–10 min at 70 °C.
3. Prepare 1 L of running buffer (MES for low molecular weight proteins or MOPS for mid-size molecular weight proteins).
4. After heating, the sample is loaded in the gel (10% or 4–12% gradient gel).
5. 200 mL 1× running buffer containing 500 µL reducing agent is placed in the inner gel chamber.
6. 600 mL 1× running buffer is placed in the outer gel chamber.

Gel electrophoresis:

Buffer type	Voltage	Expected current	Run time
Novex Bis-Tris gels with MES running buffer	200 V constant	Start: 110–125 mA/Gel	35 min
		End: 70–80 mA/Gel	
Novex Bis-Tris gels with MOPS running buffer	200 V constant	Start: 100–115 mA/Gel	50 min
		End: 60–70 mA/Gel	

After end gel electrophoresis the gel is stained with colloidal Coomassie Blue or other staining procedure compatible with MS.

8.2.5
In-Gel Digestion

Requirements

Equipment: Clean scalpel, spatula, 0.5 mL safe-lock reaction tubes.

8.2.5.1 Excision of Protein Spots/Lanes from 16-BAC/SDS-PAGE and 1D SDS-PAGE gel

Protein spots/bands of interest are excised from either the 16-BAC-SDS-PAGE or the 1D SDS-PAGE gels. A clean scalpel is used for this purpose. Cut as

close as possible to the edge of the spot/lane to avoid extra gel material. The excised spot/lane is further cut into smaller pieces of approx 1 × 1 mm. The gel particles are transferred into a 0.5-mL reaction test tube.

8.2.5.2 In-Gel Reduction, Alkylation, and Digestion

Chemicals: H_2O, acetonitrile, NH_4HCO_3, dithiothreitol, iodoacetamide, $CaCl_2$, formic acid, trypsin.

Solutions: 100 mM NH_4HCO_3 (pH 8.0), 10 mM DTT in 100 mM NH_4HCO_3, 55 mM iodoacetamide in 100 mM NH_4HCO_3, 5% (v/v) formic acid, trypsin (0.1 µg µL^{-1}), 100 mM $CaCl_2$.

Digestion buffer (prepared fresh):

	1	2
Trypsin (0.1 µg µL^{-1})	15 µL	–
NH_4HCO_3 (100 mM)	50 µL	50 µL
$CaCl_2$ (100 mM)	5 µL	5 µL
H_2O	50 µL	50 µL
Total	120 µL	120 µL

The amount of buffer is sufficient for approximately 10–15 digestions.

1. Wash the gel pieces with 150 µL ddH_2O. Incubate for 5 min at 25 °C (1.050 rpm) in a thermomixer.
2. Spin gel pieces down and remove all liquid with thin pipette tips.
3. Add 150 µL acetonitrile and incubate for 15 min at 25 °C (1.050 rpm) in a thermomixer to shrink (dehydrate) the gel pieces (they become white and stick together).
4. Spin the gel pieces down and remove all liquid.
5. Dry the gel pieces for approx 5 min in a SpeedVac.
6. Swell the gel pieces in 100 µL 10 mM DTT (the gel pieces must be covered completely). Incubate at 56 °C for 50 min to reduce the cysteine residues within the protein.
7. Spin the gel pieces down and remove all liquid.
8. Add 150 µL acetonitrile and incubate for 15 min at 25 °C (1050 rpm) in a thermomixer (until the gel pieces have shrunk).
9. Spin the gel pieces down and remove all liquid with a thin tip.
10. Incubate the gel pieces with 55 mM iodoacetamide for 20 min at room temperature in the dark to modify (alkylate) the cysteines.

11. Spin the gel pieces down and remove all liquid with a thin tip.
12. Add 150 μL of 100 mM NH_4HCO_3, incubate for 15 min at 25 °C (1050 rpm) in a thermomixer.
13. Spin the gel pieces down and add 150 μL of acetonitrile. Incubate for 15 min at 25 °C (1050 rpm) in a thermomixer.
14. Spin the gel pieces down and remove all liquid with a thin tip.
15. Shrink the gel pieces in 150 μL acetonitrile by incubating for 15 min at 25 °C (1050 rpm) in a thermomixer.
16. Spin the gel pieces down and remove all liquid with a thin tip.
17. Dry the gel pieces for 5–10 min in a SpeedVac.
18. Rehydrate the gel pieces at 4 °C in digestion buffer **1** (see above) containing trypsin for 30–45 min. Use only small amounts of digestion buffer. Check the samples after 15–20 min and add more buffer if all liquid is absorbed by the gel pieces. Add 10–20 μL of digestion buffer **2** (without trypsin) to cover the gel pieces completely and to keep them wet during enzymatic cleavage.
19. Incubate samples in incubator at 37 °C overnight.

8.2.5.3 Extraction of Peptides from In-Gel Digests

1. Prepare fresh 0.5 mL reaction tubes to collect the supernatant.
2. Spin the gel pieces down.
3. Add 10–15 μL water to the digest, so that the gel pieces are completely covered with liquid.
4. Spin the gel pieces down and incubate for 15 min at 37 °C and 1050 rpm in a thermomixer.
5. Spin the gel pieces down.
6. Add at least 50 μL of acetonitrile (a volume twice as large as the volume of the gel pieces should be added) and incubate for 15 min at 37 °C (1050 rpm) in a thermomixer.
7. Spin the gel pieces down and collect the supernatant in the new reaction tubes.
8. Add 50 μL of 5% formic acid to the gel pieces and incubate for 15 min at 37 °C (1050 rpm) in a thermomixer.
9. Spin the gel pieces down and add 50 μL of acetonitrile, then incubate for 15 min at 37 °C (1050 rpm) in a thermomixer.
10. Spin the gel pieces down and collect the supernatant and pool the extracts in the new reaction tubes.
11. Add 100 μL acetonitrile and incubate for 15 min at 37 °C (1050 rpm) in a thermomixer.
12. Spin the gel pieces down and transfer the supernatant to the pooled extracts in the new reaction tubes.
13. Evaporate the samples to dryness in the SpeedVac (about 11/2 h).

8.2.6
On-Line and Off-Line Nano Liquid Chromatography (NanoLC)

8.2.6.1 Pre- and Analytical Columns from Vendors

Requirements

C18-analytical columns: C18 PepMap 100, 75 µm ID, 3 µm 100 Å (LC Packings). PreproSil-Pur 120 C18-AQ, 3 µm and/or 5 µm (Dr Maisch GmbH). Onyx Monolithic C18, 100 µm ID (Phenomenex).

C18-pre columns: µ-Precolumn Cartridge; C18 PepMap 100, 300 µm ID (5 mm length), 5 µm 100 Å (LC Packings).

8.2.6.2 Preparation of Pre- and Analytical Nano-Flow Reverse-Phase Columns

Requirements

Chemicals and materials: Formamide, methanol, 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A), 80% (v/v) acetonitrile, 0.1% (v/v) TFA in water (solvent B), Kvasil1● (PQ Europe), fused silica capillaries (375 µm outer diameter (OD) and 75 µm inner diameter (ID) for analytical columns, 375 µm OD and 150 µm ID for pre-columns (Polymicro Technologies), MicroTight Fittings with 5 µM PEEK filter end fitting (UpChurch Scientific Inc.), MicroTight Sleeve Green (0.0155 × 0.025, UpChurch Scientific Inc.), polymer tubing PEEK Gray 1/16 × 0.015 (400 µm ID, UpChurch Scientific Inc.), reverse-phase material (e.g. Vydac MS218, 5 µm 300 Å beads (Vydac) or Reprosil-Pur 120 C18-AQ, 3 µm (Dr Maisch GmbH).

Instrumentation: Pressure vessel (for packing the columns; Bruchbuehler) connected to a high-pressure helium cylinder. Pressure valve should allow up to 200 bar (e.g. Messer Griesheim).

8.2.6.2.1 Generation of a "frit" restrictor in the fused silica capillary for analytical columns

1. Mix 88 µL Kvasil1 and 16 µL formamide in a 1.5-mL test tube.
2. Vortex rigorously for 2–3 min (the solution becomes viscous).
3. Dip one end of a 30–40-cm-long fused silica capillary (375 µm OD 75 µm ID) in the solution for 1–2 s (the solution will move upward into the fused silica by capillary action).
4. Wipe off excess solution. Polymerization is achieved by leaving the capillary at room temperature overnight or by heating at 50 °C for 2–3 h.
5. Check the frit under the microscope/binocular. It should be 2–5 mm in length. Cut the frit if it is too long.

8.2.6.2.2 Packing reverse-phase analytical columns

1. The fused silica capillary (with the frit) is inserted into the pressure vessel and the open end is placed in a reaction tube (within the pressure vessel) containing 100% methanol. The vessel is closed properly and the helium pressure is raised until the methanol flows through the capillary. This step assures that the capillary is clean before column-packing and that the frit stays intact under higher pressure.

2. Resuspend approx 5–10 mg of reverse-phase material with 500 µL methanol in a reaction tube. Place the reaction tube in the pressure vessel and the capillary in methanol slurry as above. Pack the column with the slurry according to the cleaning procedure above, but with higher pressure (up to 70 bar). In order to keep the reverse-phase material in suspension during the packing procedure, a very small magnetic stirrer should be put into the reaction tube and the entire vessel should be placed on a magnetic stirring device. Column packing can be observed under the microscope/binocular. The column should be packed for 25–20 cm. After the helium pressure has been turned off, the column should remain in the vessel overnight to slowly minimize the pressure and thus avoiding any back-flushing of sample material.

3. The open end (without the frit) is covered with a peek sleeve (400 µm ID) and is tightly fixed with a stainless nut and ferule compatible with the valve port of the nanoLC system

4. The packed column is mounted in a capillary/nanoLC system and equilibrated first with solvent B and then with solvent A for the 30 min each, with a flow rate of 400–800 nL min^{-1} (depending on the back pressure, which should not exceed 160 bar).

5. The performance of the column is tested in several test runs with a tryptic digest of a standard protein (e.g. 100 fmol BSA).

Pre-columns are made in the same way as described above for analytical columns using a fused silica with an OD of 375 µm and ID of 75 µm (for LC-MS/MS) or 150 µm (for LC-MALDI). When pre-columns for LC-MALDI are generated, a column (after end packing) with an approximate length of 2 cm is cut out from the fused silica and subsequently closed by MicroTight fittings on both sides.

8.2.6.3 On-Line NanoLC-ESI MS/MS

Requirements

Chemicals: Water, acetonitrile, formic acid.

Solutions: Solvent A, 0.1% formic acid (v/v) in water; solvent B, 100% acetonitrile (v/v), 0.1% formic acid (v/v) in water; solvent C, 0.1% formic acid

(v/v) in water; Solvent D: 10% acetonitrile (v/v), 0.15% formic acid (v/v) in water.

Instrumentation: Waters Q-TOF Ultima ESI mass spectrometer equipped with a capillary LC system and autosampler. Columns from vendors and/or self-packed pre- and analytical columns are described above.

1. Extracted peptides derived from in-gel digested synaptic vesicle proteins are dissolved in an appropriate volume of solvent D (e.g. 20–50 μL).
2. Samples (max. 6 μL) are loaded onto the pre-column with solvent C at a flow rate of 10 μL min^{-1} for 10 min.
3. Peptides are eluted onto the analytic column by backflush and subsequently separated with the following gradient with a flow rate of 180 nL min^{-1}: 7% (v/v) solvent B to 40% (v/v) solvent B for 50 min, 40% B to 80% B for 1 min, isocratic elution at 80% B for 10 min, 80% B to 7% for 1 min, isocratic equilibration at 7% B for 10 min.
4. Peptides are chosen for MS/MS analysis by performing a survey scan of the ionized species that elute from the column into the instrument (automated MS and MS/MS analysis). The settings for the survey scan have to be optimized according to the corresponding ESI instrument.
5. MS/MS spectra of the peptides are processed (smoothing, centroiding) and searched against databases using Mascot as search engine according to the above mentioned settings (see Section 8.3.4).

8.2.6.4 Off-Line NanoLC

Requirements

Chemicals: α-Cyano-cinnamic acid, water, acetonitrile, TFA (Sigma-Aldrich), o-phosphoric acid (H_3PO_4, Merck).

Solutions: Solvent A, 0.1% TFA (v/v) in water; solvent B, 80% ACN (v/v), 0.1% TFA (v/v) in water; solvent C, 3.5% ACN (v/v), 0.1% TFA (v/v) in water; MALDI matrix, 10 mg mL^{-1} HCCA in 70% ACN (v/v), 0.1% TFA (v/v) in water.

Instrumentation: Dual Gradient System (Dionex) equipped with an autosampler; LC-MALDI Spotter Probot (Dionex) with a 300-nL mixing chamber (UpChurch Scientific Inc.); MALDI-TOF/TOF 4800 analyzer (Applied Biosystems/Sciex MDS).

Q2

• Samples (complex peptide mixtures derived from in-gel digestion of synaptic vesicles) are dissolved in x μL • of 10% (v/v) acetonitrile, 0.1% (v/v) TFA and injected via the autosampler onto the pre-column with solvent C at a flow rate of 5 μL min^{-1} for 25 min.
• The desalted peptides are eluted from the pre-column and subsequently separated on the analytical column by the following "standard" gradient:

15 min 10% (v/v) solvent B, 10–60% (v/v) solvent B for 60 min, 60–100% (v/v) solvent B for 3 min, 100% (v/v) solvent B for 9 min, 100% (v/v) solvent B—10% solvent B for 1 min. The gradient can be adjusted according to the complexity of the sample.

- Fractions are spotted every 15 s onto stainless steel LC-MALDI plates (Applied Biosystems) with α-cyanocinnamic acid as matrix. Matrix is delivered with a flow rate of 0.9 μL min^{-1} and is mixed with the eluate using a T-piece mounted in before the spotter needle.
- Fully automated MALDI-MS and MS/MS analysis of the spotted fraction is performed according to the manufacturer's instructions for the MALDI instrument.
- Settings for data analysis are according to the analysis of data acquired by LC-ESI-MS/MS, except that the number of missed cleavages (see above) for the database search is higher (2–3 missed cleavages) than in the ESI analysis.

8.3
Summary and Outlook

A comprehensive proteomic analysis of highly purified synaptic vesicles has provided a detailed map of the protein constituents involved in the synaptic vesicle life cycle in addition to vesicle-associated proteins (Takamori *et al.*, 2006). These proteins include already known players in addition to novel proteins. Since the synaptic vesicle proteome is rather complex, with more than 400 identified proteins, the sample has to be fractionated before MS analysis. A combination of 1D SDS-PAGE and 16-BAC/SDS-PAGE is recommended to ensure maximum recovery of both soluble and (integral) membrane proteins. In a new study, synaptic vesicles were fractionated by a combination of 1D SDS-PAGE and 16-BAC/SDS-PAGE and subsequently analyzed by nanoLC-MS/MS; this resulted in the identification of 321 and 262 proteins, respectively (Table 8.1). Of these, 149 proteins were uniquely identified by 1D SDS-PAGE, and a further 90 proteins were uniquely identified by 16-BAC/SDS-PAGE. Even though 172 proteins were found to be common between the two analyses the relatively high number of unique proteins identified in each method emphasizes that the two fractionation strategies are complementary. As seen in Table 8.1, the proteins identified include a variety of protein classes including trafficking proteins, small endocytosis-related GTPases, transporters/channels, cytoskeleton proteins, cell surface protein, signaling molecules, metabolic enzymes, chaperones, proteasome proteins, RNA-processing proteins, and novel proteins (for a detailed description of the individual proteins see Takamori *et al.*, 2006). Interestingly, even though proteomics approaches have failed to identify all known synaptic vesicle proteins (in particular integral membrane proteins such as the low abundant transporters and the chloride channels), the total number of identified proteins

Table 8.1

	Total	Overlap	16-BAC-SDS	1D SDS
Trafficking proteins●	18	11	12	17
Endocytosis-related proteins	19	8	12	15
Small GTPases + related proteins	50	32	45	37
Other trafficking proteins	39	22	29	32
Transporter/channel	45	27	33	39
Cytoskeleton	29	12	14	27
Cell surface	24	7	14	17
Signaling molecules	38	13	25	26
Metabolic enzymes	42	15	25	32
Others	43	9	16	37
Chaperones	12	4	6	10
Proteasome	11	5	6	10
RNA processing	22	4	15	11
Novel	18	3	11	11

Q3

is still considered surprisingly high. The large number of proteins identified can be explained to some extent by proteins that are thought to associate only transiently with synaptic vesicles. Another explanation might be that heterogeneous populations of synaptic vesicles exist where not all proteins reside on the same vesicle. This phenomenon is already observed for the transporters (i.e. VGLUT and GABA) which represent a specific pool of synaptic vesicles (Takamori *et al.*, 2000a, 2000b). A proteomic analysis of different subpopulations of synaptic vesicles will therefore be helpful to elucidate such differences. Finally it might be the case that many novel synaptic vesicle proteins are still to be identified. These proteins include known proteins which have not been assigned as synaptic vesicle proteins before and proteins which have only been identified by automated genome annotation. A functional characterization of these proteins as novel synaptic vesicle proteins will play an important role in understanding the detailed mechanism of the synaptic vesicle life cycle.

Acknowledgments

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Abstract: Synaptic vesicles are storage organelles for neurotransmitters in neurons. When an action potential arrives at the nerve terminal, voltage-gated calcium channels open and synaptic vesicles undergo rapid exocytosis, releasing their neurotransmitter content into the synaptic cleft. The synaptic vesicle membrane is rapidly retrieved by endocytosis and reutilized for the reformation of further synaptic vesicles. The synaptic vesicle cycle shares basic properties with other intracellular membrane pathways. Hence, not only can synaptic vesicles be considered to be the basic minimal units of synaptic transmission but also the basic minimal units of membrane transport, and their integral protein composition serves as the basis for all the functions a trafficking vesicle must perform. The high abundance and homogeneity obtained from purified synaptic vesicles make them an ideal model for improving membrane-based proteomics, in addition to identifying novel synaptic vesicle proteins.

This chapter describes the purification and mass spectrometric analysis of synaptic vesicles and their protein components. Successful purification of synaptic vesicles, in respect to purity and amount, is the prerequisite for a comprehensive proteomic analysis. The first part of this chapter addresses this issue by focusing on the purification of synaptic vesicles from rat brain, on the reduction of contaminants, and on how to monitor the purity of the purified synaptic vesicles. The second part provides protocols for the isolation of synaptic vesicles, the separation of synaptic vesicle proteins mainly by gel electrophoresis prior to mass spectrometric analysis, and the analysis of complex protein samples by liquid chromatography coupled either on- or off-line.

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