Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1753 (2005) 23 – 33
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Review

Proteomics of β2-microglobulin amyloid fibrils

Monica Stoppini[a], Palma Mangione[a], Maria Monti[b], Sofia Giorgetti[a,c], Loredana Marchese[a], Patrizia Arcidiaco[d], Laura Verga[c], Siro Segagni[e], Piero Pucci[b], Giampaolo Merlini[a,c], Vittorio Bellotti[a,c,*]

[a]*Dipartimento di Biochimica, Università degli Studi di Pavia, via Taramelli 3b, 27100 Pavia, Italy*
[b]*CEINGE Biotecnologie Avanzate, scarl, Università di Napoli Federico II, via Comunale Margherita 482, 80145 Napoli, Italy*
[c]*Laboratorio di Biotecnologie, IRCCS Policlinico S. Matteo, p.le Camillo Golgi 2, 27100 Pavia, Italy*
[d]*Centro Grandi Strumenti, Università degli Studi di Pavia, via Bassi 21, 27100 Pavia, Italy*
[e]*Divisione di Nefrologia e Dialisi, Fondazione Salvatore Maugeri, Via Ferrata, 4, 27100 Pavia, Italy*

Received 14 June 2005; received in revised form 28 July 2005; accepted 28 July 2005
Available online 15 August 2005

Abstract

Knowledge on the chemical structure of β2-microglobulin in natural amyloid fibrils is quite limited because of the difficulty in obtaining tissue samples suitable for biochemical studies. We have reviewed the available information on the chemical modifications and we present new data of β2-microglobulin extracted from non-osteotendinous tissues. β2-microglobulin can accumulate in these compartments after long-term haemodialysis but rarely forms amyloid deposits. We confirm that truncation at the N-terminus is an event specific to β2-microglobulin derived from fibrils but is not observed in the β2-microglobulin from plasma or from the insoluble non-fibrillar material deposited in the heart and spleen. We also confirm the partial deamidation of Asn 17 and Asn 42, as well as the oxidation of Met 99 in fibrillar β2-microglobulin. Other previously reported chemical modifications cannot be excluded, but should involve less than 1–2% of the intact molecule.

Keywords: β2-microglobulin; Amyloid fibril; Fibril extraction; Proteomic analysis; β2-m modification

1. Introduction

1.1. Hypotheses on the role of β2-microglobulin modifications in amyloid deposition

Dialysis-related amyloidosis (DRA) is a pathology associated with a persistent increase of monomeric β2-microglobulin (β2-m) caused by dialysis treatment of renal failure. One of the most surprising properties of this type of amyloidosis is its strict specificity for tissues of the musculo–skeletal system in spite of the fact that the protein is ubiquitously released throughout the body from every cell expressing the MHCI complex. The specificity of deposition in ligaments, synovium, cartilage, and bones causes a clinical presentation dominated by carpal tunnel syndrome, scapulohumeral periarthritis, subchondral bone cysts, and consequent pathological fractures. The clinical features of DRA were first described by Assenat and colleagues [1] in France in 1980.

In 1985, Gejyo's group [2] identified β2-m as the protein constituent of these amyloid fibrils. The mechanism of formation of amyloid fibrils for this protein, as for all the other amyloidogenic proteins, is not clear, and there was great uncertainty about the molecular bases of amyloid deposition when the amyloidogenicity of β2-m was discovered. In particular, for β2-m, which is deposited in the wild-type form and which does not have any polymorphisms, it was hard to believe that the process of fibrillogenesis was simply an effect of high concentration.

* Corresponding author. Dipartimento di Biochimica, Università di Pavia, via Taramelli 3b, 27100 Pavia, Italy. Tel.: +39 382 502984; fax: +39 382 423108.
E-mail address: vbellot@unipv.it (V. Bellotti).

1570-9639/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbapap.2005.07.019

Given that the process of aggregation is associated in some way with the general process of ageing, it was speculated that some chemical modifications typical of protein senescence could be generated in β2-m during dialysis, thus facilitating the aggregation of the protein and amyloid formation. The observation that a minor band, defined as acidic β2-m, can be clearly appreciated in urine, serum, and dialysis fluids on native gel electrophoresis has further strengthened the idea that chemical modifications are capable of generating this "acidic" band of β2-m. β2-m is easily modified in vitro by highly reactive reagents such as oxygen radicals produced by γ irradiation. Oxidation has a dose-dependent effect on β2-m, causing not only full oxidation of methionine 99, but also oxidation of the tryptophans and formation of di-tyrosine with consequent production of a covalently linked β2-m dimer [3].

The second type of hypothesised chemical modification is deamidation of Asn 17. Odani et al. [4] showed that the acidic form (p*I* 5.22) of β2-m purified by the ultrafiltrate contains an Asp residue in position 17, generated by the deamidation of Asn. This event was not completely unexpected because Asn 17 is followed by a Gly and it is well known that the deamidation of Asn is accelerated by 30- to 50-fold when a Gly follows, because succinimide formation is facilitated.

The last chemical modification occurring in β2-m that has been claimed to be relevant in the process of amyloid formation is β2-m glycation. This modification was also suspected on the basis of its capacity to mimic the appearance of the acidic species of β2-m. The capacity of antibodies specific for proteins with advanced glycation end (AGE) products to recognise the β2-m amyloid deposits raised enormous interest in the early 1990s on the possible contribution of glycated β2-m to amyloid formation and amyloid toxicity. However, despite experimental evidence of the pro-inflammatory role of glycated β2-m [5] and a detailed characterisation of the residues [6] involved in the glycation, there is no demonstration that glycation has any role in this type of amyloidosis and it cannot be excluded that an increase of glycated material in these amyloid deposits could be attributed to entities other than β2-m, such as collagen.

1.2. An impelling need to match idealised conditions of fibrillogenesis in vitro and the in vivo biological environment

Extensive investigations of the chemical modifications associated with β2-m and its propensity to form amyloid were carried out in the years preceding the modern interpretation of the fibrillogenic process. It has been demonstrated that β2-m can generate amyloid fibrils in vitro, without any structural modification of the protein being necessary, just by creating a chemical environment in which partial unfolding occurs [7,8]. Research in the last 3 years is close to producing para-physiologic conditions of in vitro fibrillogenesis. Such conditions will probably represent more faithfully the biological environment in which amyloid deposition occurs. This is an essential step in order to match reproducible, idealised conditions of fibrillogenesis in vitro with the biological conditions of the disease in vivo, enabling the design and investigation of anti-amyloidogenic therapeutic strategies that might be effective in vivo.

It is important at this stage to scrutinise all the data regarding the chemical characterisation of β2-m isolated from natural amyloid fibrils and provide information on the chemical modifications that should be introduced in the protein used in the in vitro fibrillogenesis models.

1.3. Chemical characterisation of natural amyloid fibrils

Chemical characterisations of natural amyloid fibrils in DRA have been performed very rarely in comparison with all the studies focused on the biology, clinical features, and pathology of this form of amyloidosis. Table 1 reports the cases so far described.

The first chemical characterisation consisted in an analysis of fibrils isolated from the carpal tunnel of patients undergoing chronic haemodialysis [9]. In this case, the extraction procedure consisted in tissue homogenisation with phosphate saline buffer and successive extractions in water. The amyloid fibrils were then solubilised and purified by gel filtration with 6 M guanidine. The sequence analysis performed on the first 16 N-terminal amino acids identified the protein as β2-m.

In the same year, Gorevic's group [10] reported the sequence of the first 30 amino acids of a protein isolated from the humeral head of a patient with end-stage renal disease who had been maintained on chronic haemodialysis for 10 years. The fibrillar material was extracted in 6 M guanidine, purified with a Sephadex G-75 column equilibrated with 5 M guanidine, and subsequently analysed in SDS-PAGE. The size of the protein, its amino acid composition, and the partial amino acid sequence allowed these authors to conclude that the major constituent of the fibrils was intact β2-m.

In 1986, the same group [11] reported the full amino acid sequence of β2-m isolated from amyloid fibrils purified from bone biopsy, processed using the same procedure as mentioned above. In this case, too, they concluded that intact β2-m, and not modified protein, is the major constituent of the fibrils. Two-dimensional gel electrophoresis of partially solubilised amyloid fibrils showed electrophoretic heterogeneity of molecular weights and isoelectric points (p*I*). In fact, amyloid fibrils appeared to be composed of monomers, dimers, and higher polymers of β2-m consisting of different isoforms with p*I* of 5.7 and 5.3, typical of normal β2-m [12], and also of other isoforms with a more acidic p*I*.

Argiles et al. [13] analysed amyloid deposits surgically obtained from the carpal tunnel from 13 dialysed patients. The proteins were isolated from amyloid deposits carrying out the first steps of homogenisation in phosphate-buffered

Table 1
Summary of the different techniques reported in the literature for the analysis of amyloid fibrils

Source of natural fibrils	Extractive procedure	Analytical techniques	Results
Carpal tunnel	Phosphate saline homogenisation–H_2O extraction	N-terminal sequence, SDS-PAGE	Intact β2-m [2]
Humeral head	H_2O homogenisation–guanidine extraction	Complete amino acid sequence, 2D-PAGE	Full-length, intact β2-m [10,11]
Synovia gelatinous mass, caput femoris, bone amyloidoma	Phosphate saline homogenisation–H_2O extraction	N-terminal sequence, SDS-PAGE	N-terminal sequence of wild-type and truncated species (quantification of truncated species) [17,18]
Carpal tunnel	Phosphate saline homogenisation–guanidine extraction	N-terminal sequence, 2D-PAGE	Intact β2-m [13]
Synovia	Phosphate saline homogenisation–formic acid extraction	Mass spectrometry, SDS-PAGE, immunoblot	Monomer and dimer mass determination: intact monomer, glycated dimer [15,16]
Carpal tunnel, caput femoris	Phosphate saline homogenisation–H_2O extraction	N-terminal sequence, mass spectrometry, SDS-PAGE, 2D-PAGE	N-terminal sequence of wild-type and truncated species (quantification of truncated species) [20]

saline and the last in 6 M guanidine. Two-dimensional PAGE of the proteins that were extracted revealed the presence of different isoforms of β2-m with a p*I* lower than 5.7. Proteins isolated from the amyloid deposits were purified by gel filtration using 25 mM imidazole–HCl as the elution buffer and subsequently by chromatofocusing in imidazole buffer. The sequence analysis carried out on different purified isoforms showed that all corresponded to the intact β2-m without amino acid replacements.

Miyata et al. [14] and Niwa et al. [15] confirmed the presence of acidic β2-m isoforms in amyloid fibrils isolated from carpal tunnel connective tissue in different haemodialysis patients. Using an immunohistochemical technique with monoclonal anti-AGE antibody, these researchers demonstrated that the isoforms of β2-m are modified with AGE (advanced glycation end) products. In this case, the amyloid tissue was homogenised in 0.15 M NaCl, and then the lyophilised amyloid fibrils were dissolved in 80% formic acid for 8 h and separated by gel filtration, obtaining two isoforms of β2-m corresponding to the dimer and monomer of the protein, as demonstrated in SDS-PAGE and ESI-LC/MS [16]. The molecular weight of the β2-m dimer suggests that it is modified by different AGE structures.

In 1987, Linke et al. [17] found, for the first time, truncated β2-m species in amyloid fibrils obtained from the synovium of a patient on long-term haemodialysis. Amyloid fibrils were extracted using the classical water extraction method; lyophilised amyloid fibril concentrates were then dissolved in 80% formic acid and separated by gel filtration in 60% formic acid and 20% isopropanol using HPLC equipment. The N-terminal sequence of the two major extracted proteins of 12 and 24 kDa was the same and corresponded to the intact β2-m and a truncated isoform lacking the first six amino acids. This result was confirmed, by the same author, through the analysis of amyloid fibrils isolated, using the same procedure as that described in the previous study, from the bone and carpal synovium of seven patients on long-term haemodialysis [18]. The analysis of plasma ultrafiltrates showed the presence of only the intact β2-m. This finding, in the author's opinion, excludes the possibility that β2-m fragmentation observed in amyloid fibrils is a procedure-related artefact. This conclusion is also supported by the use of proteinase inhibitors during all steps of purification and the absence of proteinase activity in tissue extracts.

Similar results were obtained in our laboratory analysing amyloid fibrils isolated from the carpal tunnel and femoral head of six patients on long-term haemodialysis [19]. Fig. 1 reports the extraction procedure used in our laboratory, which is the classical water extraction procedure for the isolation of β2-m fibrils. The extracted material solubilised in 6 M guanidine was purified by gel filtration. The fraction containing β2-m was submitted to N-terminal sequencing and mass determination by ESI-MS, confirming that the main protein form was consistent with a full-length molecule without significant chemical modifications; other minor species were also present, deriving from the loss of the first 6, 10, and 17 N-terminal residues and loss of C-terminal residues Met 99 and the last 13 C-terminal residues. The amino acid sequencing allowed us to quantify the N-terminal truncated isoforms on the basis of the recovered phenylthiohydantoin amino acids [20]. The yield of the intact β2-m was approximately 70%, and that of the N-truncated species, approximately 30%. These values correspond exactly to the percentages calculated by Linke et al. [17] and also confirm that the main cleavage site is at the C-terminus of Lys6.

This literature review highlights discrepancies that could derive from: (1) the nature of the amyloid tissue analysed, (2) the method used for the extraction procedure, (3) the method used for the purification, and (4) the types of the analyses performed on the isolated amyloidogenic protein.

Since the pre-analytical phase seems to be particularly important in this situation, a consensus needs to be reached on the best procedure for extracting β2-m fibrils from

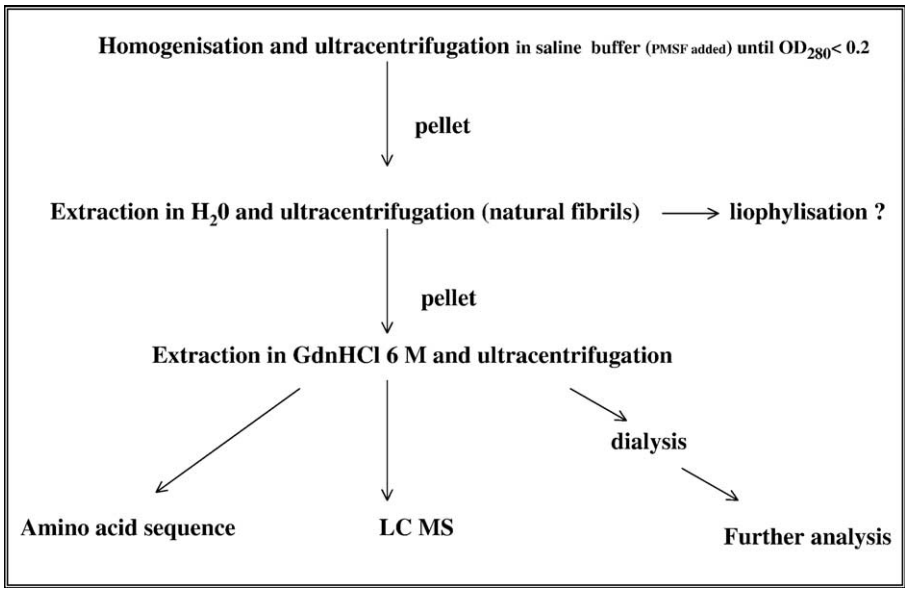

Fig. 1. Scheme of the amyloid fibril extraction procedure used.

natural sources, enabling a high yield of material with the typical ultrastructural characteristics of amyloid fibrils and without introducing any artefacts responsible for structural modifications of the amyloidogenic protein. From this point of view, we have noted that the yield of β2-m fibrils obtained with the water extraction procedure is comparable to that achieved with the other procedures, while appearing to be the least harsh procedure for the protein. Another important aspect is the stability of the proteolysed species as regards the full-length β2-m. Our studies [21], regarding in particular the isoform lacking the first six residues, revealed that this truncated species has a more flexible structure, a greater propensity to self-aggregate, and a higher tendency to precipitate in non-denaturing aqueous buffers. Taking into account these characteristics, we can assume that a loss of proteolysed species occurs during the purification procedures performed in non-denaturing conditions.

In this study, we reported a proteomic approach to the study of amyloid fibrils and identify the tissue districts in which the truncated species of β2-m are present.

2. Materials and methods

2.1. Chemicals

Immobiline Dry Strips, Dry Strip cover fluid, urea, IPG (immobilised pH gradient) buffer, and dithioerythritol (DTE) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), acrylamide, SDS, and ammonium persulfate were purchased from Bio-Rad (Hercules, CA, USA). All the other reagents were purchased from Sigma (St. Louis, MO, USA). All buffers were prepared with a Milli-Q water system (Millipore Bedford, MA, USA).

2.2. Protein extraction from ex vivo fibrils

Amyloid fibrils were extracted from amyloid deposits isolated from the femoral head of a patient submitted to hip replacement surgery. The material was homogenised in 10 ml of 10 mM Tris/EDTA, pH 8.0, containing 1.5 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$)/100 mg of tissue, and centrifuged at 60,000×*g* in an ultracentrifuge (Beckman L8-704; Beckman Instruments) for 30 min before the supernatant was removed. This step was repeated nine times until the absorbance measurement at 280 nm was less than 0.2; subsequently, the pellet was homogenised in water in the presence of 1.5 mM $PhMeSO_2F$ and centrifuged at 60,000×*g* for 30 min. Ten aqueous fractions were obtained. The yield in fibrils was monitored by microscopic analysis of the extracted material stained with Congo red.

2.3. Protein extraction from the heart and spleen of a DRA patient

Proteins were extracted from samples of spleen and heart obtained post-mortem from a patient affected by DRA who had been treated with regular haemodialysis for 20 years. The proteins were extracted using the water extraction procedure described for amyloid fibrils. After the last water extraction, the pellet still contained aggregated material, so it was finally homogenised with 6 M Gdn/HCl, and the supernatant was collected for further analysis by electrophoresis.

2.4. Plasma proteins analysis

Fresh plasma samples were obtained from two DRA patients treated for 12 and 31 years with regular haemodialysis.

An aliquot of 6.25 μl of plasma was mixed with 10 μl of a solution containing SDS (10% w/v) and DTE (2.3% w/v).

The sample was heated to 95 °C for 5 min and then diluted to 500 μl with a solution containing urea (8 M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM), and a trace of bromophenol blue. Sixty microliters (45 μg) of the final diluted plasma sample was loaded on the first dimension separation [22].

Plasma levels of β2-m were determined immunologically according to Berggard et al. [23].

2.5. Electron microscopy

Samples for transmission electron microscopy were prepared by floating the fibrillar suspension on formvar/carbon-coated grids for 2–3 min, before air drying and staining them with 2% uranyl acetate. Samples were examined in a Jeol JEM 1200 EX electron microscope operating at 80 kV.

2.6. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The first protein separation was performed using Immobiline Dry Strips, pH 3–10 (non-linear gradient). The samples (60 μg and 300 μg suitable for silver and Coomassie staining, respectively) were conditioned in a rehydration buffer: 8 M urea, 4% CHAPS, 65 mM DTE, 0.8% ampholine, and 0.002% bromophenol blue. Isoelectric focusing was performed on an IPGphor system (Amersham Biosciences). After isoelectric focusing, proteins in the IPG strips were soaked for 15 min in SDS-PAGE equilibration buffer (50 mM Tris–HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS) supplemented with 2% dithiothreitol. –SH groups were subsequently blocked with a solution containing 50 mM Tris–HCl, pH 6.8, 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, and a trace of bromophenol blue for 5 min. The second dimension separation was carried out in a vertical gradient slab gel with the Laemmli-SDS-discontinuous system, using an acrylamide gel gradient of 9–16%. Proteins were visualised using brilliant blue or a silver staining method according to previously described methods [24,25].

2.7. SDS-PAGE and Western blotting

SDS-PAGE was performed according to Laemmli [26]. After SDS-PAGE or 2D-PAGE, the proteins in the gel were transferred onto a PVDF membrane as previously described [27]. The β2-m isoforms were identified using a chemiluminescent procedure with an anti-human β2-m polyclonal antibody (Dako).

2.8. MALDI/MS analyses and protein sequencing

The spots stained by the brilliant blue were excised from the gel, destained, reduced, alkylated, and submitted to hydrolysis with endoproteinase Glu-C (SIGMA). Peptide mixtures were analysed by MALDI-time-of-flight mass spectrometry using a Voyager DE-PRO mass spectrometer (Applied Biosystems). Samples were freeze-dried and then dissolved in 10 μl of 0.2% trifluoroacetic acid; 1 μl was mixed with 1 μl of a solution of alpha-cyano-4-hydroxycinammic acid, 10 mg/ml in acetonitrile, 0.2% trifluoroacetic acid 7:3 (v:v), and the mixture was applied onto the metallic sample plate and air dried. Mass calibration was performed using a peptide standard mixture provided by the manufacturer. All mass values are reported as monoisotopic masses, and raw data were analysed using software provided by the manufacturer.

The protein sequence of the material electroeluted by the gel was determined by adsorptive biphasic column technology using an HP G1000A protein sequencer (Hewlett-Packard).

3. Results

3.1. Identification of the proteins from amyloid deposits

The amyloid fibrils that we analysed in this study were extracted, using the classical water extraction procedure, from amyloid deposits in the femoral head of a patient

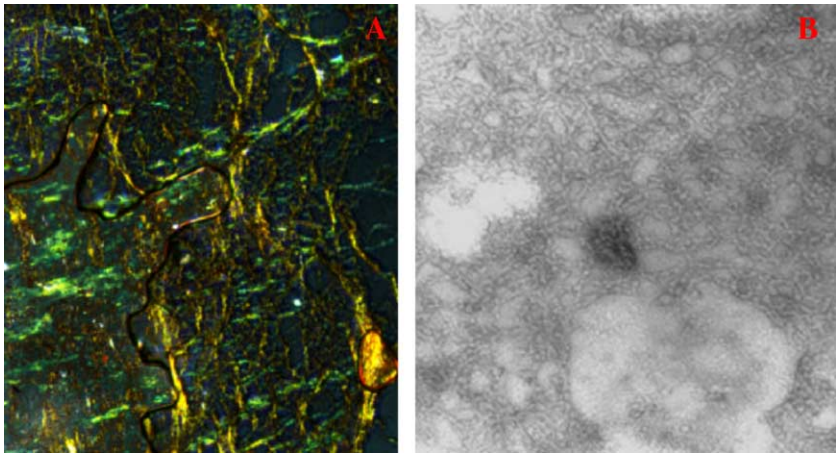

Fig. 2. Congo red staining (A) and electron microscopy of the water-extracted fibrillar material (B).

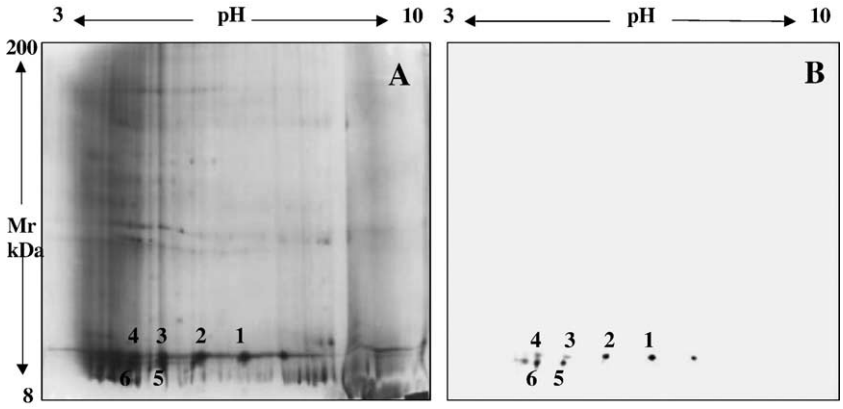

Fig. 3. Two-dimensional gel electrophoresis (A) and Western blotting (B) of water-extracted fibrils. After the 2D electrophoretic separation, β2-m was transferred onto a PVDF membrane, incubated with a polyclonal anti-human β2-m antibody, and detected by a chemiluminescent system. The numbers indicate the spots corresponding to the different isoforms of β2-m analysed by N-terminal sequencing and mass spectrometry.

affected by DRA who underwent hip replacement surgery. The presence of fibrillar material was estimated by microscopic analysis after staining with Congo red and by electron microscopy (Fig. 2).

The extracted material, without further purification, was analysed in 2D-PAGE showing marked electrophoretic heterogeneity for molecular weight and especially for p*I*. Fig. 3A illustrates a typical 2D gel electrophoresis of fibrils and Fig. 3B shows the corresponding immunoblot immunostained with a polyclonal anti-human β2-m antibody. The 2D gel displays the presence of several spots at 12 kDa and slightly lower molecular weight regions. These spots migrated at a pH ranging from 4.0 to 6.2.

Immunoblotting with anti-amyloid P component highlights the presence of a single spot at a molecular weight and isolelectric point consistent with an unmodified SAP monomer (data not shown).

All the main well-defined spots in the 2D gel were recognised by anti-β2-m antibody, suggesting that they are different isoforms of β2-m. As reported in Fig. 3, there were two main groups of β2-m species (the first group included the spots indicated as 1, 2, 3, and 4; the second one was represented by the spots indicated as 5 and 6). The first group was predicted to correspond to the full-length protein and the second to truncated β2-m species. All the main spots were submitted to N-terminal amino acid sequencing and to MALDI mapping analysis. Table 2 reports a summary of the chemical analysis. According to the prediction of their apparent molecular weight, spots 1, 2, 3, and 4 had an N-terminal sequence consistent with the full-length molecule. The N-terminus in spots 5 and 6 started with Ile 7. We did not find any other N-terminus in all the spots that we sequenced. The protein characterisation produced by the MALDI mapping approach confirmed these data and added some more information about the length and chemical modifications of these polypeptides. We can state that all the analysed species terminate at Met 99, and, therefore, we can rule out that the main components are cleaved at the C-terminal. The presence of the cleaved form 7–99 is consistent, and its representation in the fibrils, according to Coomassie staining, is even more abundant than we previously believed [19]. Furthermore, these analyses allowed us to identify two chemical modifications. The first is Met 99 oxidation to methionine sulphone, which might be related to manipulation of the sample. The second is Asn deamidation, most likely involving Asn 17 but also Asn 42. This is a partial phenomenon, not certainly essential for the fibrillar con-

Table 2
N-terminal sequencing and MALDI/MS analyses of the gel spots reported in Fig. 3A

Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6
IQRTPKIQV 1–99[a]	IQRTPKIQV 1–99[a]	IQRTPKIQV 1–99[a]	IQRTPKIQV 1–99[a]	IQVYSRHPA 7–99[a]	IQVYSRHPA 7–99[a]
Peptide 1–10	Peptide 1–10	Peptide 1–10	Peptide 1–10	No data	
					Peptide 7–36, partial deamidation
	Peptide 11–36, small degree of deamidation	Peptide 11–36, partial deamidation	Peptide 11–36, partial deamidation		
Peptide 37–50, partial deamidation	Peptide 37–50, partial deamidation	Peptide 37–50, partial deamidation	Peptide 37–50, partial deamidation		Peptide 37–50, partial deamidation
Peptide 51–69	Peptide 51–69				
Peptide 70–77	Peptide 70–77				
Peptide 78–99, partial oxidation of Met 99	Peptide 78–99, partial oxidation of Met 99	Peptide 78–99, partial oxidation of Met 99	Peptide 78–99, partial oxidation of Met 99		Peptide 78–99, partial oxidation of Met 99

[a] Fragments identified by N-terminal sequencing.

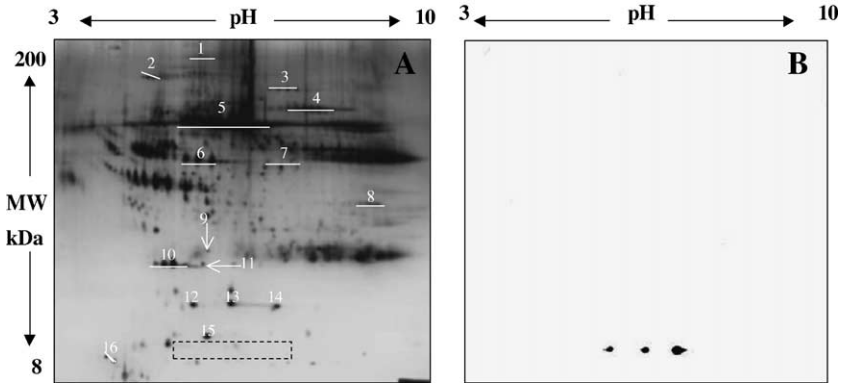

Fig. 4. Two-dimensional gel electrophoresis (A) and Western blotting (B) of proteins isolated from the plasma of a patient affected by DRA and treated by haemodialysis for 12 years. Western blotting was performed as previously described [27]. In Panel A, the proteins identified for comparison with the 2D-PAGE database are indicated by numbers. The comparison demonstrated the absolute overlapping of our map with that reported in the data bank. All proteins identified are reported in Table 3. The dotted box indicates the region computed for β2-m and confirmed by the immunoblotting (B).

version and involves the two Asn followed by Gly and not, for example, the Asn 83 followed by a His. It cannot be excluded that the deamidation of these two residues might enhance the amyloidogenic propensity of β2-m, as suggested by Radford's group [28], for Asn 17 and not yet explored for Asn 42.

3.2. Identification of proteins from plasma

Circulating plasma β2-m in two DRA patients treated by haemodialysis for 12 (patient A) and 31 years (patient B) was analysed by 2D-PAGE. The concentration of plasma β2-m was 18 mg/l in patient A and 20 mg/l in patient B (normal level, <3 mg/L).

Silver-stained 2D-PAGE (Figs. 4A and 5A) of the two patients' plasma showed a pattern overlapping with the two-dimensional plasma map reported in the data bank (http://www.expasy.ch/ch2d), demonstrating the accuracy of our method. A group of proteins identified by gel matching is reported in Table 3. The black dotted box in Figs. 4A and 5A represents the region around the p*I* and molecular weight values computed for β2-m, where one would expect to see the protein on the gel. This identification was confirmed by immunodetection, in which β2-m was visualised by using a polyclonal anti-human β2-m antibody (Fig. 4B and Fig. 5B).

Immunoblotting revealed the presence of at least three isoforms of β2-m with the same molecular weight corresponding to 12 kDa and different p*I* ranging from 5.2 to 6.2. No proteolysed isoforms were detectable in the plasma of the two patients.

3.3. Identification of proteins from the heart and spleen of a DRA patient

In an effort to verify whether patients with musculo–skeletal manifestations of β2-m amyloidosis have systemic amyloid infiltration, we analysed the protein material extracted from the heart and the spleen of a patient with severe musculo–skeletal amyloidosis who died after 20 years of haemodialysis. Tissue samples were homogenised following the classical water extraction procedure used for amyloid fibrils. Eight aqueous fractions were obtained and analysed by SDS-PAGE (Fig. 6B). After the eighth water

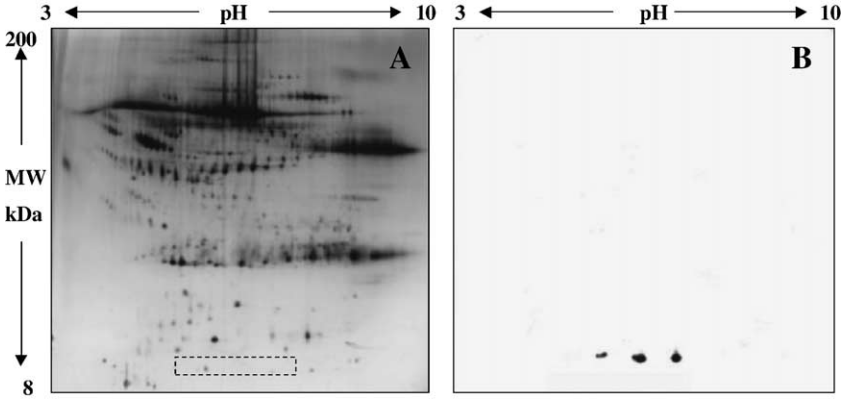

Fig. 5. Two-dimensional gel electrophoresis (A) and Western blotting (B) of proteins isolated from the plasma of a patient affected by DRA and treated by haemodialysis for 31 years. The black dotted box in Panel A represents the region computed for β2-m, where one would expect to see the protein on the gel, confirmed by the immunodetection (B).

Table 3
Results of the identification analysis for proteins indicated in Fig. 4A for comparison with the plasma map reported in the data bank

Number	Protein	p*I*	MW (kDa)
1	α-2 macroglobulin	5.3–5.6	186.0–190.0
2	Ceruloplasmin	5.0–5.2	123.0–160.0
3	Complement factor B	5.9–6.3	100.0–105.0
4	Transferrin	6.3–6.6	75.2–85.8
5	Albumin	5.5–5.9	66.3–67.5
6	Fibrinogen β chain	5.0–5.6	49.0–51.0
7	IgM heavy chain μ(intermediate segment)	5.9–6.15	48.5–48.8
8	IgG heavy chain γ(intermediate segment)	6.7–7.3	37.6–38.1
9	Serum Amyloid Protein	5.5	26.4
10	Apolipoprotein A-I	5.0–5.2	23.0–23.15
11	proApolipoprotein A-I	5.5	23.6
12	Haptoglobin α2 chain	5.4	16.9
13	Haptoglobin α2 chain	5.68	17.1
14	Haptoglobin α2 chain	6.08	16.9
15	Transthyretin	5.52	13.8
16	Apolipoprotein C-II	4.5–4.6	9.98–9.8

extraction, the pellet still contained aggregated material visible in SDS-PAGE (Fig. 6B). It was homogenised in 6 M Gdn/HCl in order to solubilise the aggregated material. After centrifugation, the supernatant was collected and analysed by SDS-PAGE and immunoblotting (Fig. 6D), yielding a good amount of soluble β2-m well visible in SDS-PAGE stained by Coomassie and in Western blots immunostained with anti-β2-m antibody.

The materials from both the aqueous extraction and the 6 M Gdn/HCl solubilisation were also analysed in 2D gel electrophoresis transferred onto PVDF membranes and immunostained (Figs. 6C and E, respectively). It is noteworthy that in both cases, all the visualised spots corresponded to the full-length β2-m with different p*I*; spots corresponding to the truncated species were not visible.

All the extracted material was found to stain negatively with Congo red, and it did not show characteristic birefringence by polarising microscopy, demonstrating the absence of amyloid fibrils in the aggregates present in this tissue (Fig. 6A).

The aggregates present in the spleen of this patient were submitted to the same procedures; the results of the analyses were identical (Fig. 7).

4. Discussion

A review of the literature regarding the characterisation of β2-m amyloid fibrils highlights a few points that should probably be considered for future experimental strategies. In all the reports we reviewed, the amount of amyloid fibrils

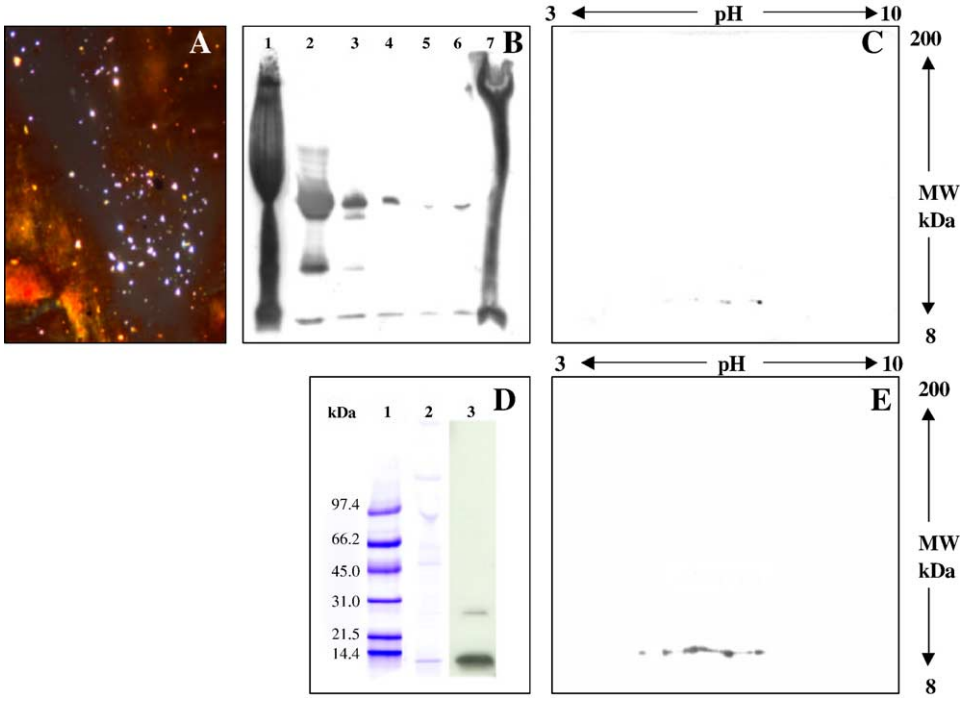

Fig. 6. Analysis of the proteins extracted from the heart of a patient with DRA who underwent haemodialysis for 20 years. (A) Congo red staining of one heart section visualised under polarised light. (B) Western blotting developed with anti-β2-m antibody after SDS-PAGE separation. Lane 1: amyloid fibrils extracted from amyloid deposits. Lanes 2, 3, and 4: material extracted in 10 mM Tris/EDTA, pH 8.0. Lanes 5 and 6: material extracted in the two last aqueous extractions. Lane 7: pellet obtained after the aqueous extractions. (C) Western blotting developed with anti-β2-m antibody after 2D-PAGE separation of the material obtained in the last aqueous extraction. (D) Electrophoretic analysis of the material solubilised in 6 M Gdn/HCl. Lane 1: standard for molecular weight. Lane 2: SDS-PAGE in reducing conditions stained by Coomassie blue. Lane 3: Western blot immunostained with a polyclonal anti-human β2-m antibody after SDS-PAGE. (E) Western blotting developed with anti-β2-m antibody after 2D-PAGE of the material solubilised in 6 M Gdn/HCl.

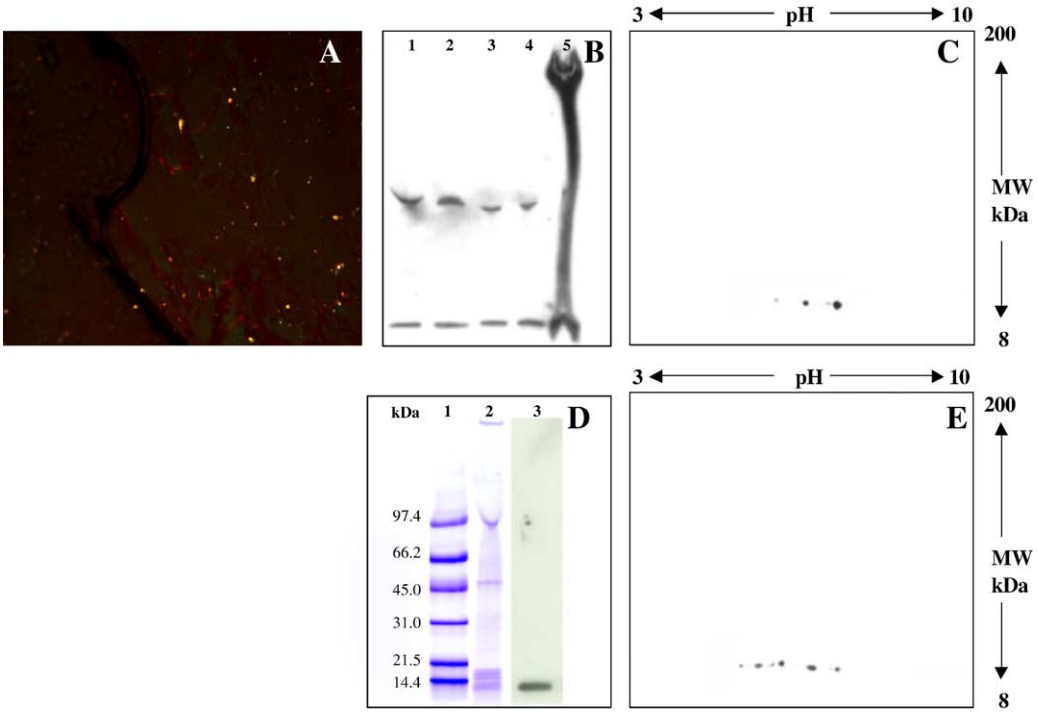

Fig. 7. Analysis of the proteins extracted from the spleen of a patient with DRA who underwent haemodialysis for 20 years. (A) Congo red staining of a spleen section visualised under polarised light. (B) Western blotting developed with anti-β2-m antibody after SDS-PAGE. Lanes 1 and 2: material extracted in 10 mM Tris/EDTA, pH 8.0. Lanes 3 and 4: material extracted in the two last aqueous extractions. Lane 5: pellet obtained after the aqueous extractions. (C) Western blotting developed with anti-β2-m antibody after 2D-PAGE separation of material obtained in the last aqueous extraction. (D) Electrophoretic analysis of the material solubilised in 6 M Gdn/HCl. Lane 1: standard for molecular weight. Lane 2: SDS-PAGE in reducing conditions stained by Coomassie blue. Lane 3: Western blot developed with a polyclonal anti-human β2-m antibody after SDS-PAGE. (E) Western blotting developed with anti-β2-m antibody after 2D-PAGE of the material solubilised in 6 M Gdn/HCl.

purified from ex vivo samples from patients affected by DRA was always very small. This is probably because the type of tissue involved by this form of amyloidosis is extremely difficult to homogenise and very rich in insoluble fibrous material.

Probably, as a consequence of this intrinsic difficulty, the procedures adopted by various laboratories for the fibril extraction are extremely heterogeneous. In several cases and, in particular, for the procedures that imply the use of a chaotropic agent, it is impossible to verify the presence of real fibrils in the extracted material. However, we have clearly shown in this study that β2-m can accumulate as insoluble aggregates even in extra-skeletal tissues such as the spleen and heart. The detection of non-fibrillar β2-m aggregates in these organs is a novel observation and suggests that peculiar structures are present in the skeletal tissue, where they might catalyse the correct assembly of β2-m in fibrillar polymers.

Recent data suggest that glycosaminoglycans, in particular heparin ([29], Sebastiani et al. paper in preparation) and collagen ([30], Relini et al. paper in preparation), could have such a role; interestingly, both these macromolecules are highly represented in the target tissue of DRA.

The data we have reported and reviewed provide the state of knowledge about various types of post-translational modifications of fibrillar β2-m. Oxidation appears to be a very limited phenomenon that only involves methionine 99, but no other residues susceptible to more drastic oxidation reactions such as Tyr and Trp. The deamidation involves the two Asn followed by Gly in the sequence, Asn 17 and Asn 42, but the majority of the protein is not deamidated. We did not find any glycation products in the fibrillar protein, and careful review of the literature suggests that the only evidence of glycation was provided by Niwa on the minority dimeric form of β2-m purified from amyloid material isolated through a denaturing procedure [16]. The reports regarding proteolysis are also conflicting because, particularly in the early reports in which the amino acid sequencing was carried out with the purpose of identifying the amyloidogenic protein, no other species than the full-length form was found. However our data and Linke's data are very similar, and the discrepancies with the early observations are probably related to the abundance of the available material. It is instructive to compare the pattern of our two-dimensional electrophoresis with that reported by Argiles et al. [13], who achieved a very nice separation of β2-m amyloid fibrils with this technique. The spots we have now ascribed, without any doubt, by amino acid sequencing and mass spectrometry to the fragment lacking the six N-terminal residues, were also present in their analysis, with very similar molecular weights and isoelectric points.

In conclusion, we can state that the truncated form of β2-m is a real entity in natural amyloid fibrils. We were surprised by the lack of this fragment in the insoluble β2-m that we extracted from the spleen and heart of the patient who had suffered from severe musculo–skeletal amyloidosis and died after 20 years of uninterrupted haemodialysis. The proteomic approach that we have used allows us to exclude that the truncated species of β2-m circulates in plasma. All these data raise a dilemma, which is still unsolved for many other amyloid diseases, about whether the proteolytic cleavage occurs before or after the formation of the fibrils.

Nevertheless, we hope that the information that we have summarised here might be useful in the future for merging the biochemical studies carried out with pure in vitro and ex vivo material.

Acknowledgements

This work was supported by funds from MIUR-Italian Ministry of Education, University and Research (FIRB RBNE01S29H_02; COFIN 2002058218_003; 2003051399_004), CARIPLO Foundation, and IRCCS Policlinico San Matteo.

References

[1] H. Assenat, E. Calemard, B. Charra, G. Laurent, J.C. Terrat, T. Vanel, Hemodialysis: carpal tunnel syndrome and amyloid substance, Nouv. Presse Med. 9 (1980) 1715.

[2] F. Gejyo, T. Yamada, S. Odani, Y. Nakagawa, M. Arakawa, T. Kunitomo, H. Kataoka, M. Suzuki, Y. Hirasawa, T. Shirahama, A.S. Cohen, K. Schmid, A new form of amyloid protein associated with chronic hemodialysis was identified as beta 2-microglobulin, Biochem. Biophys. Res. Commun. 129 (1985) 701–706.

[3] C. Capeillere-Blandin, T. Delaveau, B. Descamps-Latscha, Structural modifications of human beta 2 microglobulin treated with oxygen-derived radicals, Biochem. J. 277 (1991) 175–182.

[4] H. Odani, R. Oyama, K. Titani, H. Ogawa, A. Saito, Purification and complete amino acid sequence of novel beta 2-microglobulin, Biochem. Biophys. Res. Commun. 168 (1990) 1223–1229.

[5] T. Miyata, R. Inagi, Y. Iida, M. Sato, N. Yamada, O. Oda, K. Maeda, H. Seo, Involvement of beta 2-microglobulin modified with advanced glycation end products in the pathogenesis of hemodialysis-associated amyloidosis. Induction of human monocyte chemotaxis and macrophage secretion of tumor necrosis factor-alpha and interleukin-1, J. Clin. Invest. 93 (1994) 521–528.

[6] T. Miyata, R. Inagi, Y. Wada, Y. Ueda, Y. Iida, M. Takahashi, N. Taniguchi, K. Maeda, Glycation of human beta 2-microglobulin in patients with hemodialysis-associated amyloidosis: identification of the glycated sites, Biochemistry 33 (1994) 12215–12221.

[7] H. Naiki, N. Hashimoto, S. Suzuki, H. Kimura, K. Nakakuki, F. Gejyo, Establishment of a kinetic model of dialysis-related amyloid fibril extension in vitro, Amyloid 4 (1997) 223–232.

[8] V.J. McParland, N.M. Kad, A.P. Kalverda, A. Brown, P. Kirwin-Jones, M.G. Hunter, M. Sunde, S.E. Radford, Partially unfolded states of beta(2)-microglobulin and amyloid formation in vitro, Biochemistry 39 (2000) 8735–8746.

[9] F. Gejyo, S. Odani, T. Yamada, N. Honma, H. Saito, Y. Suzuki, Y. Nakagawa, H. Kobayashi, Y. Maruyama, Y. Hirasawa, M. Suzuki, M. Arakawa, Beta 2-microglobulin: a new form of amyloid protein associated with chronic hemodialysis, Kidney Int. 30 (1986) 385–390.

[10] P.D. Gorevic, T.T. Casey, W.J. Stone, C.R. DiRaimondo, F.C. Prelli, B. Frangione, Beta-2 microglobulin is an amyloidogenic protein in man, J. Clin. Invest. 76 (1985) 2425–2429.

[11] P.D. Gorevic, P.C. Munoz, T.T. Casey, C.R. DiRaimondo, W.J. Stone, F.C. Prelli, M.M. Rodrigues, M.D. Poulik, B. Frangione, Polymerization of intact beta 2-microglobulin in tissue causes amyloidosis in patients on chronic hemodialysis, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 7908–7912.

[12] P.W. Hall, E.S. Ricanati, C.V. Vacca, Characterization of human beta2-microglobulin by isoelectric focusing, Clin. Chim. Acta 77 (1977) 37–42.

[13] A. Argiles, M. Garcia-Garcia, J. Derancourt, G. Mourad, J.G. Demaille, Beta 2 microglobulin isoforms in healthy individuals and in amyloid deposits, Kidney Int. 48 (1995) 397–405.

[14] T. Miyata, O. Oda, R. Inagi, Y. Iida, N. Araki, N. Yamada, S. Horiuchi, N. Taniguchi, K. Maeda, T. Kinoshita, Beta 2-microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis, J. Clin. Invest. 92 (1993) 1243–1252.

[15] T. Niwa, S. Miyazaki, T. Katsuzaki, N. Tatemichi, Y. Takei, T. Miyazaki, T. Morita, Y. Hirasawa, Immunohistochemical detection of advanced glycation end products in dialysis-related amyloidosis, Kidney Int. 48 (1995) 771–778.

[16] T. Niwa, M. Sato, T. Katsuzaki, T. Tomoo, T. Miyazaki, N. Tatemichi, Y. Takei, T. Kondo, Amyloid beta 2-microglobulin is modified with N epsilon-(carboxymethyl)lysine in dialysis-related amyloidosis, Kidney Int. 50 (1996) 1303–1309.

[17] R.P. Linke, H. Hampl, S. Bartel-Schwarze, M. Eulitz, Beta 2-microglobulin, different fragments and polymers thereof in synovial amyloid in long-term hemodialysis, Biol. Chem. Hoppe-Seyler 368 (1987) 137–144.

[18] R.P. Linke, H. Hampl, H. Lobeck, E. Ritz, J. Bommer, R. Waldherr, M. Eulitz, Lysine-specific cleavage of beta 2-microglobulin in amyloid deposits associated with hemodialysis, Kidney Int. 36 (1989) 675–681.

[19] M. Stoppini, P. Arcidiaco, P. Mangione, S. Giorgetti, D. Brancaccio, V. Bellotti, Detection of fragments of beta2-microglobulin in amyloid fibrils, Kidney Int. 57 (2000) 349–350.

[20] V. Bellotti, M. Stoppini, P. Mangione, M. Sunde, C. Robinson, L. Asti, D. Brancaccio, G. Ferri, Beta2-microglobulin can be refolded into a native state from ex vivo amyloid fibrils, Eur. J. Biochem. 258 (1998) 61–67.

[21] G. Esposito, R. Michelutti, G. Verdone, P. Viglino, H. Hernandez, C.V. Robinson, A. Amoresano, F. Dal Piaz, M. Monti, P. Pucci, P. Mangione, M. Stoppini, G. Merlini, G. Ferri, V. Bellotti, Removal of the N-terminal hexapeptide from human beta2-microglobulin facilitates protein aggregation and fibril formation, Protein Sci. 9 (2000) 831–845.

[22] G.J. Hughes, S. Frutiger, N. Paquet, F. Ravier, C. Pasquali, J.C. Sanchez, R. James, J.D. Tissot, B. Bjellqvist, D.F. Hochstrasser, Plasma protein map: an update by microsequencing, Electrophoresis 13 (1992) 707–714.

[23] I. Berggard, A.G. Bearn, Isolation and properties of a low molecular weight beta-2-globulin occurring in human biological fluids, J. Biol. Chem. 243 (1968) 4095–4103.

[24] B.R. Oakley, D.R. Kirsch, N.R. Morris, A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels, Anal. Biochem. 105 (1980) 361–363.

[25] D.F. Hochstrasser, M.G. Harrington, A.C. Hochstrasser, M.J. Miller, C.R. Merril, Development of polyacrylamide gels that improve the separation of proteins and their detection by silver staining, Anal. Biochem. 173 (1988) 424–435.

[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[27] M. Stoppini, V. Bellotti, P. Mangione, G. Merlini, G. Ferri, Use of

anti-(beta2 microglobulin) mAb to study formation of amyloid fibrils, Eur. J. Biochem. 249 (1997) 21–26.
[28] N.M. Kad, N.H. Thomson, D.P. Smith, D.A. Smith, S.E. Radford, Beta(2)-microglobulin and its deamidated variant, N17D form amyloid fibrils with a range of morphologies in vitro, J. Mol. Biol. 313 (2001) 559–571.
[29] S. Yamamoto, I. Yamaguchi, K. Hasegawa, S. Tsutsumi, Y. Goto, F. Gejyo, H. Naiki, Glycosaminoglycans enhance the trifluoroethanol-induced extension of beta 2-microglobulin-related amyloid fibrils at a neutral pH, J. Am. Soc. Nephrol. 15 (2004) 126–133.
[30] S. Giorgetti, A. Rossi, P. Mangione, S. Raimondi, S. Marini, M. Stoppini, A. Corazza, P. Viglino, G. Esposito, G. Cetta, G. Merlini, V. Bellotti, Beta2-microglobulin isoforms display an heterogeneous affinity for type I collagen, Protein Sci. 14 (2005) 696–702.