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Conservation of infectivity in purified fibrillary extracts of scrapie-infected hamster brain after sequential enzymatic digestion or polyacrylamide gel electrophoresis

(scrapie-associated fibrils/scrapie amyloid protein/enzymes)

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ABSTRACT **Infectious extracts of scrapie-infected hamster brain enriched for scrapie-associated fibrils and scrapie amyloid protein (PrP) were partially denatured and subjected to either polyacrylamide gel electrophoresis with subsequent isolation of the PrP band or sequential enzymatic digestion with deglycosidase, phospholipase, proteinase, and several different nucleases. Infectivity measurements of these various specimens revealed a convincing association between infectivity and scrapie amyloid protein, with or without its sugar chains and disulfide bonds, and did not support the hypothesis that nucleic acid is involved in replication.**

Numerous attempts to demonstrate or infer the presence of an infectious nucleic acid in viruses that cause the transmissible spongiform encephalopathies have failed (1–13); however, infectivity has consistently been associated with scrapie-associated fibrils (SAFs) and their subunit amyloid molecule [scrapie amyloid protein (PrP)] derived from a normal host protein [scrapie amyloid precursor protein (PrP^c)] (12, 14–18).

We have conducted infectivity studies on partially denatured and enzymatically digested infectious brain extracts before and after subjecting them to attack by a variety of nucleases and have also succeeded in introducing enough virus into gels to permit protein band infectivity measurements: the results of both types of experiments furnish additional evidence that scrapie virus is a nucleic acid-free replicating protein.

MATERIALS AND METHODS

Scrapie Virus. The 263K strain of scrapie virus (fourth passage level in our laboratory) was used to infect golden Syrian hamsters that were sacrificed when terminally ill, and their brains were removed and stored at −70°C until processed. The procedure for extracting SAFs from brain tissue has been described in detail (19): successive centrifugations in the presence of 1% lauroyl sarcosinate/10% sodium chloride/proteinase K (5 μg/ml) resulted in fibrillary extracts with 40–50 μg of scrapie amyloid protein per g of original brain tissue and 10^8–$10^{9.5}$ mean lethal dose (LD_{50}) infectivity units per 33-μl vol. An additional extract, prepared to minimize the effect of proteinase activity, was similarly processed except for the inclusion of a proteinase inhibitor cocktail (see below) in the initial homogenization step and the exclusion of proteinase K from the protocol.

Enzymes. *N*-Acetyl-β-glucosaminidase A and B; β-glucuronidase; carboxypeptidase A, B, and Y; chondroitinase ABC; collagenase; DNase II; hyaluronidase; leucine aminopeptidase; lipoprotein lipase; micrococcal endonuclease; neuraminidase; phospholipase A_2, B, and C; proteinase A and K; RNase A and T_2; and trypsin were all purchased from Sigma; endoglycosidase H and F were purchased from Calbiochem; DNase I and RNase H were purchased from Bethesda Research Laboratories; and glycopeptidase F was purchased from Boehringer Mannheim. Phosphatidylinositol phospholipase C (PI-PLC) and reagents for verifying its activity—variable surface glycoprotein (VSG) antigen and anti-cross-reacting determinant (CRD) antibody—were kindly donated by Martin Low (Department of Physiology, Columbia University College of Physicians and Surgeons, NY).

Chemical Reagents. Acrylamide, bisacrylamide, ammonium persulfate, SDS, and TEMED were purchased from Bio-Rad; Tris buffer was from Bethesda Research Laboratories; *N*-lauroylsarcosine (Sarkosyl) and 2-mercaptoethanol (2-ME) were from Sigma; glycine was from Schwarz/Mann; sulfobetaine 3-14 was from Calbiochem; and magnesium chloride and calcium chloride were from Mallinckrodt. The proteinase inhibitor mixture used in one experiment was composed of EDTA, phenylmethylsulfonyl fluoride (PMSF), and Bestatin (Sigma), antipain, leupeptin, and pepstatin A (Fluka); EDTA was used at a final concentration of 2 μg/ml and PMSF was at 50 μg/ml, and the other four reagents were at 5 μg/ml.

Enzyme Digestion Protocols. Individual enzyme digestions were performed on duplicate samples of 50 μl containing 25 μg (1 μM) of protein, mixed with an equal vol of 0.1 M Tris-buffered enzyme solution at a pH optimal for enzyme activity (as specified by the supplier) in Eppendorf vials, and agitated continuously for either 2 hr or 16 hr at 37°C. Sequential enzyme digestions were performed on initial extract volumes of 300 μl containing 150 μg (6 μM) of protein in 0.1 M Tris buffer, the pH of which was adjusted as needed with small amounts of NaOH or HCl to achieve optimal activity for each successive enzyme, and all digestions were carried out at 37°C with constant agitation for a minimum of 4 hr. Except for proteinase K, enzyme concentrations were always well in excess of the minimum number of units needed for the complete conversion, in 1 hr or less, of 1 mM quantities of standard control substrates (assayed by the suppliers); glycosidase and PI-PLC concentrations were equal to or greater than those already specifically established for scrapie amyloid protein (20–22); proteinase K was used to

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Abbreviations: SAF, scrapie-associated fibril; PI-PLC, phosphatidylinositol phospholipase C; VSG, variable surface glycoprotein; CRD, cross-reacting determinant; 2-ME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride.

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achieve partial proteolysis at a final concentration of 100 μg/ml for 1 hr (15).

Four experiments were carried out in which infectivity titers were estimated by incubation periods in animals inoculated with a 10^{-2} dilution of the treated extracts. Each experiment began with a glycosidase digestion (endoglycosidase H, endoglycosidase F, or glycopeptidase F), followed by PI-PLC, and then by proteinase K. The proteinase-treated specimen was divided for parallel digestions with micrococcal endonuclease, DNase I, RNase A, and RNase H.

A final experiment was performed in which infectivity was measured by an endpoint dilution assay: the extract was boiled for 5 min in the presence of 0.15% SDS and 1% 2-ME, and then sulfobetaine 3-14 was added in a final concentration of 1% to inhibit the denaturing action of SDS on enzyme proteins, before sequential incubations for 18 hr with glycopeptidase F (5 units) and 6 hr with PI-PLC (4000 units). The divided extract was then separately incubated for 18 hr with micrococcal endonuclease (5 units), DNase I (300 units), or RNase A (5 units). Proteinase K treatment was not included in this experiment, because the already partially denatured protein was found to be extremely sensitive to the action of proteinase K, and a subthreshold partial proteolytic digestion step could not be reliably reproduced.

Numerous controls to verify enzyme potency on standard substrates (albumin, casein, fetuin, VSG, RNA, and DNA), and to check the absence of contaminating proteinase activity in nonproteinase enzymes, were run under identical conditions in parallel with the enzyme-treated brain extracts. Aliquots of all samples were frozen at the conclusion of each step (PMSF was also added to samples treated with proteinase K) and held at −70°C. After thawing, the volumes of treated preparations were corrected for the various dilutions imposed by the addition of enzymes and buffers to equivalence with the original untreated sample before use in infectivity titrations.

Gel Electrophoresis Protocols. A 200-μl sample of brain extract containing 100 μg of protein was added to 200 μl of standard sample buffer containing 5% 2-ME, 2% SDS, 10% (vol/vol) glycerol, and 0.0005% bromphenol blue dye, buffered with 0.5 M Tris to pH 7.0. The mixture was boiled for 2 min and then loaded onto a vertical 1.5-mm-thick polyacrylamide minigel (4% stacking gel and 12% resolving gel), using a two-dimensional comb with 1 reference well. Additional samples and molecular size markers were loaded onto a second (1.0-mm-thick) gel with a 10-well comb and run at the same time. Electrophoresis was performed for 35 min at 100 mV in a Mini-Protean II dual slab cell (Bio-Rad), at which time the tracking dye had just reached the lower gel margins. The reference well was cut away and, together with part of the duplicate gel, was stained with silver and realigned with the unstained gel to permit localization of the 27- to 30-kDa protein band. The gel was then cut into four horizontal strips: the uppermost strip (15 mm) included all of the stacking gel and the upper margin of the resolving gel; lower strips divided the remainder of the resolving gel into three portions: from about 100 to 35 kDa (18 mm), from 35 to 20 kDa (12 mm, containing the protein band), and from 20 kDa to the lower edge of the gel (20 mm). Each strip was minced, ground with a mortar and pestle or Tenbroeck homogenizer as a 10% (wt/vol) suspension in PBS, sonicated for 30 sec at a dial setting of 6, using a no. 419 1/8-inch microtip probe and model W200-R sonicated (Heat Systems/Ultrasonics), and stored at 4°C until inoculated into hamsters.

A similar experiment was performed with the proteinase-inhibited brain extract that contained both the 27- to 30-kDa scrapie amyloid protein and its configurationally altered 33- to 35-kDa precursor protein. The two protein bands were separated into narrow adjacent upper (40–30 kDa) and lower (30–20 kDa) gel strips for comparative infectivity measurements.

Silver Staining and Immunoblotting. Silver stains and Western immunoblots were performed by standard procedures. Scrapie amyloid protein was detected in immunoblots by using a previously described rabbit polyclonal primary antibody to a fibril preparation that had been extracted according to the same protocol as the present experiment (23).

Electron Microscopy. Several 10-μl samples from each sonicated preparation were dropped onto freshly glow discharged, 300-mesh Formvar-coated copper grids. The grids were air dried without wicking, washed five times in distilled water, stained with 2% phosphotungstic acid for 20 sec, and examined under a Philips 300 transmission electron microscope at 60 kV.

Virus Infectivity Measurements. Endpoint dilution titrations were carried out in 4-week-old weanling female LGV golden Syrian hamsters (Harlan Sprague Dawley); 33 μl of serial 1:10 dilutions of each specimen was inoculated intracerebrally into groups of four to eight hamsters. Animals were observed for 10 months after inoculation, and the brains from a random selection of dead animals were examined histologically for the presence of spongiform change. Virus infectivity titers were calculated according to the method of Reed and Muench (48), and are expressed as $\log_{10}$ LD_{50} per 33-μl inoculum volume.

RESULTS

Enzyme Digestions. In experiments limited to single enzyme incubations, none of the enzymes listed above, with activities directed against various carbohydrate moieties, phospholipids, peptides, structural proteins, DNA, or RNA, prolonged the incubation periods of inoculated animals beyond the 77- to 81-day range of untreated controls, even after 16-hr incubations at concentrations equimolar with the amount of scrapie amyloid protein in the extract sample. Under the same conditions, proteinase K prolonged the mean incubation period to 96 days, giving an estimated 2 log reduction of infectivity.

In later experiments that used successive incubations with deglycosidases, PI-PLC, proteinase K, and various nucleases, no difference in estimated infectivity was observed between samples taken before and after the final nuclease digestion: all animals in each of four experiments became ill within 1–2 days of the 74- to 77-day range of untreated controls, yielding estimated infectivity levels of 7–8 log units in both prenuclease- and postnuclease-treated specimens.

The results of these preliminary experiments, based on incubation period infectivity estimates, led to a final experiment in which infectivity was measured by the more accurate method of full endpoint dilution assays. Following an initial SDS/2-ME denaturation step that produced a moderate alteration in fibril morphology as viewed by electron microscopy, incubation with glycopeptidase F produced multiple lower molecular weight bands in both the scrapie amyloid protein (Fig. 1, lanes 1–3), and a control glycoprotein (Fig. 2*A*, lanes 1 and 2), but no reduction of infectivity (Table 1).

Subsequent incubation with a large excess of PI-PLC was not effective in separating the protein from its terminal phospholipid membrane anchor: it neither changed its molecular mass (Fig. 1, lane 4 compared to lane 2), nor exposed the CRD epitope in VSG that is visible after PI-PLC treatment of many similarly anchored compounds (24), including the amyloid precursor protein present in normal cells (22, 25). Thus, instead of a positive reaction to anti-CRD antibody, as seen in the immunoblot of a control VSG from *Trypanosoma brucei* (Fig. 2*B*, lane 1), the amyloid protein sample showed

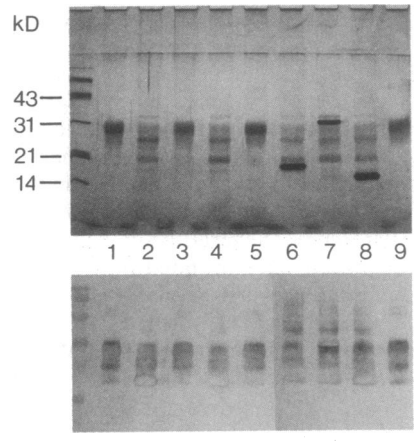

FIG. 1. Silver-stained polyacrylamide gel (*Upper*) and corresponding Western immunoblot (*Lower*) of successive samples in the course of a sequential enzymatic digestion of a scrapie-infected hamster brain SAF extract containing scrapie amyloid protein. Untreated extract (lane 1); after partial SDS denaturation and incubation with glycopeptidase F (lane 2) or control buffer (lane 3); after further incubation with PI-PLC (lane 4) or control buffer (lane 5); after further parallel incubations with micrococcal endonuclease (lane 6), DNase I (lane 7), RNase A (lane 8), or control buffer (lane 9). The dense bands in lanes 6–8 of the silver-stained gel are micrococcal endonuclease (18 kDa), DNase I (30 kDa), and RNase A (14 kDa) proteins.

no staining either before or after incubation with PI-PLC (lanes 2 and 3).

Subsequent incubation with nucleases, shown to be free of contaminating proteolytic activity, and to be active against RNA and DNA control specimens, did not significantly reduce the infectivity in any of the treated samples (Table 1).

PAGE. In preliminary experiments to determine the effect of boiling and PAGE sample buffer constituents on the

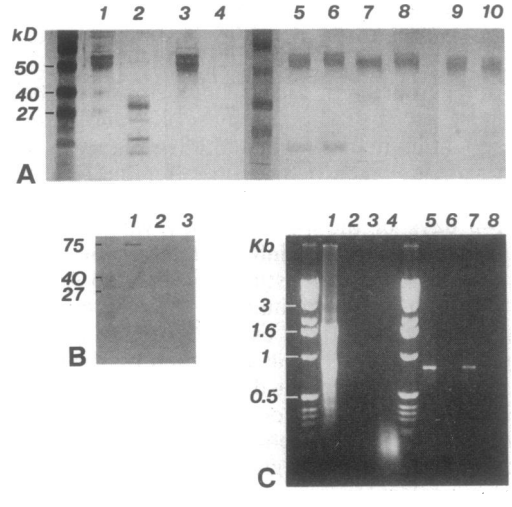

FIG. 2. Enzyme control preparations. (*A*) Silver-stained polyacrylamide gel of the glycoprotein fetuin: before and after incubation with glycopeptidase F (lanes 1 and 2); before and after incubation with proteinase K (lanes 3 and 4); before and after incubation with micrococcal endonuclease (lanes 5 and 6), DNase I (lanes 7 and 8), and RNase A (lanes 9 and 10). (*B*) Immunoblots stained with anti-CRD antibody of VSG (lane 1), and SAF extract before (lane 2) and after (lane 3) incubation with PI-PLC. (*C*) Ethidium bromide-stained agarose gel of RNA extracted from scrapie mouse brain (lanes 1–4) and a DNA fragment of the *PrP* gene extracted from a Creutzfeld–Jacob disease patient (lanes 5–8): untreated (lanes 1 and 5), and after incubation with micrococcal endonuclease (lanes 2 and 6), RNase A (lanes 3 and 7), or DNase I (lanes 4 and 8). Molecular mass markers are included for each test. Kb, kilobases.

Table 1. Infectivity titers of partially denatured fibrillary extracts of scrapie-infected hamster brain subjected to sequential enzymatic digestion

Sequential treatment	Infectivity titer, $\log_{10}$ LD_{50}: Enzyme-treated extract	Infectivity titer, $\log_{10}$ LD_{50}: Buffer-treated extract*
None	7.7	
Boiling for 3 min in 0.15% SDS/1% 2-ME		
+ glycopeptidase F	7.5	8.0
+ phospholipase C†	7.5	8.3
+ micrococcal endonuclease	8.0	
+ DNase I	7.6	8.3
+ RNase A	8.0	

*Specimen run in parallel with treated sample, using the same buffer mixtures without enzymes.
†Specific for PI-PLC.

morphologic appearance and infectivity levels of fibrillary brain extracts, aliquots were mixed with equal volumes of water, 2% SDS, 5% 2-ME, or 2% SDS plus 5% 2-ME, and either left at room temperature or boiled for 2 min.

None of the treatments carried out at room temperature caused any apparent change in the numbers or morphologic appearance of the fibrils, or any significant lengthening of the incubation periods in inoculated animals. Treatments that included boiling resulted in a reduction in the number of fibrils (particularly evident in the specimens mixed with SDS), and a fuzzy appearance to some of the residual intact fibrils. Incubation periods were slightly longer than in the unboiled specimens or untreated controls, but in no case equivalent to more than a 1 log loss of estimated infectivity, which is at the threshold of significance for bioassays. Two subsequent experiments using endpoint dilution titrations showed reductions of 0.6 and 1.5 log units with respect to the untreated control specimens, leaving residual infectivity levels of 8–9 log units in the specimens subjected to boiling in SDS/2-ME.

Having determined that much of the infectivity in fibrillary brain extracts can withstand boiling in an SDS/2-ME mixture (which implies that the integrity of disulfide bonds is not required for replication), we boiled another sample with standard PAGE loading buffer that contained the same concentrations of SDS and 2-ME used in the preliminary experiments and carried out a routine PAGE. The results of this experiment are presented in Table 2 (infectivity data), Fig. 3 (electron photomicrographs), and Fig. 4 (silver-stained gel and corresponding immunoblot).

Several points are noteworthy: (*i*) as in the preliminary experiments, the SDS/2-ME boiling step produced no significant change in infectivity; (*ii*) essentially all of the input infectivity was recovered in the gel; (*iii*) ≈10% of this

Table 2. Infectivity titers of fibrillary extracts of scrapie-infected hamster brain subjected to SDS/PAGE

	Infectivity titer/ 33-μl inoculum, $\log_{10}$ LD_{50}	Specimen vol, μl	Total infectivity in specimen, infectious units × 10^6
Untreated extract	9.5	67	6325*
Boiled in SDS/2-ME	9.0	67	2000*
Stacking gel	6.5	5400	538
100–35 kDa	4.5	6900	7
35–20 kDa	7.5	5300	5376*
20–1 kDa	4.5	8100	8

*The difference between these values (≤0.5 $\log_{10}$ unit) is not significant.

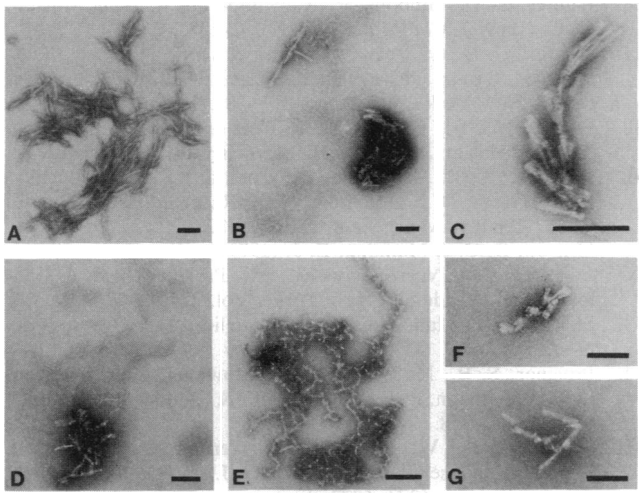

FIG. 3. Electron photomicrographs of SAF in hamster brain extracts, illustrating fibril morphology in untreated (*A*) and treated (*B–G*) preparations. Samples were boiled 5 min in distilled water (*B*) or 1% SDS (*C*), boiled in PAGE sample buffer containing 1% SDS and 2.5% 2-ME (*D* and *E*), or loaded on homogenized stacking gel after SDS/PAGE (*F* and *G*) (homogenized strips of the resolving gel contained too much polyacrylamide matrix debris for suitable examination). (Bars = 100 nm.)

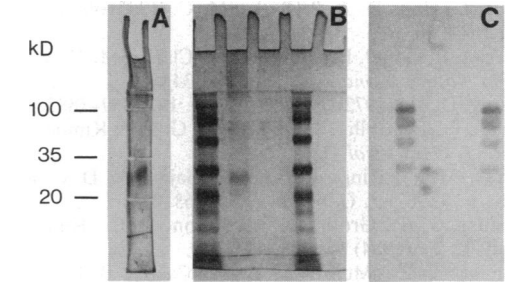

FIG. 4. (*A*) Silver-stained reference lane of bulk gel that was loaded with SAF extract and later divided for infectivity titrations. The approximate molecular masses at which the visible transverse cuts were made are indicated on the left. (*B*) Silver-stained gel. (*C*) Corresponding immunoblot run in parallel with the gel that was titrated. Molecular mass markers bracket an aliquot of the same SAF extract used for infectivity measurements (left lanes), and an extract from normal, uninfected brain (right lanes).

infectivity was retained in the uppermost stacking part of the gel, probably in partially denatured fibrils unable to penetrate the gel matrix (Fig. 3 *F* and *G*); and (*iv*) almost all of the remaining infectivity was recovered in the 20- to 35-kDa strip of resolving gel containing the 27- to 30-kDa scrapie amyloid protein (nearly 5000-fold more than in the adjacent sections).

In the experiment on proteinase-inhibited fibril extracts containing both the scrapie amyloid protein and its precursor, strips encompassing each protein contained approximately equal amounts of infectivity.

DISCUSSION

Almost a quarter of a century ago, claims that scrapie virus replicated without a nucleic acid, based on its unusual resistance to inactivation by ultraviolet radiation (1), sparked a controversy between conservatives, whose instincts were to defend the basic tenets of molecular genetics by searching for a viral nucleic acid, and romantics, who were intrigued by the possibility of a heretical form of replication.

The effect of enzymes on scrapie infectivity has been periodically examined in the past, using crude brain tissue suspensions (26–29), partially purified brain extracts (30–32), and, most recently, highly purified extracts enriched for SAFs and scrapie amyloid protein (11, 12, 15, 17, 30, 33). None of the tested enzymes except proteinases reduced the infectivity of these preparations.

Our sequential enzyme digestion experiments were based on the premise that a putative nucleic acid involved in agent replication might be protected by a coating layer of amyloid protein and so be resistant to the action of nuclease. The protein was therefore subjected (with or without prior denaturation) to a sequence of enzymes that could alter or damage its structure before incubation with nucleases. Although the phospholipid terminus remained intact, we did achieve partial deglycosylation, and exposure to SDS/2-ME or proteinase K degraded a large proportion of protein-containing fibrils. In spite of these treatments, no significant reduction of infectivity was detected after subsequent incubation with nucleases.

Our gel electrophoresis experiments grew out of the realization that the infectivity of hamster fibril extracts could withstand exposure to SDS and 2-ME, although earlier reports had shown that exposure of the scrapie agent to 1% SDS, either at room temperate for 1 hr (32) or boiled for 2 min (34), caused a 3–4 log unit loss of infectivity. Even in extracts exposed to lower concentrations of SDS, >90% of the infectivity had failed to enter composite polyacrylamide agarose gels (30, 31). We do not know why such losses occurred in these studies, unless the hamster agent is more resistant than the mouse agent to this treatment (32), or unless incubation period assays did not give a true indication of endpoint dilution titers (30, 31, 34): our results were consistently reproduced in a series of five separate experiments.

The data presented in this paper do not nourish the hypothesis of a nucleic acid component to the infectious agent but do provide evidence linking infectivity both to the 27- to 30-kDa scrapie amyloid protein and its configurationally altered 33- to 35-kDa precursor. Two independent studies by other investigators in our laboratory have reached the same conclusion by entirely different methods (35, 49).

After more than 20 years of unrewarded effort in numerous laboratories to detect an infection-specific nucleic acid (1–13), and despite the logical impossibility of precluding the existence of something that has not been found, it would seem appropriate as a purely practical matter to accept what has been discovered and move ahead with it, letting whatever theoretical objections that attend the idea of a replicating protein work themselves out in the course of time. Molecular genetic studies have already linked at least three different mutations in the gene encoding this protein to the expression of disease in animals (36–39) and in humans (40–46). The most important problems facing us now are to identify the physiologic function of the normal precursor protein, to characterize the configurational difference between its noninfectious and infectious forms, and to determine what genetic or environmental factors trigger this conversion. Since the efficiency of extracting the precursor protein from normal brain is at best only 1–2 μg of protein per g of tissue (47), the accumulation of sufficient quantities for experimental manipulation, particularly crystallography, poses a formidable but not insurmountable task.

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