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Production, Purification and Oxidative Folding of the Mouse Recombinant Prion Protein

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ABSTRACT. The method leading to overexpression of the full-length mouse recombinant prion protein (mrPrP 23-231) in the cytoplasm of *E. coli* as a his-PrP fusion protein and its effective purification using affinity chromatography is described. A typical yield of the method was 8–10 mg his-mrPrP per L of the bacterial culture. The purity of purified protein was >95 %. The purified his–mrPrP was converted to a soluble form and its folding to α-helical and β-sheet conformations was studied. The properties of differently folded mrPrP were determined by measuring their circular dichroism spectra, partial resistance to cleavage by proteinase K and by centrifugation in sucrose gradient.

Abbreviations

BSE	bovine spongiform encephalopathy	PK	proteinase K
CD	circular dichroism	PMCA	protein misfolding cyclic amplification
CJD	Creutzfeldt–Jacob disease	PMFS	phenylmethanesulfonyl fluoride
DTT	1,4-dithiothreitol	PrP	prion protein
GPI	glycosylphosphatidyl-*myo*-inositol	PrPC	cellular prion protein
HAT	*his* affinity tag	PrPSc	abnormally folded form of cellular prion protein
IPTG	isopropyl β-D-thiogalactoside	rPrP	recombinant PrP
mPrP	mouse prion protein	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
mrPrp	mouse recombinant prion protein	TSE	transmissible spongiform encephalopathies

Prion protein is a key player in the group of neurodegenerative disorders (so-called TSE – transmissible spongiform encephalopathies), such as bovine spongiform encephalopathy (BSE) in cows, scrapie in sheep, kuru and Creutzfeldt–Jacob disease (CJD) in humans (Prusiner 1981; Fischer *et al.* 1996; Horwich *et al.* 1997). TSE can arise sporadically or they can be of infectious or genetic origin (Dormond *et al.* 2002). The characteristic feature of the illness is an accumulation of abnormally folded (scrapie, PrPSc) form of cellular prion protein in the brain (Taylor *et al.* 2002). According to the "protein only" hypothesis (Prusiner 1982) the causative agent of the disease is a misfolded prion protein which serves as a template for conversion of normal cellular prion protein (PrPC) into the abnormal pathological form.

To understand the molecular mechanism of the prion disorders it is necessary to investigate the mechanism of PrPC conversion to PrPSc. The study of this process is very difficult *in vivo*, and *in vitro* such studies are hampered by difficulties and low yields of the purification of PrPC from the biological material. Production of recombinant PrP (rPrP) can overcome these difficulties by providing sufficient amounts of the protein. Most of prepared rPrPs have been cloned and expressed in the bacterial expression systems which explains why most biochemical experiments were done on unglycosylated rPrP. The lack of glycosylation and the lack of the GPI anchor have been shown to have no negative effect on the infectivity of PrPSc (Chesebro *et al.* 2005). However, the glycosylation may play a role at the level of folding by stabilizing a given PrP conformation (Ermonval *et al.* 1997). Prion protein is a glycoprotein with its highest level of expression on the neurons. No chemical differences or changes in the amino acid sequence have so far been detected between PrPSc and PrPC. These isoforms differ in their biophysical and biochemical properties. NMR structures have been reported for mouse (Riek *et al.* 1997), Syrian hamster (Donne *et al.* 1997), human (Zahn *et al.* 2000), sheep, pig, cat, dog (Lysek *et al.* 2005), chicken, turtle, frog (Calzolai *et al.* 2005) and elk (Grossert *et al.* 2005) recombinant cellular form of the prion proteins. All these studies revealed that the C-terminus is folded in defined structural domains while the N-terminal half of the molecule is unstructured. The tertiary structure of these proteins from different species is almost identical: three α-helical regions and one

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short two-stranded antiparallel β-sheet. Two of the helices are connected by a single disulfide bond between Cys-179 and Cys-214 (numbers correspond to mouse prion protein, mPrP). mPrP is synthesized as a 253-amino-acid-residue polypeptide which is post-translationally processed by cleavage of N- and C-terminal signal sequences (Stahl *et al.* 1991).

Two glycosylation sites have been found in the protein (Asn-181 and Asn-107); this is in agreement with observation of di-, mono- and unglycosylated forms of PrP (Chesebro *et al.* 2005; Lawson *et al.* 2005).

PrPC is a membrane protein which is attached to the membrane by C-terminal glycosylphosphatidyl-*myo*-inositol (GPI)-anchor. It exists in a monomeric form, contains a high percentage of α-helices and is sensitive to cleavage by PK (Prusiner 1998; Caughey *et al.* 2001; Glockshuber 2001). On the other hand, PrPSc is a β-sheet-rich structure, forms insoluble aggregates in the form of the prion rods or amyloid deposits and its structured C-terminal part has increased resistance to digestion by PK.

The recombinant α-helical form of PrP can be converted *in vitro* to a β-sheet conformation by using the novel technology of protein misfolding cyclic amplification (PMCA) which is analogous to DNA amplification by PCR and is potentially important for research (Saa *et al.* 2005) or by a cell-free system composed of substantially purified constituents. This conversion is selective and requires the presence of pre-existing PrPSc (Kocisko *et al.* 1994).

The four reported cases of a transmission of a disease by blood transfusion to humans (Llewelyn *et al*. 2004; Peden *et al.* 2004; *HPA Press Statement* 2007) stressed the need of a deeper and better understanding of a blood related prion pathogenesis, such as interaction of PrPSc with PrPC expressed on blood cells, *e.g.*, platelets (Holada *et al.* 2006).

In the present study we used the full-length mrPrP(23–231) as a model for studying the folding of the protein to α- and β-conformations. We have constructed fusion HAT-mrPrP, overexpressed it in *E. coli* and purified it from inclusion bodies by affinity chromatography in the denatured form. The critical step for the production of the recombinant PrP is a folding to the correct conformations. This was achieved by conversion of the denatured form to the soluble one, followed by oxidative folding of the soluble form into two distinguishable conformations. The purified HAT-mrPrP was characterized by measuring the circular dichroism spectra, by testing the resistance of the PrP to cleavage by PK and by its ability to form aggregates.

MATERIAL AND METHODS

Cloning of the mrPrP. The gene for mPrP(23–231) was amplified from cDNA (kindly provided by Dr. Stopka) using 5′-oligonucleotide containing *Ple*I (*New England Biolabs*) restriction site (5′-GGA GTC TGC AAA AAG CGG CCA AAG CC-3′) and 3′-oligonucleotide containing *Sac*I (*New England Biolabs*) site (5′-GGA GTC CTT AGT AAT AGG CCT GGG ATT CC-3′). Plasmid pHAT10 (*Clontech*) used as an expression vector contains a sequence encoding 24 amino acid residues of the *his* affinity tag (HAT) in the insertion site. The sequence of the cloned gene was verified by dideoxy sequencing.

Expression of the HAT-mrPrP. The vector was transformed into *E. coli* (BL-21) by classical heat shock for overexpression. One-hundred μL of the chemically competent cells prepared by standard methods was thawed on ice and 1 μL of the purified plasmid (c = 0.3 mg/mL) was added. The competent cells were left on ice for 20 min, followed by a 5-min shocking at 37 °C. The cells were then left on ice for 2 min and medium warmed to 37 °C was added. The transformed cells were left to grow at 37 °C for 30 min and 50 μL of the cell mixture was inoculated into a Petri dish with Luria–Bertani (LB) medium containing ampicillin (100 μg/mL) as a selection marker.

The bacterial culture was grown in 5 mL inoculum overnight at 37 °C and consecutively transferred to fresh medium (in a 1 : 100 volume ratio). Freshly inoculated bacterial culture was grown to A_{600} of 0.6. The expression of HAT-mrPrP was induced by adding IPTG to a final concentration of 1 mmo/L. The bacteria were harvested after 4 h by centrifugation (4000 ***g***, 15 min, 4 °C).

Purification of HAT-mrPrP. The bacterial pellet was resuspended in 7 mL of 20 mmol/L Tris-HCl (pH 7.4) and sonicated on ice with *Dynatech* Sonic Dismembrator ARTEK 300 with the biggest tip at 50 % output thrice for 30 s with a 90-s rest period. The inclusion bodies were collected by centrifugation (18 000 ***g***, 45 min, 4 °C) and dissolved in 8 mol/L urea–50 mmol/L phosphate buffer–150 mmol/L NaCl (pH 7.0) for 1 h at room temperature.

After subsequent centrifugation at 18 000 ***g*** for 1 h at room temperature, the supernatant containing the urea extract of HAT-mrPrP was loaded onto a column containing resin with immobilized Co^{2+} (BD talon; *Clontech*) and washed with five bed volumes of equilibrating buffer (50 mmol/L phosphate buffer–8 mol/L urea, pH 7.0). HAT-mrPrP was eluted by 10 mL of equilibration buffer containing 150 mmol/L imidazole and 1 mL fractions were collected.

The purity of HAT-mrPrP was analyzed on Coomasie Brilliant blue-stained SDS-PAGE gels and by Western blots.

The protein concentration was determined by measuring absorbance at 280 nm using calculate HAT-mrPrP molar absorption coefficient (ε_{280} = 63 450 L mol^{-1} cm^{-1}) and HAT-mrPrP molar mass (M = 25.3 kDa).

Anti-PrP monoclonal antibodies. The mixture of AG4 (*TSE Resource Centre*) detecting the N-terminal half of mPrP (epitope 31–51) at 1 : 1000 and 6H4 (*Prionics*) detecting the C-terminal half (epitope 144–152) at 1 : 5000 was used for detection of mrPrP. The mixture was used to detect possible cleavage fragments of the purified HAT-mrPrP.

Western blot. The samples were separated on 10 % SDS-PAGE gels and blotted on the 0.2-μm nitrocellulose membrane (*Bio-Rad*). The membranes were blocked with Tris-buffered saline–0.05 % Tween 20 containing 5 % dry milk. The blot was incubated with primary antibody overnight at 4 °C. The alkaline phosphatase-conjugated goat antimouse IgG (*BioSource*) was used as secondary antibody. The antibody diluted 1 : 2000 was incubated with the blot for 1 h at room temperature. The 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium (BCIP/NBT; *Chemicon*) was used as substrate for alkaline phosphatase.

Folding of HAT-mrPrP using oxidative conditions. Purified HAT-mrPrP in elution buffer was reduced by adding 100 mmol/L DTT (final concentration) for 90 min at room temperature. Subsequently, DTT, urea and imidazole were removed by desalting. One mL micropipette tips were loaded with Sephadex G-25 Fine (*Amersham*) equilibrated with 20 mmol/L acetate buffer (pH 4.0). Redundant buffer was discarded after centrifugation (500 ***g***, 15 min, room temperature) and 300 μL of reduced HAT-mPrP was loaded per tip. Samples were let to soak for 5 min and desalted HAT-mrPrP was collected after centrifugation (the same as *above*). Soluble reduced HAT-mrPrP was oxidized by air oxygen by stirring for 16 h in an open tube at room temperature. Biochemical and biophysical properties of oxidized and reduced HAT-mrPrP were determined.

Measurement of circular dichroism spectra. Far-UV CD spectra were recorded on *Jobin-Yvon* Mark VI (*France*) instrument at room temperature in 20 mmol/L acetate buffer (pH 4.0). The optical path length was 0.5 mm. In general, the spectra were averages of four computer-controlled scans taken with the time constant of 2 s and step size 0.5 nm. The spectra are expressed as molar ellipticity per residue using the protein concentration 0.5 mg/mL. Protein concentration of oxidized and reduced HAT-mrPrP was always 0.5 mg/mL.

Sucrose gradient fractionation of the HAT-mrPrP fibrils. The fractionation was performed on 10–60 % sucrose step gradient at 200 000 ***g*** for 1 h at 4 °C. Sucrose was dissolved in the buffer used for the oxidative folding. The samples of oxidized and reduced HAT-mrPrP were left to stay for 2 d at 4 °C to allow the formation of the fibrils. Samples were loaded on the top of the gradient and after the ultracentrifugation 0.75 mL fractions were collected by the injection needle from the bottom of the tube.

Dependence of proteinase K activity on pH. The testing of PK activity was done with QuantiCleave Protease Assay kit (*Pierce*). PK uses native casein that has been treated with succinanhydride to block primary amines on the surface of the protein. In the presence of a proteinase the succinylated casein is cleaved and exposes primary amines. These amines react with 2,4,6-trinitrobenzenesulfonic acid to produce color product the intensity of which is measured at 450 nm. The resulting increase in absorbance correlates with the activity of proteinase in the sample. The cleavage was performed in 20 mmol Tris (pH 7.0 and 8.0) or in 20 mmol/L acetate buffer (pH 5.0 and 6.0) with or without an addition of Ca^{2+} (5 mmol/L). The cleavage was carried out for 30 min at 37 °C and A_{450} was measured.

Cleavage of HAT-mrPrP by PK. Two μL of the oxidized and reduced HAT-mrPrP at a concentration of ≈0.3 mg/mL was incubated with different concentrations of PK (50, 5, 0.5 μg/mL) in 18 μL of 0.2 mol/L Tris (pH 8.0) for 30 min at 37 °C. The digestion was stopped by the addition of PMFS to a final concentration of 1 mmol/L. The samples were analyzed by Western blot using 6H4 monoclonal antibody to detect the C-terminal half (epitope 144–152) which is known to be partially resistant to cleavage by PK.

RESULTS AND DISCUSSION

Expression of the recombinant prion protein. In our first attempt to obtain the mrPrP(23–231) we used pMal C (*New England Biolabs*) expression vector but we were unable to produce sufficient amounts of protein using standard conditions either in soluble form or in inclusion bodies. The yield detected on Coomassie Blue-stained SDS-PAGE gels was poor. Neither increasing the concentration of IPTG from 1 to 4 mmol/L with a step of 0.5 nor decreasing the temperature from 37 to 25 °C or prolonging the time of the expression from 4 h to overnight had a positive effect. After induction of the expression, the cells rapidly stopped growing probably due to the toxicity of the fusion protein. Similar observations were reported earlier by Mehl-

horn *et al.* (1996). To overcome this problem we changed the vector to pHAT10 (*Clontech*). The mrPrP was expressed with N-terminal HAT into the cytosol of *E. coli* under the control of T7 promoter–*lac* operator system (Studier *et al.* 1986). The histidine tail was composed of 24 amino acids (KDHLI HNVHK EEHAH AHNK) and contained an enterokinase cleavage site (DDDDK) behind the last lysine of HAT. To avoid possible loss of the plasmid due the toxicity of the inclusion bodies the expression was performed with freshly transformed *E. coli* cells.

In this system we obtained large amounts of mrPrP (8–10 mg/L of the bacterial culture) accumulated in the inclusion bodies (Fig. 1). After the sonication, the inclusion bodies were collected by centrifugation and solubilized in 8 mol/L urea (*see Materials and Methods*). The purity of mrPrP in urea extract

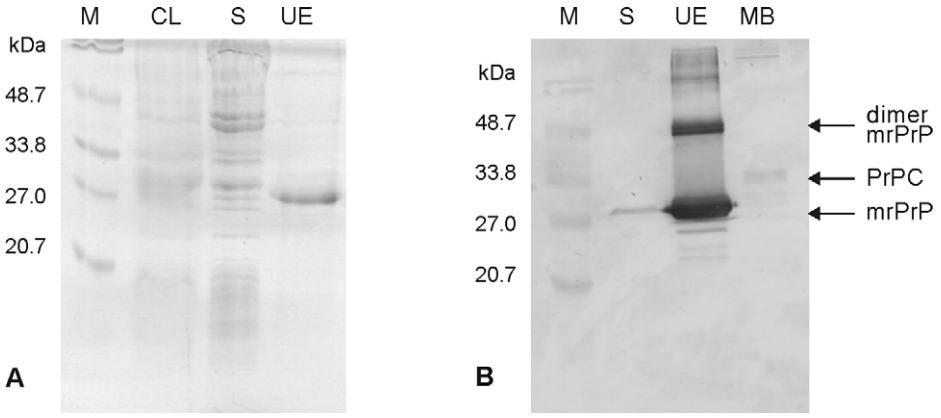

Fig. 1. HAT-mrPrP is expressed into inclusion bodies. **A**: 12.5 % SDS-PAGE stained with Coomassie Brilliant blue. **B**: Western blot analysis; PrP was detected with the mixture of monoclonal antibodies AG4 (1 : 1000) and 6H4 (1 : 5000). M – molar-mass standards (*BioRad*), CL – cell lysate of production culture, S – supernatant after sonication of induced *E. coli* (BL-21 cells), UE – urea extract of pellet after sonication of induced cells, MB – mouse brain homogenate..

of inclusion bodies was about 80 %. The urea extract was loaded on the metal-ion affinity-chromatography column, and mrPrP was purified in a one step using HAT. The recovery of mrPrP was approximately 70 % of the amount present in solubilized inclusion bodies. Purity of eluted proteins was analyzed by Coomassie Blue-stained SDS-PAGE gels and by Western blots (Fig. 2). Both methods revealed the formation of a dimer of the HAT-mrPrP which is in accordance with previously published data (Riley *et al.* 2002). The purity of eluted fractions was also checked by colloid silver-stained SDS-PAGE gels. No additional bands were visible in comparison with Coomassie Blue-stained gels (*data not shown*), suggesting a high purity of the prepared mrPrP (>95 %). The purification procedure showed a good reproducibility and can be successfully used as an easy one-step procedure with a high yield of purified recombinant protein.

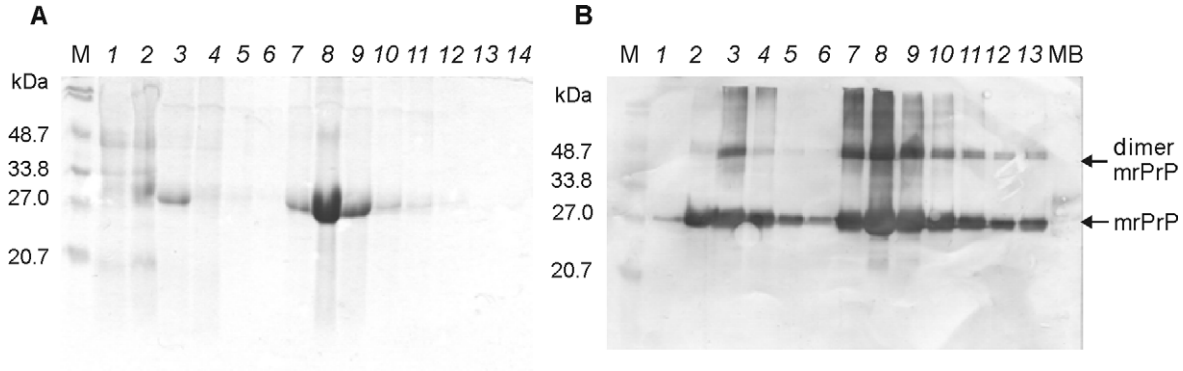

Fig. 2. Purification of HAT-mrPrP on immobilized metal ion affinity chromatography. **A**: 12.5 % SDS-PAGE stained with Coomassie Brilliant blue; *6–14* – eluted 1 mL fractions number *1–9* by 150 mmol/L imidazole. **B**: Western blot analysis of purification; *6–13* – eluted 1 mL fractions number *1–8* by 150 mmol/L imidazole; for the PrP detection *see* Fig. 1. M – molar-mass standards (*BioRad*), MB – mouse brain homogenate, *1* – uninduced cells, *2* – induced cell, *3* – urea extract of PrP, *4* – flow through column, *5* – wash of the column.

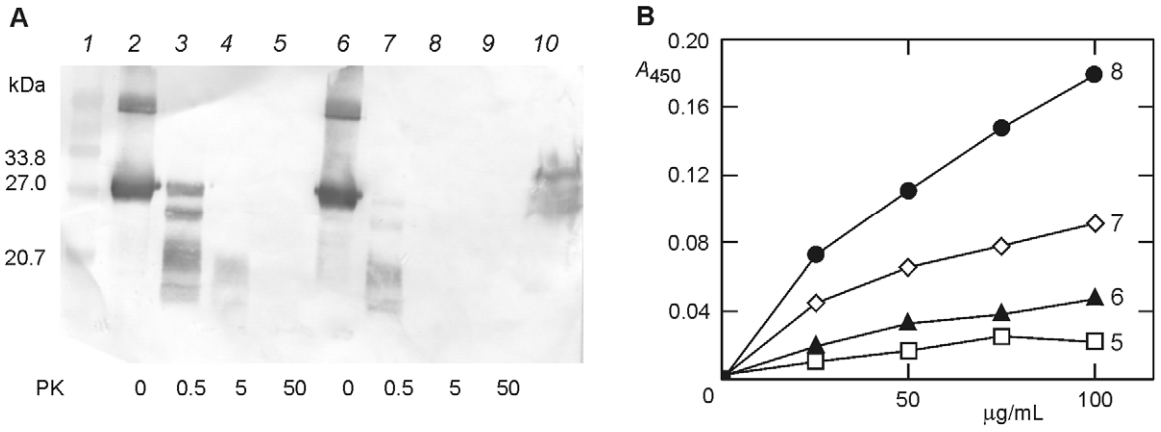

Fig. 3. Testing of the sensitivity of differently folded HAT-mrPrP to cleavage by PK (at concentrations of 0, 0.5, 5, 50 μg/mL). **A**: Western blot analysis of the HAT-mrPrP cleaved by the PK; *1* – molar-mass standards (*BioRad*), *2* – oxidized HAT-mrPrP without PK, *3–5* – oxidized HAT-mrPrP cleaved with different concentrations of PK, *6* – reduced HAT-mrPrP without PK, *7–9* – reduced HAT-mrPrP cleaved with different concentrations of PK; *10* – mouse brain homogenate. Oxidized HAT-mrPrP displayed increased resistance to PK treatment. **B**: Dependence of the proteolytic activity of PK (μg/mL) on pH (*numbers at curves*). Since the HAT-mrPrP was folded in 20 mmol/L acetate buffer (pH 4.0) it was necessary to determine the activity of PK at low pH with succinylated casein as substrate. The cleavage exposed primary amines that reacted with 2,4,6-trinitrobenzenesulfonic acid to produce a color product the intensity of which was measured at 450 nm.

Folding and characterization of HAT-mrPrP. The crucial step before measuring the CD spectra is a conversion of the insoluble purified mrPrP to a soluble form. We used desalting in tips (*see Material and Methods*) that permitted to obtain imidazole–urea–DTT-free, undiluted and soluble HAT-mrPrP. We tested a range of conditions of folding of the HAT-mrPrP to α-helical and β-sheet conformations. Different approaches to folding have been described: using Cu^{2+} ions (Wong *et al.* 2000), SDS (Leffers *et al.* 2005), or glutathion (Lu *et al.* 2001). After addition of the above chemicals, almost complete precipitation of mrPrP occurred. We determined that the highest solubility of HAT-mrPrP was achieved under acidic conditions; therefore, we used pH 4.0 for desalting the samples. At the physiological pH, the mrPrP precipitated and the yield of the desalting was low. The near-UV CD spectra of the oxidized mrPrP showed β-sheet content with some amount of α-helices and random coils. The reduced mrPrP showed more α-helical structure with a contribution of the random coils. The shape of the spectra could suggest that uncleaved HAT may influence the secondary structure of the mrPrP because the mean residue ellipticity is less negative than the values published in the literature (Lu *et al.* 2001) (Fig. 4). Our observation of β-sheet folding of the oxidized mrPrP is in contrast with published reports. Possible explanation of this difference may lay in the presence of HAT in the molecule of our mrPrP.

We used an assay based on known different susceptibility of α- and β-conformations to cleavage by PK as another tool for characterization of folded HAT-mrPrP. The pathogenic β-sheet-rich conformation is known to have increased resistance of the folded core [C-terminal part of the protein PrP(90-231)] to proteolysis. On the other hand, α-helical normal conformation of PrP is completely cleaved by PK. Because our folding experiments were done at pH 4.0 (necessitated by the high solubility of the HAT-mrPrP) the pH dependence of the PK activity was tested. PK had very low enzyme activity at acidic pH (Fig. 3B) with no significant effect due to an addition of Ca^{2+} (*data not shown*). For this reason it was necessary to change the pH of the cleavage buffer to a weak alkaline. Solubilized HAT-mrPrP was converted from pH 4.0 (20 mmol/L acetate) to pH 8.0 by the addition of excess 0.2 mol/L Tris (pH 8.0). Treated samples were separated by SDS-PAGE, blotted and the cleavage products were detected by monoclonal antibody 6H4 which detects an epitope in the C-terminal half of the molecule of HAT-mrPrP. The treatment of the reduced HAT-mrPrP with PK (5 μg/mL) led to a complete digestion of the protein which is in a good agreement with the spectroscopic measurements showing an α-helical structure combined with some unstructured part. In contrast, the oxidized HAT-mrPrP treated with the same concentration of PK showed partial resistance to the digestion which also corresponds with the spectroscopic data showing a β-sheet combined with random coil (Fig. 3A). We did not observe the formation of high-molar-mass bands on nonreducing SDS-PAGE gels (*data not shown*), suggesting that increased resistance to PK was not due to the formation of disulfide bond stabilized multimers of oxidized HAT-mrPrP.

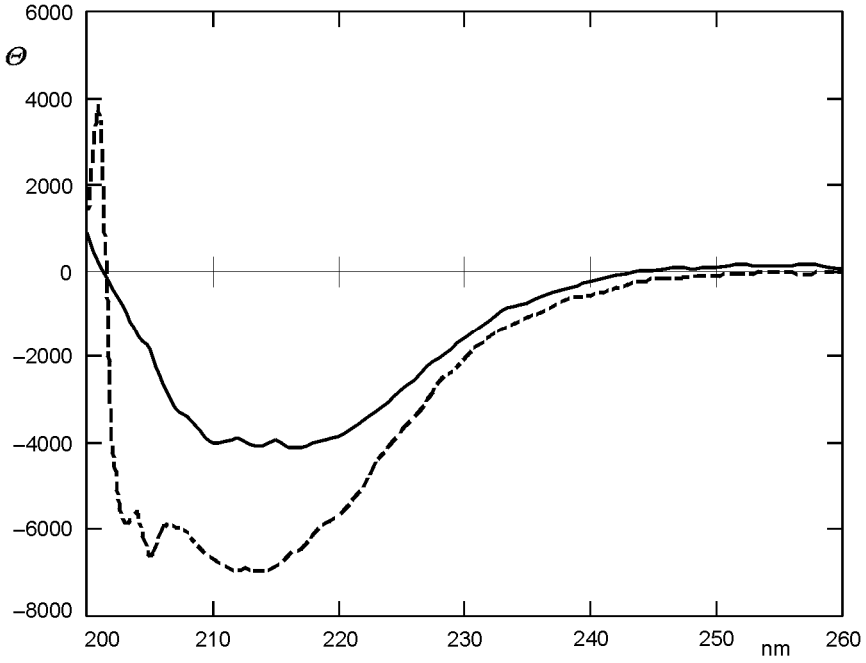

Fig. 4. Far-UV circular dichroism (molar elipticity Θ, $^\circ cm^2 dmol^{-1}$) spectra of HAT-mrPrP(23-231); the spectra were recorded at 0.5 mg/mL HAT-mrPrP in 20 mmol/L acetate buffer (pH 4.0) at room temperature; *solid line* – oxidized HAT-mrPrP, *dashed line* – reduced HAT-mrPrP.

PrPSc (β-sheet form) is known to form fibrils. By using centrifugation on a sucrose gradient it is possible to distinguish between a monomeric soluble form of PrP and its fibrils. PrPC (α-helical form) is distributed after centrifugation in a low concentration part of the sucrose gradient while the β-sheet form is distributed throughout the whole sucrose gradient (Fig. 5).

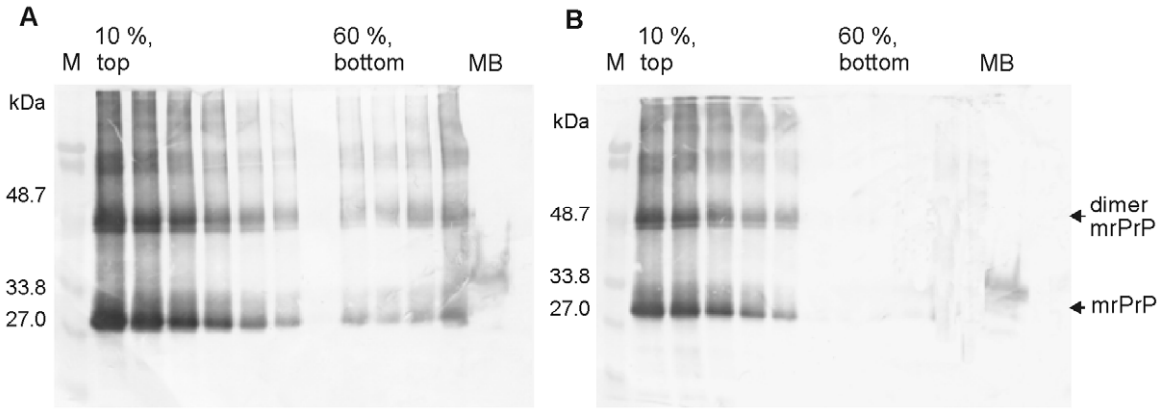

Fig. 5. Fractionation of differently folded HAT-mrPrP in sucrose gradient (10–60 %); 60 μg aliquots of the oxidized (**A**) and reduced (**B**) HAT-mrPrP were loaded on the top of the sucrose gradient and ultracentrifuged. Western blots of collected fractions were developed with the mixture of monoclonal antibodies AG4 (1 : 1000) and 6H4 (1 : 5000). Oxidized HAT-mrPrP was distributed throughout the whole sucrose gradient; reduced HAT-mrPrP was distributed only in low concentration of the gradient. M – molar-mass standards (*BioRad*), MB – mouse brain homogenate.

All these observations support the successful folding of the HAT-mrPrP to a native-like soluble α-helical conformation and a β-sheet containing an abnormal-like conformation.

In conclusion we have described an effective and reproducible method of the overproduction and purification of HAT-mrPrP that yields mg quantities of the protein. Furthermore, we have been able to convert the insoluble HAT-mrPrP to the soluble form and to fold it into α- and β-conformations which was confirmed by CD spectroscopy, PK treatment and also by analysis of fibril formation.

The HAT-mrPrP so prepared can be used in studies of its interactions with cellular binding partners or for developing conformation specific PrP monoclonal antibodies. The uncommon sequence of HAT should allow the detection of HAT-mrPrP and distinguish it from PrPC in complex biological samples. The ability of HAT-mrPrP in β-sheet conformation to bind to blood cells expressing different levels of PrPC is the subject of ongoing studies. These studies could shed some light on the mechanism of PrPSc distribution in blood of TSE infected donors.

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