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Production of recombinant human β_2-microglobulin for scintigraphic diagnosis of amyloidosis in uremia and hemodialysis

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Amyloid of β_2-microglobulin (β_2m) origin can be diagnosed using ^{131}I-radiolabelled-β_2m scintigraphy in patients with uremia and hemodialysis treatment. As the tracer β_2m is isolated from another patient, it carries the common risks, including viral infections such as Hepatitis B, C and HIV, which are associated with human plasma products.

In order to exclude these risks we have produced recombinant human β_2m (rhβ_2m) in *Escherichia coli*. The expression vector pASK40ΔLβ_2m(His)$_5$ contains a C-terminal (His)$_5$-tag for purification via immobilized metal ion affinity chromatography (IMAC). Size exclusion chromatography on a Superose 12 column represents the second step of purification.

The isolated rhβ_2mH$_5$ reacted in an immunochemically identical manner to native human β_2m, and showed a single band of ≈ 11.8 kDa in Western blot analysis and revealed a single spot in two-dimensional gel electrophoresis. Mass spectrometry analysis revealed a single peak at the expected molecular mass of 12 415.8 Da. Uniformity was further proven by crystallization and N-terminal amino-acid sequence analysis.

The rhβ_2mH$_5$ protein was then produced under conditions that allow the intravenous use in humans. Intraveneously applied indium-111-labelled rhβ_2mH$_5$ was monitored in hemodialysed patients with and without known β_2m-amyloidosis. The tracer was localized specifically to particular areas known to contain amyloid.

Thus, this rhβ_2mH$_5$ preparation is suitable for detecting amyloid-containing organs of the β_2m-class *in vivo* and fulfils the requirements of a tracer for common use. Finally, the use of indium-111 instead of iodine-131 has reduced the radioactive load and resulted in higher resolution.

Keywords: amyloidosis; recombinant β_2-microglobulin; scintigraphy; indium-111; whole-body diagnosis.

Amyloidosis of β_2-macroglobulin (β_2m) origin is a serious disease in patients with long-standing uremia and hemodialysis treatment. It is seen to occur with increasing prevalence with the duration of dialysis, with age of the patient and with the kind of dialyzer used [1–3]. As this class of amyloidosis causes severe osteo-articular symptoms with carpal tunnel syndrome and bone erosions which lead to pathologic bone fractures, early diagnosis is needed in order to achieve a timely therapeutic intervention.

As early bioptic diagnosis has not yet been established, minimal invasive scintigraphic diagnosis has been reported using ^{131}I-labelled β_2m [4] which was confirmed by others [5–7]. However, the tracer β_2m in all studies was isolated from urine or uremic ultrafiltrate of other patients. Although these selected donor patients had been carefully scrutinized for any possible transmissible disease, there still remains a risk, including viral infections such as Hepatitis B, C and HIV, inherent in all human plasma products.

As such a tracer may only be applicable in a limited and strictly controlled setting, it cannot be commonly applied and its commercial distribution is not feasible due to the remaining risks. The same applies to the human amyloid-P component scintigraphy which has been used to diagnose various amyloidoses including β_2m-amyloidosis [8–10].

To avoid the mentioned risks and increase security we have expressed the human precusor β_2m as a recombinant protein. Here, we present its production, isolation and characterization. Finally, we illustrate the applicability of this protein as a tracer for scintigraphic identification and the localization of anatomical sites containing amyloid of the β_2m-class.

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Abbreviations: β_2m, β_2-microglobulin; IMAC, immobilized metal ion affinity chromatography; IPTG; isopropyl thio-β-D-galactoside;

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MATERIALS AND METHODS

Construction of the expression plasmid pASK40ΔLhβ_2m(His)$_5$

Human β_2-microglobulin (β_2m) was produced in *Escherichia coli* as a recombinant polypeptide with five histidine residues attached to the carboxyterminus.

RNA of the human lymphoblastoid B cell line LG2 was isolated and transcribed into cDNA as previously described [11]. One μL cDNA was amplified by PCR (5 cycles 1 minute annealing at 46 °C, 1 minute elongation at 72 °C and 25 cycles with an annealing temperature of 66 °C) with a 5′-oligonucleotide (5′-GCGCTCGGTACCTATCCAGCGTACTCC-3′) containing a *Kpn*I recognition sequence (underlined) with ATC encoding the first amino-acid, isoleucine, of the mature β_2m polypeptide. The 3′-primer (5′-GTCAAGCTTATCAGTGATGGTGATGGTGCATGTCTCGATCCCACTT-3′) has a *Hin*dIII recognition sequence (underlined) and adds five histidine

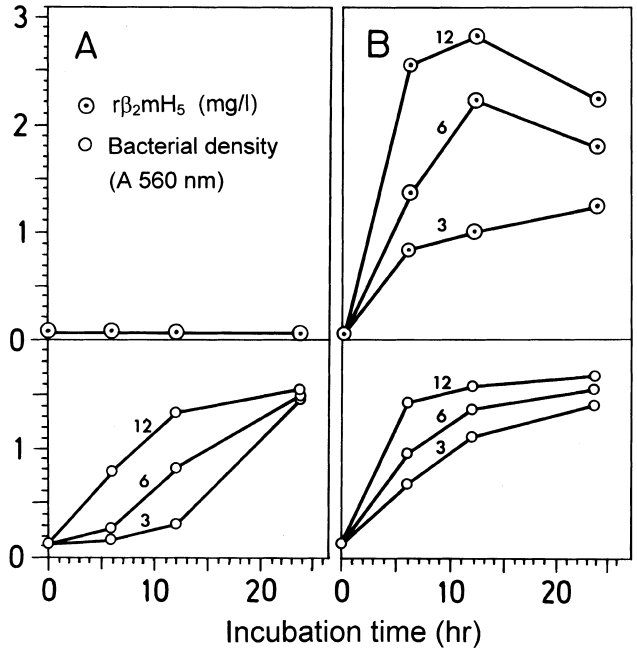

Fig. 1. Synthesis of rhβ$_2$mH$_5$ under different culture temperatures (A, 37 °C; B, 22 °C) and various durations of incubation. Quantitation of bacterial cell density at 560 nm (*y*-axis) and the concentration of rhβ$_2$mH$_5$ using radial immunodiffusion. The *x*-axis indicates the length of incubation of the bacterial culture at 37 °C, and the numbers next to the curves reveal the time in hours after induction with IPTG.

residues to the C-terminal methionine and two consecutive translational termination codons. The amplified fragments were digested with *Kpn*I and *Hin*dIII, purified by agarose gel electrophoresis and ligated into the vector pASK40 cut with *Kpn*I and *Hin*dIII. Restriction enzymes, T4 ligase, *Taq* polymerase, and reverse transcriptase were purchased from Boehringer Mannheim, Germany. The recombinant subclones were sequenced to confirm the inserted β$_2$m sequence. The N-terminal sequence of mature β$_2$m was fused to the *ompA* signal peptide by deleting the sequence between the *ompA* signal peptide sequence and the ATC of the β$_2$m sequence via *in vitro* mutagenesis [12]. Site-directed mutagenesis was performed with a single-stranded template, and the oligonucleotide β$_2$m–deletion: 5′-GAGTACGCTGGATGGCCTG-GCGTA-3′ and Klenow polymerase (Boehringer Mannheim) following standard protocols. *In vitro* generated double-stranded plasmid was transformed into JM83 bacteria [12]. Colonies were transferred to Hybond N$^+$ filters (Amersham Buchler, Braunschweig, Germany) and screened by hybridization with the ^{32}P-labelled β$_2$m–del oligonucleotide following the protocol of Cate *et al.* [13].

Expression of rhβ$_2$m

The protein rhβ$_2$mH$_5$ was expressed basically following [12]. In brief, a culture grown agitated in LB-medium (10 g tryptone, 5 g yeast extract, both from Difco Lab., Augsburg, Germany; 10 g NaCl in 1 L of demineralized H$_2$O) supplemented with 100 μg·L^{-1} ampicillin (Sigma, Deisenhofen) at room temperature overnight was added to a 200-mL and later a 2-L culture, and incubated in the same manner. At a bacterial density of D_{560} = 0.6 isopropyl thio-β-D-galactoside (IPTG, Sigma) was added to a final concentration of 1 mM. After 9 h incubation at room temperature the cells (D_{560} = 1.5) were collected by centrifugation at 8000 ***g*** (Sorvall ultracentrifuge, rotor SS34) for 30 min at 4 °C. In order to optimize the procedure, the temperature and time of incubation, without and with IPTG, have been varied. As shown in Fig. 1, cells were grown at 37 °C or 22 °C (room temperature) for 6, 12 or 24 h before they were induced by IPTG for another 3, 6 or 12 h. Cells were harvested and the concentration of rhβ$_2$H$_5$ determined by radial immunodiffusion [14].

Isolation using immobilized metal ion affinity chromatography (IMAC)

A chelating Sepharose Fast Flow column (15 mL, Pharmacia Biotech GmbH, Freiburg, Germany) was regenerated using 50 mM EDTA in 1 M NaCl at pH 8.0 and subsequently washed with bidistilled water. The column was then loaded with 20 mL of ZnCl$_2$ (1 mg·mL^{-1}) and washed again with water. After equilibration with 50 mM Tris/HCl and 1 M NaCl at pH 8.0 the supernatant of the bacterial lysate was applied and run in the same buffer. The cells from a 10-L culture were resuspended in 50 mL of 50 mM Tris/HCl, 1 M NaCl, pH 8.0, passed twice through a French press (Aminco, SCM Instruments Inc, Urbana, IL, USA) at 920 bar, and centrifuged at 35 000 ***g*** for 30 min at 10 °C (Sorvall). The supernatant was cleared from the remaining DNA and RNA by benzonase (1 IU·mL^{-1} in 1 mM MnCl$_2$; Merck, Darmstadt, Germany) before chromatography. The column was washed with Tris/HCl buffer, preeluted with 10 mM and subsequently with 15 mM imidazole (Sigma) before the imidazole gradient of 15–200 mM was started for eluting the rhβ$_2$mH$_5$ (Fig. 2). The rhβ$_2$mH$_5$ was identified, quantitatively monitored and compared with native β$_2$m isolated from urine (purchased from Sanderson Co., Sussex, UK) and from an uremic ultrafiltrate [15] using two-dimensional double immunodiffusion [16] and radial immunodiffusion [14]. Fractions containing rhβ$_2$mH$_5$ were collected and concentrated via Centricon-3 (Amicon GmbH, Witten, Germany) to approximately 3 mg·mL^{-1} and applied to a Superose 12 column (Pharmacia GmbH) run in 0.75 M NH$_4$HCO$_3$, pH 8.0. The effluent was fractionated and

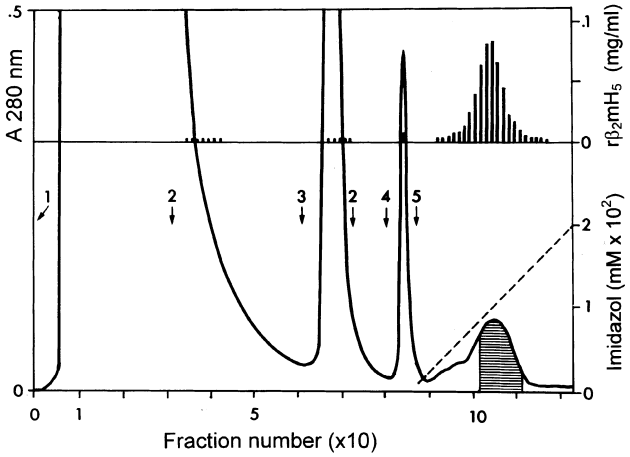

Fig. 2. First step of isolating of rhβ$_2$mH$_5$ from bacterial lysate using immobilized metal ion affinity chromatography. 1, Application of lysate; 2, Tris/HCl buffer-wash; 3 and 4, preelution with 10 and 15 mM imidazole; 5, elution with an imidazole gradient (10–200 mM). Fractions were collected (cross-hatched) after quantitation of rhβ$_2$mH$_5$ using radial immunodiffusion (upper panel).

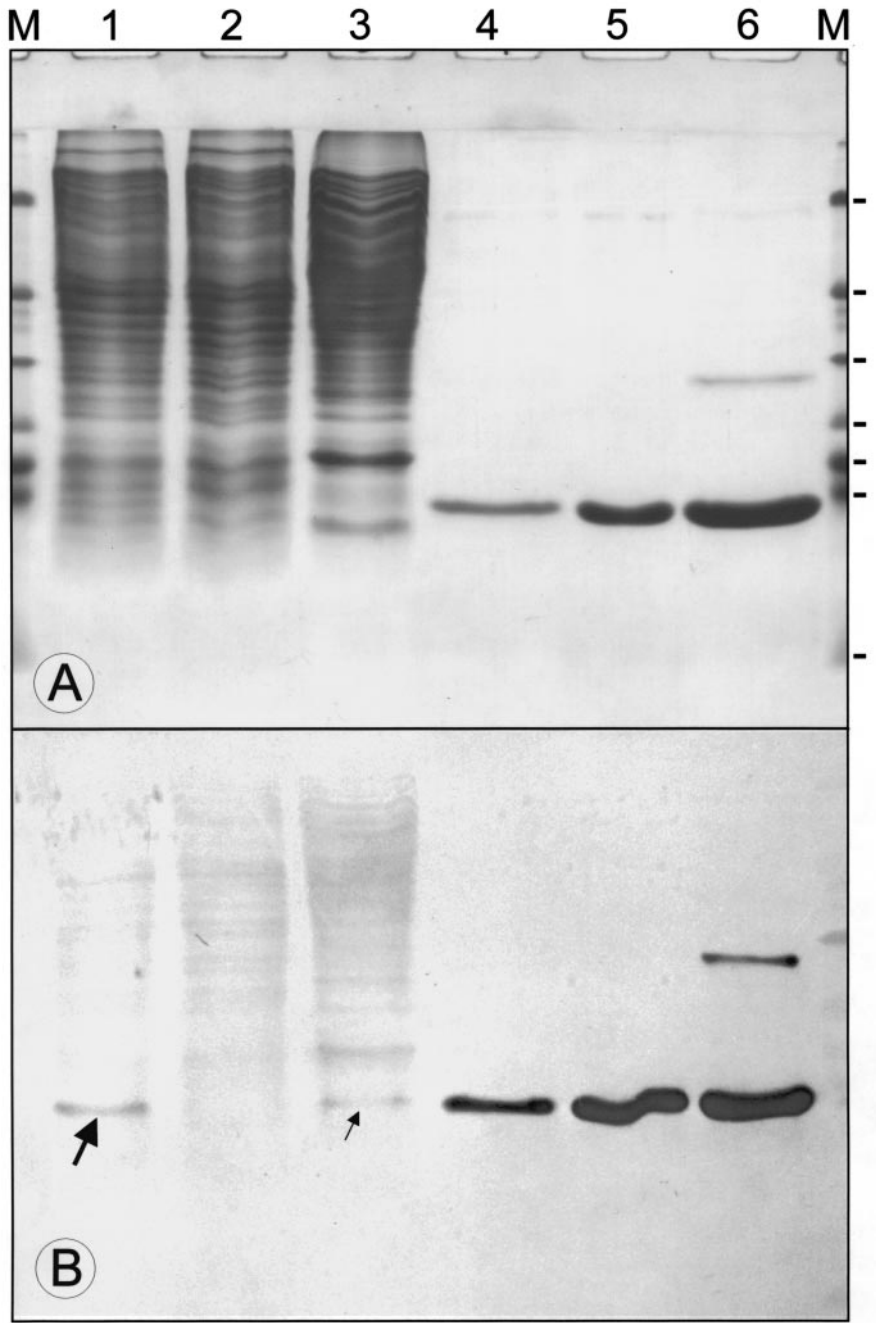

Fig. 3. Flat bed polyacrylamide gel (12.5%) electrophoresis illustrating steps in isolating rhβ_2mH_5 from a bacterial lysate. Anode is at the bottom. Staining was with Coomassie Blue. Lanes: 1, bacterial lysate; 2, effluent (first peak in Fig. 2); 3, preelution with 15 mM imidazole (third peak of Fig. 2); 4 and 5, eluate containing 5 and 10 μg of rhβ_2mH_5 (fourth peak of Fig. 2) 6, lyophilized after gel filtration (Fig. 5, fraction 27–28); M, molecular mass markers, from top to bottom: bovine serum albumin (66 kDa), ovalbumin (44 kDa), Bence–Jones protein (24 kDa), soybean trypsin inhibitor (20 kDa), equine myoglobin (17.3 kDa), cytochrome *c* (12.3 kDa), insulin B-chain (3.5 kDa). Immunodetection of rhβ_2mH_5 with rabbit anti-β_2m after electrotransfer to a nitrocellulose membrane. Traces of rhβ_2mH_5 are identified in the bacterial lysate (large arrow) and in the 15 mM imidazole preeluate (small arrow). Only monomeric (lane 4 and 5) and additional dimeric rhβ_2mH_5 are visible (lane 6).

quantitatively monitored [14]. Fractions containing rhβ_2mH_5 were collected, concentrated in the same way and stored frozen. Part of the protein preparation was dialyzed against water and lyophilized for analysis.

Quality control

Uniformity of the isolated rhβ_2mH_5 was probed using polyacrylamide gel electrophoresis [17], Western blotting [18] using a rabbit anti-β_2m serum (Dakopatts, Glostrup, Denmark), two-dimensional electrophoresis in polyacrylamide (charge–size, using isoelectric focusing and sizing in SDS [19]) as well as electrospray mass spectrometry on an API III triple quadrupole mass spectrometer (Sciex, Toronto, Canada). For further details see Fig. 3. N-terminal amino-acid sequence analysis was performed using a model 477A protein sequencer (Applied Biosystems, Weiterstadt, Germany) according to standard protocols provided by the company.

Lyophilized rhβ_2mH_5 was dissolved at different concentrations in a variety of buffers. Crystallization was attempted with various precipitants [20] in a hanging drop at 4 °C for several months per set.

Patient

A 51-year-old male patient, with chronic glomerulonephritis and a hypoplastic left kidney, finally resulting in anuria, underwent home-dialysis from age 23 for 13 years followed by center dialysis for another 11 years. A first renal transplantation at age 47 was not successful. A second

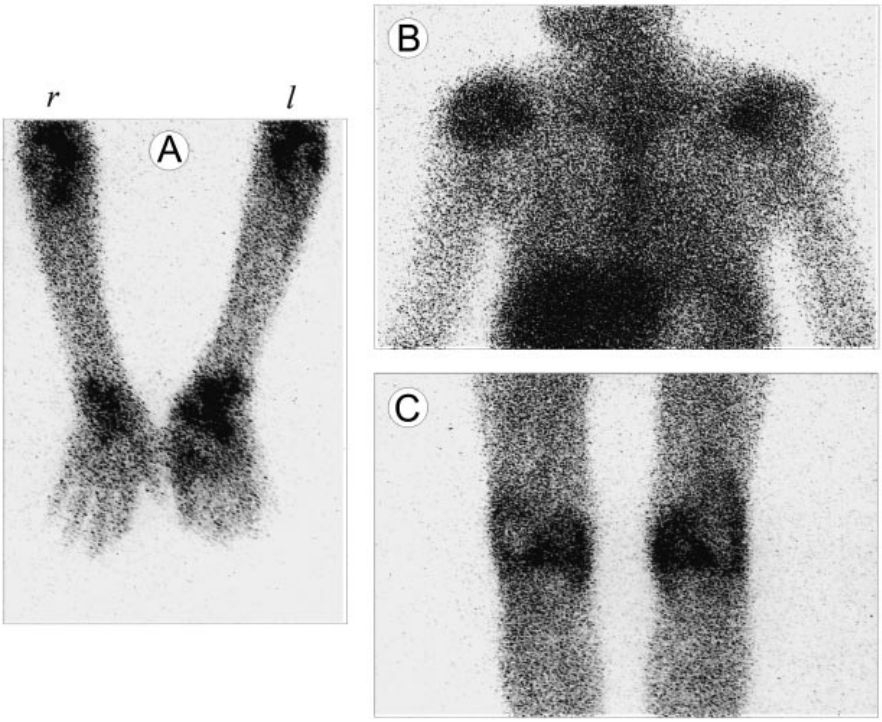

Fig. 4. Whole body ^{111}In-labelled rhβ_2mH_5 scintigraphy in a patient with Aβ_2m amyloidosis. Demonstrations of significant tracer uptake particular in areas with amyloid deposits. Orientation in all images: r, right; l, left. (A) Palmar scan image of both hands and forearms with articular tracer uptake. (B) Ventral image of shoulders (uptake of tracer by the hepatic reticulo-endothelial system is unspecific and not indicative for amyloid).Ventral image of knees with intense tracer uptake.

renal transplantation at age 50 has been successful until the present. At age 30, seven years after the start of dialysis, amyloidosis of the Aβ_2m-class was diagnosed by a fine needle biopsy of a radiolucent bone defect in the *os naviculare* of the left wrist. At that time, spondylarthropathy, periarthritis humoeroscapularis and bilateral carpal tunnel syndrome were present, further correlating the diagnosis of Aβ_2m amyloidosis.

Whole body scintigraphy with iodine-131-labelled β_2m

An iodine-131-labelled natural β_2m scintigraphy revealed additional amyloidosis in the knee and shoulder regions already at the age of 40.

Whole body scintigraphy with indium-111-labelled rhβ_2mH_5

At age 50, the local Ethics Commission at the Medical Faculty of the University of Hannover, Germany, approved the current study, and the patient consented to undergo a second scintigraphy using the recently improved method employing rhβ_2mH_5 described here. The transformed *E. coli* clone and the protocol of production and isolation was handed over to Boehringer Co (today *Roche-Pharma*) Penzberg, Germany which produced this protein under GMP (general medical practice) conditions that allow the diagnostic application to humans as described previously [21]. The rhβ_2mH_5 was radiolabelled with ^{111}In via the covalently bound chelator diethylentriamin-pentacetic acid (DTPA) (Aldrich) [22]. At day zero, 70 MBq ($\approx$ 70 μg) of ^{111}In-DTPA-rhβ_2mH_5 was intravenously injected. A low-flux dialysis at day one and a high-flux dialysis at day three followed in order to remove circulating tracer for minimal background tracing. Whole body scintigraphy and additional images of selected joint regions were carried out on day three. Regional images are shown in Fig. 4.

RESULTS

Identification of the correct DNA sequence

Plasmids of positive clones were analyzed by restriction enzyme digestion and DNA sequencing for the presence of the correct β_2m sequence fused to the *ompA* signal peptide. One clone, showing the identical sequence to the wild type β_2m, ΔLhuβ_2m$(His)_5$ was used to produce recombinant human β_2m in *E. coli*.

Improving the expression of rhβ_2mH_5

Initial experiments with bacterial cells induced with IPTG at 37 °C resulted only in a marginal expression of rhβ_2mH_5 or no expression at all, while cell growth and production of rhβ_2mH_5 greatly improved at room temperature, i.e. 22 °C (Fig. 1). Therefore, the optimal expression was examined under different conditions as detailed in Fig. 1. The highest expression of almost 3 mg·L^{-1} of bacterial medium was measured when cells were grown for 6 h at 37 °C and induced with IPTG for 12 h at 22 °C.

Expression and isolation

Using optimal expression conditions rhβ_2mH_5 was produced in several different volumes including a fermenter of 150 L. One example isolating rhβ_2mH_5 using IMAC from approximately 30 g of bacterial cells (wet weight) is shown in Fig. 2. The preeluting steps using 10 mM and subsequently 15 mM (or only 15 mM) of imidazole primarily removed proteins which immediately preceded the peak representing rhβ_2mH_5, thus yielding a protein of at least 90% purity with a purification factor of approximately 1000 using IMAC. Quantitative determination of rhβ_2mH_5 revealed that the effluent of the imidazole gradient contained the majority of the rhβ_2mH_5 as the unbound proteins of the effluents of both prewash eluates and the regeneration step were found to be free or almost free of rhβ_2mH_5. Only the 15 mM preeluting fraction contained a trace

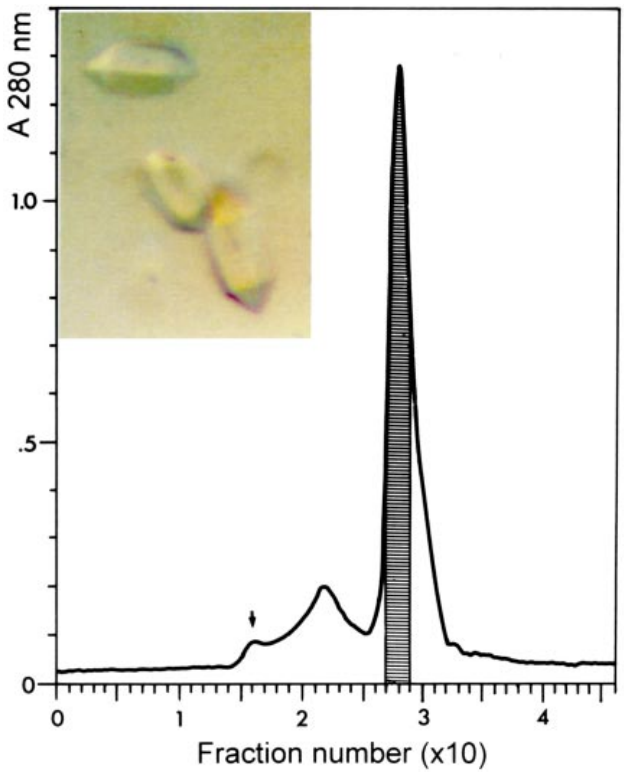

Fig. 5. Second step of isolating rhβ$_2$mH$_5$ using Superose 12 in 0.75 M ammonium carbonate, pH 8.0. Collected fractions are cross-hatched. Void volume is indicated by an arrow. Inset: crystals of rhβ$_2$mH$_5$ (length approximately 0.2 mm).

of rhβ$_2$mH$_5$ (Fig. 3, lane 3 in B, small arrow). A further isolation step included size-exclusion chromatography (Fig. 5). The major protein of 11.8 kDa was immunochemically shown to represent rhβ$_2$mH$_5$. The main protein was collected as indicated (cross-hatched fractions) and analyzed as shown below. From 30 g of packed cells approximately 8 mg of pure protein was isolated.

Characterization

The isolated rhβ$_2$mH$_5$ was examined using polyacrylamide gel electrophoresis together with other effluents and eluates. As shown in Fig. 3A, lane 4 and 5 the purified rhβ$_2$mH$_5$ represents a single band at 11.8 kDa. Western blot analysis in Fig. 3B identified this protein as rhβ$_2$mH$_5$. Aside from this 11.8-kDa band, a dimer (23 kDa) appeared after size-exclusion chromatography and lyophilization (Fig. 3B, lane 6). This dimer was detected neither in the original cell lysate (Fig. 3A, lane 1) nor after IMAC (Figs 3 and 5). Overloading of the polyacrylamide gel allowed to estimate the purity of the rhβ$_2$mH5 (not shown). The isolated protein had at least 98% purity.

In two-dimensional polyacrylamide gel electrophoresis (isoelectric focussing and sizing), only one single spot was visible, a feature which indicates uniformity (not documented). The isoelectric point was found to be at 6.8. The higher value as compared to the native protein of 5.9 is most likely due to the pentahistidine tail added for easy purification.

The N-terminal amino-acid sequence was identical to the one of the native protein in the first 27 amino-acid residues determined and also identical up to position 39, except for 3 residues that could not be determined (33, 35 and 38, marked with * below) and two uncertainties in position 28 (serine) and 36 (glutamic acid) (as indicated with s and e, respectively). The missing amino acid in position 35 is consistent with the presence of cysteine. The sequence of rhβ$_2$mH$_5$ obtained is: IQRTP KIQVY SRHPA ENGKS NFLN* YVsGF HP*DI eV*L.

Electrospray-mass spectrometry (Fig. 6) resulted in a single peak of 12 415.8 Da which is two protons less than the calculated value of 12 417.9 Da of the reduced form, in good agreement with the assumption of an intact disulfide bridge. Thus, the rhβ$_2$mH$_5$ had exactly the predicted mass value.

Crystallization experiments resulted in crystals under one condition (0.1 M Tris/HCl, 0.2 M ammonium sulfate, 2.0 M K/Na phosphate, pH 6.5, at 4 °C.). These crystals (approx. 0.2 mm length, Fig. 5, inset), however, did not allow the establishment of the X-ray structure. We are in the process of improving the crystals by varying the conditions including hysteresis experiments which had been performed with the expectation to possibly improve the packing of rhβ$_2$mH$_5$ in these crystals [23,24].

Whole body scintigraphy using ^{111}In-DPTA-rhβ$_2$mH$_5$

As shown in Fig. 4, scintigraphic labelling occurred in anatomical regions known to contain amyloid [2–7]. In

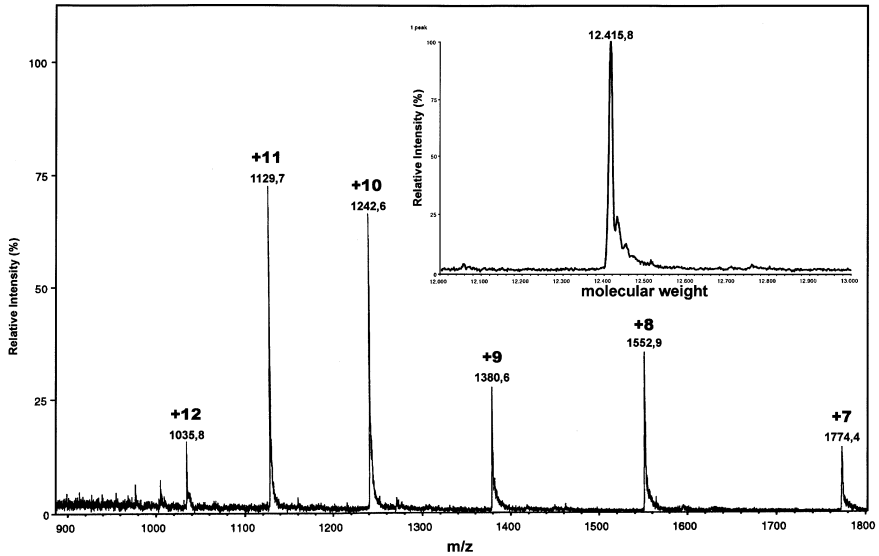

Fig. 6. Electrospray-mass spectrometry of rhβ$_2$mH$_5$. Numbers on top of the peaks represent charge states. Inset: computer-reconstructed correct mass of rhβ$_2$mH$_5$.

Fig. 4a, a palmar scan image of both hands and forearms revealed pronounced tracer accumulation in both elbow regions, both wrists and metacarpo-phalangeal joints on the left. Figure 4b shows a ventral image of the shoulders with bilateral positive tracer uptake, nonspecific tracer uptake by the liver and, to a lesser extent, in the spleen is also visible. This uptake by the reticulo-endothelial system is most likely unspecific for amyloid as it occurs with other ^{111}In-labelled proteins and with free indium as well. The traced regions are identical to those identified with the first imaging using radio-iodine-β_2m (see above) and conform with the characteristic distribution of Aβ_2m amyloid.

DISCUSSION

In order to reduce the potential risks of plasma proteins for patients and to standardize the tracer for better resolution we produced and characterized rhβ_2mH_5. Expressed in transformed *E. coli* with a vector introducing a correct removal of the *ompA* signal peptide and adding the carboxyterminal pentahistidine tail, this protein could be isolated to nearly absolute purity by a two step procedure. We found no indication of any interference of the pentahistidine tail that could have altered properties of this rhβ_2mH_5 except for the pI. The mild purification procedure allowed the isolation of rhβ_2mH_5 in an apparently functional state in agreement with findings on functional immunoglobulin domains which were also produced with a $(His)_5$-tag [24].

The rhβ_2mH_5 was homogeneous according to charge and mass. There was only one single N-terminal amino-acid sequence without any hint of another peptide proving identity with the natural protein without degradation during the isolation procedure. The correct sequence was also supported by results obtained by mass spectrometry which identified the expected molecular mass. The lack of two protons on the rhβ_2mH_5 mass as compared to the calculated mass is consistent with the intact disulfide bridge (as in the native protein). Our rhβ_2mH_5 was stable in physiological buffers, it could be frozen and lyophilized. It could be crystallized, although its internal packing was not sufficiently ordered to establish its three-dimensional structure. This rhβ_2mH_5 reacted immunochemically [14,16] identical to human native β_2m isolated from ultrafiltrate or urine.

Most importantly, this rhβ_2mH_5, after ^{111}In-labeling, revealed an identical scintigraphic distribution and fixation pattern in the same patient diagnosed with human ^{131}I-labeled isolated from ultrafiltrate years before. This also includes the uptake in the spleen and liver which is also observed in other In-labeled proteins. Thus, this new tracer is as specific for organs containing Aβ_2m-amyloid as natural β_2m as no tracer was fixed in organs lacking amyloid and no fixation above background has been noted in patients without amyloidosis [21].

The recombinant tracer will not only increase safety for the patient by excluding human plasma proteins with all the inherent risks (hepatitis B and C, HIV and others), but will also reduce considerably the radioactive load for each examination (by 50–70%) by improving the labelling technique using the radioactive tracer ^{111}In [21,22]. Another important advantage is the higher resolution of the whole body scintigraphy which improves the diagnostic value of this reagent. This new tracer has been applied to a series of patients with excellent results [21]. Thus, ^{111}In-rhβ_2mH_5 scintigraphy has the potential to serve as a valuable tool world-wide for the early, specific and non-(or mininal-)invasive diagnosis of Aβ_2m amyloidosis in patients with uremia and end stage renal diseases.

Finally, this rhβ_2mH_5 can also be used as a model protein for clarifying important questions concerning the pathogenesis of β_2m amyloidosis *in vitro*. It seems to be well suited as it excludes the presence of any other human plasma protein that could unintenionally interfere with experiments investigating the amyloidogenesis *in vitro*.

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