Search for peptidic “middle molecules” in uremic sera: isolation and chemical identification of fibrinogen fragments

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Abstract

According to the “middle molecule” (MM) hypothesis, the uremic solutes ranging from 500 to 5000 Da are insufficiently eliminated by conventional hemodialysis and may act as uremic toxins. However, because of the methodological difficulties of MM purification, their chemical analysis is complicated and the precise structure of these molecules remains obscure. In the present study, a new micro-preparative procedure including SDS electrophoresis and liquid chromatography was applied for isolation of MM peptides from uremic sera. Microsequencing and MS/MS analyses of these peptides showed that most of the identified MM (22 out of 23) represented the N- and C-terminal fragments of the α- and β-chains of fibrinogen. The obtained data provide new information on the precise structure of fibrinogen fragments accumulating in uremic serum as MM.

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1. Introduction

Uremia is a syndrome caused by severe glomerular insufficiency, which is associated with disturbances in tubular and endocrine functions of the kidney. Uremic syndrome resembles a systemic intoxication, therefore, the search for putative uremic toxins has been the subject of intensive investigation. Three major groups of solutes accumulate in the blood of the affected patients: low molecular weight compounds (<500 Da) [1], “middle molecules” (MM) ranging from 500 to 5000 Da [1–4], and large molecules (>5000 Da) [1,5–7]. Small molecules accumulate in uremic blood as free water-soluble substances (e.g. creatinine, urea, uric acid) and as protein-bound compounds (such as hippuric acid, homocysteine, indoxyl sulphate) [1,5]. Whereas the small water-soluble molecules are easily removed by conventional hemodialysis, the larger molecular compounds are retained and may exert toxic effects. Increased amounts of different proteins ranging from 5000 to about 30,000 Da have been demonstrated in uremic blood [1,5–15]. These
include parathyroid hormone [1,7], β-2 microglobulin [13], granulocyte inhibitory proteins [8,9], degranulation inhibiting proteins [10,12], and chemotaxis inhibiting protein [15]. MM have been generally assumed to be of peptidic origin [16–23]. Although the analysis of hemofiltrate from uremic patients provided valuable information on circulating human peptides [24,25], the precise chemical structure of MM retained in the blood of these patients remains obscure.

Early studies have shown that analysis of MM is complicated by difficulties in obtaining the middle molecular weight fraction by the commonly used gel filtration technique. In fact, the MM fraction isolated by this method was significantly contaminated by small uremic molecules [4]. Later, combinations of different separation techniques, including size exclusion, ion exchange, affinity and reversed phase chromatographies, were applied to study the putative higher molecular mass uremic toxins [8,10,15,23]. In such purification protocols, considerable amounts of starting uremic material were needed, and the experiments were usually carried out by using the extracorporeal ultrafiltrates of dialysis patients, rather than blood samples. Notably, the purification was often guided by biological or immunoassays to screen for some specific compounds presumed to be responsible for functional disturbances in uremic state. Such investigations are very useful to test one or another hypothesis, but are less effective for systematic search for MM.

In contrast to most studies performed using extracorporeal ultrafiltrates, our study was aimed to search for uremic MM peptides in sera of hemodialysis patients. In this approach, comparison of normal and uremic serum samples enabled the elucidation of solutes abnormally elevated in uremia. Our study included development and application of the appropriate small-scale preparative techniques suitable for isolation of the middle-size peptides from small amounts of the collected sera. Use of highly sensitive protein microsequencing and mass spectrometry techniques allowed chemical identification of the uremic peptides in minute quantities of the sample. The data provide new direct evidence for accumulation of the middle-sized fibrinogen fragments in uremic blood, which probably comprise the majority of peptidic MM in uremia.

2. Experimental

2.1. Serum samples

Blood serum samples were obtained from six healthy persons and from 18 uremic patients. The patients were maintained on conventional hemodialysis by using biocompatible cellulose tri-acetate 1.5 or 1.9 m² membranes, with measured \( k_t/V \) 1.35–1.45. Blood samples were taken from patients prior to heparin administration. Both control and uremic blood samples were handled and treated under the same conditions. The prepared serum samples were stored at \(-20^\circ C\) until used.

2.2. Ultrafiltration and separation on Sep-Pak C18 cartridges

Serum samples (8–10 ml) were ultrafiltered by using Biomax-30 Ultrafree 4 ml centrifugal devices with high-flux polyethersulfone membranes, nominal molecular weight limit 30 kDa (Millipore, Bedford, MA, USA). The obtained ultrafiltrates were acidified with 1% trifluoroacetic acid (TFA) up to final concentration of 0.1% TFA and passed through a prepared Sep-Pak Classic C18 cartridges (Waters, Milford, MA, USA). Three to five milliliters of serum ultrafiltrate was loaded per cartridge. The cartridge was washed with 0.1% TFA (5 ml); the elution was performed with 20% acetonitrile–0.1%TFA (2.5 ml) and afterwards with 80% acetonitrile–0.1%TFA (2.5 ml). The eluted material was pooled and lyophilized.

2.3. Micro-preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Serum material obtained following ultrafiltration and separation Sep-Pak C18 cartridges (as described above), was dissolved in 100 μl SDS-PAGE sample buffer (pH 6.9) containing 6% SDS, 125 mM Tris, 4 mM Na₂EDTA, 6 M urea, and 0.2 M sucrose. Electrophoresis was performed in the NuPAGE Electrophoresis System on 10% bis-Tris gels (1 mm thick, 10 wells) (Novex, San Diego, CA, USA). Rainbow colored molecular mass markers, ranging from 4 to 46 kDa (Amersham, Buckinghamshire, UK), were applied in the far left and far right wells of the gel.
central five slots were filled with the sample. The elec-
trophoresis was run with MES/SDS running buffer
containing 0.05 M 2-(N-morpholino)ethane sulphonic
acid, 0.05 M Tris Base, 3.415 mM SDS and 1.025 mM
EDTA (Novex). After the completion of run, the gel
section containing the serum material was cut out
at the position between tracking dye (bromophenol
blue) and Mr 5000. The gel was minced and extracted
with 500 µl of 0.05 M Tris HCl buffer (pH 6.8) con-
taining 0.1% SDS, 0.1 mM EDTA, 5 mM DTT, and
0.2 M NaCl (room temperature, 2 h). The extraction
procedure was repeated two more times. The obtained
extracts were pooled and purified from SDS by precip-
itation with guanidine [26–28]. The “control gel” sec-
tion (containing no serum material) was treated in the
same way.

2.4. Size exclusion chromatography (SEC)

Samples were run on a Fractogel™ TSK HW-40(F)
column (210 mm × 11 mm, i.d.) (Merck, Darmstadt,
Germany) equilibrated with an aqueous 50% acetonitrile
in 0.1% TFA. Elution was performed with the
same solvent. For the determination of the void vol-
ume and the exclusion limit of the column, the MW
markers ranging from 14 to 200 kDa and from 3 to
45 kDa (Rainbow) were run on the column, and the
effluent fractions (1 ml) were collected, lyophilized
and analyzed by SDS-PAGE. Riboflavin (Sigma) was
used to define the total volume of the column. Serum
material obtained following ultrafiltration and sepa-
rating Sep-PaK C18 cartridges (as described above)
was collected, lyophilized and analyzed by SDS-PAGE. Riboflavin (Sigma) was
used to define the total volume of the column. Serum
material obtained following ultrafiltration and sepa-
rating Sep-PaK C18 cartridges (as described above)
was loaded on the column for group separation:
the material excluded from the column (void vol-
ume) and the included material were collected and
lyophilized.

2.5. Reversed phase high performance liquid
chromatography (RP-HPLC)

HPLC was carried out on a Vydac 218 TP (All-
tech, Deerfield, IL, USA) reversed phase C18 col-
umn (250 mm × 4.6 mm, i.d., pore diameter 300 Å,
particle size 5 µm). Samples separated either by
micro-preparative SDS-PAGE, or by SEC, were
applied onto the HPLC column and eluted using
a linear gradient from 0 to 50% acetonitrile in
0.1% TFA over 50 min. A flow rate of 0.8 ml/min
was maintained. The elution was monitored by
UV absorbance at 220 nm. The elution time of
unretained solute (ammonia solution), t₀ = 3.7,
was determined and used for the calculation of
k’ values. The eluted fractions were collected and
lyophilized.

2.6. Amino acid sequence analysis (Edman
degradation)

The amino acid sequence analysis was performed
using an Applied Biosystem Procise protein se-
quencer run in pulsed liquid mode according to the
manufacturer’s protocol. PTH-amino acids were iden-
tified on-line from a 5 pm PTH-amino acid standard.
Data base searches were done against the Swiss Prot
Database.

2.7. Fast atom bombardment mass spectrometry
(FAB MS)

FAB MS was performed in a High Resolution Mag-
netic Sector AutoSpec FISONS VG (Manchester, UK)
instrument by using glycerol as a matrix.

2.8. Surface enhanced laser desorption ionization
mass spectrometry (SELDI MS)

SELDI mass spectra were recorded in the pos-
tive ion mode using a PBS II Protein Chip Ar-
ray reader (Ciphergen Biosystems Incumbent, Fre-
mont, CA), a linear laser desorption/ionization-time
of flight mass spectrometer with time lag fo-
cusing. Samples were re-suspended in 100 µl of
50% aqueous acetonitrile containing 0.1% TFA
and 1 µl spotted to an H4 chip surface, which
is a C-16 carbon surface and exhibits character-
istics similar to that of reversed phase columns.
The surface had been pre-washed with the aque-
ous 50% acetonitrile, 0.1% TFA prior to use.
The sample spots were allowed to dry and then
washed extensively with 5% acetonitrile to remove
any interfering material and unbound molecules.
Alpha-cyano-4-hydroxy-cinnamic acid (CHCA) ma-
trix added prior to analysis on the PBS II Protein
Chip reader. The PBS II was previously calibrated
with ACTH 1–24 (2933.5 Da) and bovine insulin
(5733.6 Da).
2.9. Liquid chromatography/tandem mass spectrometry (LC/MS/MS)

The samples were also assayed by LC/MS/MS using a Finnigan (San Jose, CA) LCQ Classic mass spectrometer and a Shimadzu LC-10ADVP HPLC system (Kyoto, Japan). The samples were injected onto a 0.32 mm × 100 mm BetaBasic-18 (ThermoHypersil-Keystone, Bellefonte, PA) packed-capillary that had been equilibrated with 10% acetonitrile containing 0.1% formic acid. Retained analytes were eluted by increasing the acetonitrile concentration to 60% at 1.25% per minute using a linear gradient, flow rate 10 μl/min. The mass spectrometer was operated in the data-dependent mode using a custom protocol that generated MS/MS data from the five most intense ions in the spectrum. The tandem mass spectral data were submitted to the MS/MS ion search component of the Mascot search engine (Matrix Science, London, UK) [29]. No enzyme or missed cleavages were specified in the search against the Swiss Prot database, and all charge states were considered. Only those sequences with statistically significant scores and suitable MS/MS spectra were deemed acceptable for protein identification.

3. Results

Serum samples obtained from healthy subjects and uremic patients were ultrafiltered, separated on Sep-Pak C18 cartridges, and afterwards run by slab gel SDS-PAGE (Fig. 1a). Serum material of Mr < 5000 was extracted from gel, purified from SDS and separated by reversed phase HPLC (Fig. 2). An increase in the amount of UV absorbing material was observed in uremia. The most prominent peaks characteristic to uremic state, but absent in normal serum, were collected for further analysis. No middle-size molecular species were found by FAB MS analysis of the uremic peaks I (k’ = 4.1), II (k’ = 4.5) and III (k’ = 5.3) (Fig. 2A). Peak III gave a spectrum confirming the attendance of phenylacetylglutamine (264 Da) reported by us previously [27]. SELDI mass spectrometric analysis of the IV–VII (k’ from 6.1 to 8.6) revealed the molecular ions ranging from about

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**Fig. 1.** Flowchart of methodology used for isolation of peptidic MM.
Fig. 2. RP-HPLC of serum material prepared by using consecutively ultrafiltration, separation on Sep-Pak C18 cartridges, and micro-preparative SDS-PAGE (Fig. 1a). (A) Samples were obtained from healthy person B.A. (a), and uremic patient N.B. (b), initial serum volumes 0.7 ml. HPLC was carried out using a linear gradient from 0 to 50% acetonitrile in 0.1% TFA, flow rate 0.8 ml/min, sensitivity 0.3 AUFS. (B) Samples were obtained from uremic patients A.P. (a), R.H. (b), M.H. (c), and S.R. (d), the initial serum volumes of 5, 3.5, 2.3 and 2.7 ml, respectively. HPLC was carried out using a linear gradient from 0 to 50% acetonitrile in 0.1% TFA, flow rate 0.8 ml/min, sensitivity 0.3 AUFS (a)–(c) and 0.53 AUFS (d).

520 to 1500 Da (Fig. 3). Amino acid sequencing (Edman degradation) and MS/MS analyses showed that the majority of the peptides were derived from the α-chain of fibrinogen (FIBA peptides) and β-chain of fibrinogen (FIBB peptides) (Table 1). The FIBA peptide sequences corresponded to the residues starting at the positions 21, 22, 24 or 26 and ending at the positions 32–34 of the α-chain of fibrinogen. Two sequences matching the C-terminal portion of α-chain (601–606 and 605–613) were also demonstrated. FIBB peptides showed sequences starting at the positions 32 or 33 and ending at positions 39, 41 or 43 of the β-chain of fibrinogen. The uremic peaks IV–VII containing the middle molecular weight substances were found in all the uremic patients tested (n = 18), however, the relative levels of these peaks were different in sera of different uremic patients (Fig. 2B).

In an alternative separation procedure (Fig. 1b), reversed phase HPLC was preceded by fractionation of serum material on a FractogelTM HW-40(F) column in aqueous organic media. The defined exclusion limit of this column corresponded to about 4–5 kDa, void volume 9 ml, total volume 19 ml. The HPLC analysis of the excluded material (void volume) showed no substantial difference between normal and uremic sera (data not shown). The HPLC profiles of the included material demonstrated several fractions present in uremia, but absent in normal state.
Fig. 3. SELDI MS of the uremic peaks IV–VII (Fig. 2B) obtained from the uremic patients A.P., R.H., M.H., and S.R.
Fig. 3. (Continued).
Table 1
Amino acid sequences of MM peptides

<table>
<thead>
<tr>
<th>Sample (fraction)</th>
<th>Peptide sequences identified</th>
<th>Source (position)*, MW ID method (MS or Edman)</th>
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<td>EGDRLAEKGGGV</td>
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<td>RMEYVSLGV</td>
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<td>Edman</td>
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<td>FIBA (605–613), 930 Edman</td>
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<td>MS</td>
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<td>SGEEGDFLAEGGGGV</td>
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<td>S.R. (VII)</td>
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</table>

The peptides were isolated from sera of uremic patients (samples A.P., R.H., M.H., and S.R.) by using consecutively SDS-PAGE and RP-HPLC.

* The positions are indicated by using the numbering system from Swiss Prot for the precursor forms.

The peptides were isolated from sera of uremic patients (samples A.P., R.H., M.H., and S.R.) by using consecutively SDS-PAGE and RP-HPLC.

4. Discussion
Application of the appropriate small-scale purification techniques and sensitive analytical methods is critical for examination of MM accumulating in blood of uremic patients. Two alternative separation procedures have been applied in this work by employing either micro-preparative SDS-PAGE or SEC with a subsequent RP-HPLC. The consecutive SDS-PAGE and RP-HPLC technique was previously developed by us and found to be useful for purification of some
Fig. 4. RP-HPLC of serum material obtained by using consecutively ultrafiltration, separation on Sep-Pak C18 cartridges, and gel filtration on a Fractogel™ TSK HW-40(F) column (Fig. 1b). Samples were obtained from normal subject R.K. (a) and uremic patients S.M. (b) and P.A. (c), initial serum volumes 2.5, 3, and 11 ml, respectively. HPLC was carried out using a linear gradient from 0 to 50% acetonitrile in 0.1% TFA, flow rate 0.8 ml/min, sensitivity 0.85 AUFS.

proteins, peptides and related compounds present in small quantities of tissue or blood samples [26,30–35]. The micro-preparative electrophoretic technique is thought to be more effective for purification of serum peptides from other serum constituents, as compared with SEC which was used in our alternative separation scheme. After completion of the electrophoretic run, serum peptides were extracted from the SDS-gels and afterwards treated with guanidine hydrochloride to remove SDS, which was necessary prior to subsequent RP-HPLC [26,27]. However, even though most of the small and medium-size peptides remain soluble following this SDS precipitation procedure and could be successfully separated by RP-HPLC [28], the possibility also exists that some peptides can co-precipitate with SDS and become lost by this technique. Therefore, we used an alternative purification scheme, where all the peptide material (albeit more contaminated with other serum components) was recovered from Fractogel™ HW-40(F) column and afterwards was separated by RP-HPLC. In fact, SEC was performed in aqueous organic media, i.e. by using volatile elution solvent water/acetonitrile/TFA, which can be easily removed by lyophilization without need of dialysis, and therefore, is beneficial in manipulations with small quantities of material containing small and medium size molecules. We believe that these two alternative separation procedures complement each other, and by using both of them, we are able to study essentially all of the medium size peptides abnormally elevated in uremia.

Clinical experience and biochemical data show that hemodialysis patients may suffer from both thrombotic and bleeding tendencies, which are probably related to disturbances in the thrombin and plasmin activities [36–40]. The reasons for these disturbances are multifactorial and thought to be caused by uremia per se as well as by the hemodialysis procedure [6,7,38,41,42]. Uremic patients display evidence of complement activation and release of cytokines and tissue factor, which may initiate coagulation [41–43]. In addition to the fibrinolytic activity of plasmin, this protein is also capable of inducing activation of complement and triggering the release of factors stimulating clot formation (such as tissue factor and phospholipids), thus making both the bleeding and thrombotic tendencies more intensive [44–46]. The subnormal platelet aggregation and bleeding tendency is often explained by the defective conformational changes in the platelet membrane glycoprotein GP IIb-IIIa upon platelet activation [47,48]. Other studies suggest that small and middle-size plasma molecules may also affect the platelet aggregation process [49]. According to one of the recent hypotheses, some unknown fibrinogen fragments could accumulate in blood of uremic patients and occupy the GP IIb-IIIa receptor, which may inhibit the normal binding of intact fibrinogen to platelets and lead to reduced platelet aggregation and bleeding [48,49]. In fact, the increased levels of fibrinogen degradation products, including fibrinopeptide A, were demonstrated in uremic blood plasma [36,38,39,43,47,50–52]. However, most of these studies were carried out by using
Fig. 5. SELDI MS of the uremic peaks A–D (Fig. 4) obtained from the uremic patients S.M. and P.A.
immunoassays, such as radioimmunoassays, ELISA, and Western blotting, and the data on precise chemical structure of middle-size fibrinogen fragments were lacking. On the other hand, a number of circulating medium size fibrinogen fragments have been chemically identified in the extracorporeal ultrafiltrates from patients with end-stage renal failure. Those included middle molecular size fragment of the \( \beta \)-chain of fibrinogen (123–127) [17] and numerous fragments resulting from proteolytic cleavage at C-termini of the \( \alpha \)-chain of fibrinogen [24], as well as N-terminal fragments of fibrinogen B containing the thrombin cleavage and polymerization sites [25].

In contrast to the studies employing large amounts of extracorporeal fluids, our study was performed using small blood samples and was aimed in elucidation only of those medium-sized peptides whose amount was abnormally elevated in uremia comparing to healthy state. Several prominent peptide-containing fractions were found in each of the uremic samples tested, whereas all the control serum samples contained only the traces of such material. Twenty of the identified molecules matched the sequences found in the N-terminal domains in the \( \alpha \)- and \( \beta \)-chains of fibrinogen and apparently represented the catabolic products of fibrinopeptides A and B which are known to be cleaved from fibrinogen by thrombin. Two of the identified fibrinogen fragments were derived from

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Fig. 6. Total Ion Chromatogram of the uremic peak B (Fig. 4) obtained from the uremic patient P.A.
Table 2  
Amino acid sequences of MM peptides

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<th>Sample (fraction)</th>
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</tbody>
</table>

The peptides were isolated from sera of uremic patients (samples S.M. and P.A.) by using consecutively SEC and RP-HPLC.

The positions are indicated by using the numbering system from Swiss Prot for the precursor forms.

the C-terminal portion of the α-chain, which is prone to the fibrinolytic action of plasmin. Since both the control and uremic blood samples were handled and treated under the same experimental conditions, it is unlikely that the presence of high amounts of the identified fibrinopeptide fragments in uremic versus normal sera could be caused by in vitro cleavage processes. The obtained data provide new information on the precise structure of MM accumulating in uremic blood. It is intriguing to know whether these fibrinogen fragments could contribute to the platelet dysfunction in uremia.

In conclusion, the applied micro-separation and analytical methods were efficient for isolation and chemical identification of the MM peptides in small samples of uremic sera. The majority of the uremic MM peptides identified by mass spectral amino acid sequence analyses represented the catabolic products of fibrinopeptides A and B. The abnormal accumulation of these MM peptides may be caused by the imbalance in thrombin and plasmin activities, insufficient renal/dialyser clearance and/or impaired protein catabolism in uremia. Further studies are needed to understand whether these molecules play an active role in the development of disturbances observed in uremia.

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