# 3 Material and Methods

## 3.1. Materials

Amyloid ß petide 1-40 and 1-42 was kindly provided by Michael Beyermann and Prof. Michael Bienert, FMP, Berlin, Germany. Hank's Balaced Salt Solution (HBSS), trypsin, DNAse, Dulbecco´s modified Eagle´s medium (DMEM) were all purchased from Life Technologies, Gibco BRL, Eggenstain, Germany. Fetal calf serum (FCS), Penicillin, Streptomycin, bovine serum albumin (BSA), normal goat serum (NGS), horse serum (HS), and 0.1% Triton X-100, biotin, LPS from *Escherichia coli* K-235, IL-1, IL-18 and IL-6 recombinant proteins were all from Sigma-Aldrich, Deisenhofen, Germany. *Griffonia simplicifolia* isolectin B4 was from Vector Labs, Burlingame, USA. Streptoavidin-Cy2/ Cy3 was from Dianova, Hamburg, Germany. $\alpha$2M and anti-$\alpha$2M antibody were purchased from Calbiochem, Darmstadt, Germany. Protein A-sepharose CL4B was from Amersham Pharmacia, Freiburg, Germany. MICROCON concentrator (100 kD MW cut off) was from Millipore GmbH, Schwalbach, Germany. Anti-TNF$\alpha$, -IL-6, -total IL-12, -MIP-1$\alpha$, -MIP-1ß, -MCP-1 and -KC were purchased from R&D Systems, Wiesbaden, Germany. Streptavidin-HRP, ELISA-Grade was from Biosource, Camarillo, CA, USA.

## 3.2. Cell Culture

Animals were kept and treated according to the Guidelines for Animal Care at the MDC Berlin. Microglial cells were prepared from cerebral cortices of newborn Naval Medical Research Institute (NMRI) mice (purchased from Tierzucht Schönwalde GmbH, Schönwalde, Germany) as desribed previously (Prinz and Hanisch, 1999). The tissue (brain dissected from the brain stem, cerebellum and olfactory bulb) was carefully freed of blood vessels and meninges, washed two times with HBSS, incubated for 5-8 min with 1% trypsin and 0,05% DNAse (in HBSS), washed two more times with HBSS and carefully dissociated with fire-polished pipette. Mixed glial cells were then cultured in DMEM supplemented with 10% FCS and antibiotics (Penicillin-Streptomycin), with change of medium every third day. After 10 to 14 days of primary cultivation, microglia were separated from the underlying astrocytic monolayer by gentle shaking of the bottles for one hour at 37°C in a shaker-incubator (100 rpm). Microglial cells in the

supernatant were washed once and plated in 96-well plates (at a density of $10^4$ cells per well), 24-well plates (4-5 x $10^4$ cells per well) or Petri dishes (2 x $10^6$ cells per dish). Microglial cells were allowed to settle for 20 min. Non-adhesive cells were removed by washing in phosphate-buffered saline (PBS). A minimum of two days elapsed before use of microglia for experiments. The purity of microglial cultures was confirmed by staining with microglial marker *Griffonia simplicifolia* isolectin B4 ($IB_4$) (Vector Labs, Burlingame, USA) (Draheim et al., 1999). Routinely prepared cultures contained more than 95% microglia.

### 3.2.1 Medium

The culture medium, Dulbecco´s modified Eagle Medium (DMEM) with 10% FCS used in primary microglial cultures consisted of:

| | |
|---|---|
| DMEM | |
| Penicillin | 50 U/ml |
| Streptomycin | 50 µg/ml |
| L-Glutamin | 2 mM |
| Fetal Calf Serum (FCS) | 0,1 ml/ml |

### 3.2.2 Staining of microglial cells by Griffonia simplicifolia isolectin B4

Lectin-histochemical labelling of microglia cells was performed using *Banderia simplicifolia* isolectin ILB4 directly coupled to fluorescein-isothiocyanate (ILB4-FITC, Sigma) or ILB4 (Vector Labs) coupled to biotin (diluted 1:50) (Sigma), in combination with streptoavidin-Cy2 or Cy3 (diluted 1:200) (Dianova) all dissolved in 0.1M phosphate buffer, pH 7.1 (PB) containing 0.2% bovine serum albumin (BSA), 0.2% normal goat serum (NGS), 0.2% horse serum (HS), and 0.1% TritonX-100 (all from Sigma). Briefly, cultures were fixed in 4% paraformaldehyde in PBS for 5 min followed by two washes in PBS, then incubated with ILB4-FITC or biotinylated ILB4 (dilution 1:30) for 20 min. In order to visualize cell-bound lectin samples were additionally incubated with streptavidin-Cy2/Cy3 or streptavidin-Bodipy 1-10 µg/ml (Molecular Probes, Invitrogen

GmbH, Karlsruhe, Germany) for 2 h at 37°C. Cells were further analyzed under fluorescence microscope (Carl Zeiss, Jena, Germany).

### 3.3 Microglia staining in the retinal organotypic culture

In order to reveal the morphological features of the microglial cells within the cultured retina tissue before and after stimulation with LPS, lectin-histochemical labelling of microglia was performed with tomato lectin directly coupled to fluorescein-isothiocyanate (FITC) (Sigma). Retinal tissue was prepared according to the protocol of Mertsch et al., 2001. Eyes were obtained from 10 days old mice (NMRI, Tierzucht Schönwalde GmbH). Animals were enucleated immediately after cervical dislocation. Eyes were placed in ice-cold preparation medium (minimum essential medium (MEM), pH 7.35, containing 1% of a 200 mM glutamine solution (all from Gibco BRL Life Technologies, Scotland). Eyeballs were opened by slitting at the ora serrata and the retinae were removed. Retinae were flattened with the ganglion cells up onto pretreated filters situated in 6-well plates (Becton, Dickinson, Heidelberg, Germany), each containing 1 ml of ice-cold cultivation medium consisting of a mixture (3:1 v/v, pH 7.40) of MEM and Hank's balanced salt solution (HBSS, containing 2% 500 mM HEPES) supplemented with 25% horse serum, 2% glutamine, 1 μg/ml insulin (all from Gibco), 0.8 μg/ml vitamin C, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all from Sigma) and 2.64 mg/ml glucose (Braun Melsungen, Melsungen, Germany). After inserting the filters into the cultivation medium, medium covering the retina was carefully removed to avoid floating of the retinae. Cultivation was performed at 35°C and 2% $CO_2$. Medium was changed every day. After 8 days in culture, $10^{-7}$ g/ml of LPS was added to the culture medium for 20 h. At day 8 (just prior to the stimulation with $10^{-7}$ g/ml of LPS) and at day 9 (after the LPS stimulation), the retinae were fixed in 4% paraformaldehyde (PFA) and immersed in 25% sucrose solution dissolved in phosphate buffer (PB) (containing 20 mM $Na_2HPO_4/NaH_2HPO_4$, pH 7.4) by overnight shaking at 4°C. After repeatedly freeze-thawed, the retinae were washed in PB and incubated in permeabilization buffer (PB containing 1% triton X-100 and 2% bovine serum albumin, BSA, all from Sigma) for 1 h. The retinae were then treated with blocking solution (PB containing 0.1% triton, 2% BSA, 2% normal goat serum, NGS, and 2% horse serum, HS, all from Sigma) for 1h

and incubated overnight with FITC-labelled tomato-lectin (1:200) dissolved in 0.1 M PB containing 0.1% triton, 0.2% BSA, 0.2% NGS and 0.2% HS. After washing with PB, retinae were flattened and mounted in Citifluor-glycerol solution (Plannet GmbH, Wetzlar, Germany). Cells were observed with a fluorescence microscope (Zeiss Axioplan, Carl Zeiss, Jena, Germany) and photographed with Kodak EPL 400/TMAX 400.

### 3.4 Reverse transcriptase polymerase chain reaction (RT-PCR)

For detection of cytokine mRNA induction, microglial cells (2 x $10^6$ per Petri dish (6cm), were treated with LPS (100 ng/ml) for 1, 2, 4 and 8 hours. RNA was isolated using Trizol reagent (Gibco BRL, Life Technologies, Maryland, US). Briefly, 2 ml of Trizol reagent was used for the homogenization of the cells, with the lysate being pipetted several times to facilitate disruption. The RNA was extracted by adding chloroform (0.2 volume equivalents) and precipitated using 1 volume of aqueous phase and 1 volume of isopropanol. The RNA was washed twice in 75% ethanol. Up to 5 µg of the isolated RNA was then used for cDNA synthesis. RNA was incubated with 1 µl of random primers (200/ 400 ng/µl) in the volume of 11 µl, at 70 °C for 10 min. The mixture was then kept for 1 min on ice. The reaction mixture consisting of 1 µl of 10 mM dNTP mix, 4 µl of 5x Superscript RT buffer, 0.5 µl of RNAsin 10 U and 2 µl of 100 mM DTT (all purchased from Gibco, BRL) was added to the RNA/ random primer mixture and left for 5 min at room temperature. The reaction was started by addition of 1 µl of SuperScriptRTranscriptase (200 U, Gibco BRL). The final mixture was incubated for 50 min at 42 °C, followed by 15 min at 70 °C in order to stop the reaction. In order to degrade the RNA of DNA-RNA hybrids mixture was additionally treated with 1 µl of 2 U/µl of *E.coli* RnaseH (Gibco, BRL) at 37°C for 20 min. The RT-PCR for cytokines was carried out with a CytoXpress Mouse Cytokine Sepsis Set 2 Kit (BioSource, California, USA). The reaction mixture of distilled water (30.5 µl), 10x mSEP2G MPCR buffer (5 µl), 10x mSEP2G MPCR primers (5 µl), Taq DNA polymerase (5 U/µl, 0.5 µl), cDNA (5 µl) and dNTP (3.12 mM, 4 µl). The PCR thermocycle profile was setup as suggested in the manufacturer's instructions. After an initial denaturing step at 96 °C for 1 min, temperature profile of denaturation step at 96 °C (1 min) and annealing step at 56 °C (4

min) was run for 2 cycles, It was followed by 28 cycles of the temperature profile: denaturation step at 94 °C for 1 min and annealing step at 56 °C for 2.5 min. For the final step, reaction mixture was incubated at 70 °C for 10 min, followed by soak at 20 °C. RT-PCR products were analyzed by 2% agarose gel electrophoresis. Products were identified as the bands of the sizes (bp) 351 (TNFα), 294 (IL-1ß), 237 (IL-12p40), 453 (IL-6), 538 (IL-10), and 658 (glyceraldehyde-3-phosphate dehydrogenase, GAPDH, as house-keeping gene).

### 3.5 Preparation of Aß peptides

Amyloid ß peptide 1-40 and 1-42 used for cell stimulation experiments were synthesized and provided by the Laboratory of Prof. Bienert (FMP, Berlin). Peptides dissolved in distillated water at 1 mM concentration were sterilized by filtration (Millex-GV-Filter Units- pore size 0.45, Millipore, Schwalbach, Germany), and either immediately further diluted to the final concentrations and applied to the cells ("fresh" samples), or kept 7 days at 37°C and then diluted to the final concentrations and used for cell stimulation ("aged" samples). Aß 1-40 and Aß 1-42 peptides were also combined, in ratios 1:5, 1:1, 5:1, 10:1, in concentration range from 0.0625 µM to 20 µM (for details, Fig. 5). As for the single peptides, the mixtures were also used either freshly prepared, or left for incubation 7 days at high concentration and only then diluted and applied onto the cells. Presence of mono-/ oligomers or/and fibrillar protein forms in certain peptide preparations were further investigated by size exclusion chromatography and electron microscopy in the Laboratory of Prof. Bienert.

#### 3.5.1 Size exclusion chromatography

20 µl of Aß 1-40/ Aß 1-42 mixture samples (10 µM: 10µM) (after 1, 3 or 7 days of incubation) were applied to a precalibrated Superdex 75 HR 10/30 chromatographic gel column. Calibration was performed using oCRH (for monomers) and $Cys^{o}$- oCRH (for dimmers), at the concentration 1 mg/ml (dissolved in 0.5 M $NaHCO_3$). 50 mM phosphate buffer at pH 5.5 was used as the running buffer. Columns were eluted at a flow rate of 0.8 ml/min and peptides detected by UV absorbance at 220 nm.

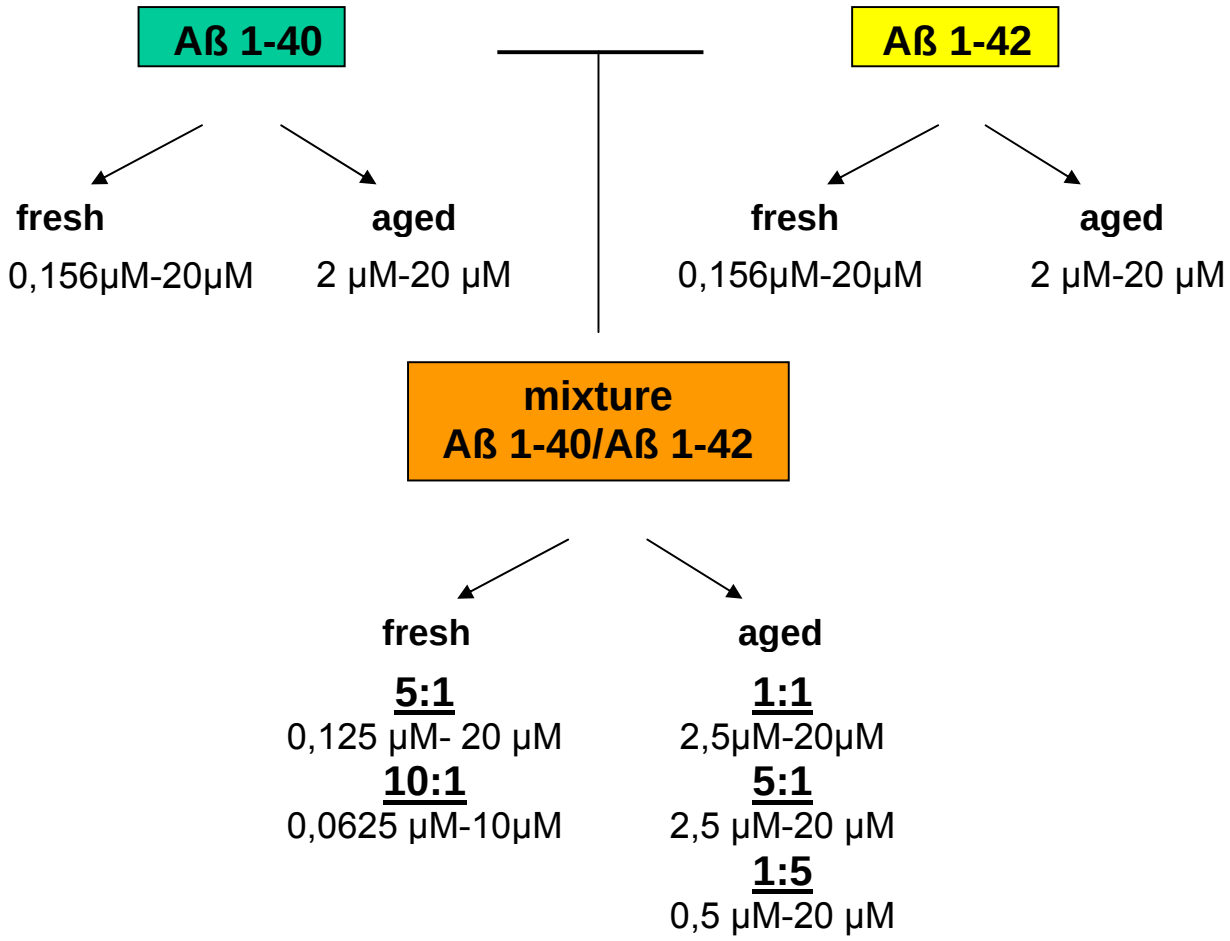


**Figure 5. Aß peptide preparation protocol.** Aß 1-40 and Aß 1-42 peptide batches, differently incubated (non-incubated, immediately used after dissolving in sterile water – "fresh", or 7 days incubated at 37°C – "aged"), alone or combined (in ratio of 1:1, 1:5, and 5:1, 10:1), at concentrations ranging from 0,5 µM to 20 µM, later tested for their potency to induce microglial release activity.

### 3.5.2 Electron microscopy

The Aß 1-40/ Aß 1-42 mixture (10 µM: 10µM), aged for 7 days was dissolved in 80% trifluoroethanol (TFE)/20% water (v/v) at a concentration of 1 mg/ml. A 5 µl aliquot was adsorbed for 60s onto 300-mesh copper grids coated with carbon film. Excess liquid was removed with filter paper. The air-dried sample on the grid was negatively stained with 5 µl of freshly prepared 2 % (w/v) uranyl acetate in water. Specimens were examined with 902A electron microscope (Zeiss, Oberkochen, Germany).

### 3.6 Chronic stimulation of microglial cells

In different stimulation experiments, primary microglial cultures (plated in 24 or 96 well plates, 4-5 x $10^4$ or $10^4$ cell per dish, respectively) were incubated with various test substances dissolved in DMEM/FCS, for 24h or 48h. All solutions were sterile filtrated before exposure of the cells. Following stimulation, cell culture supernatants were collected and stored at -70°C for cyto- and chemokine measurements and NO assay, whereas the cells were washed and stored at –20°C for protein assay. In order to optimize a protocol for Aß induced cyto/chemokine release in microglia, cells were exposed to freshly prepared or aged Aß 1-40 and Aß 1-42 peptide alone (in concentration range 0.156-20 µM), or to mixtures of both as described before, for 48 h. In co-stimulation experiments, 10 µM Aß mixtures (1:1) were combined with recombinant IL-1, IL-18 or IL-6 (Sigma), all at final concentration of 10 ng/ml, or with $\alpha$2M (Calbiochem) (10 nM - 500nM). $\alpha$2M was differently pre-treated depending on an experimental design.

### 3.7 Cytokine and chemokine quantification in Enzyme-Linked Immunosorbent Assays (ELISAs)

Analysis of cytokine (TNF$\alpha$, IL-6 and total IL-12 (collecting the IL-12 forms p70, p40, and $p40_2$)) and chemokine (MIP-1$\alpha$, MIP-1ß, MCP-1 and KC) content of the cell culture supernatants was carried out using mouse-specific antibody pairs and mouse protein standards designed for ELISA application in accordance with the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). Primary antibodies were incubated over the night at room temperature (RT) in Nunc-Immuno$^{TM}$ Plate, MaxiSorp$^{TM}$Surface (NagleNuncInternational, Denmark) 96 well-plates in concentrations: 0,8 µg/ml for TNF$\alpha$, 1 µg/ml for IL-6, 1.25 µg/ml for IL-12, 1 mg/ml for MIP-1$\alpha$, 1 µg/ml for KC. The plates were washed 4 times with washing buffer (PBS + 0,05% Tween 20). After 1 h incubation with block solution containing 1% BSA (Bovine Serum Albumin, FLUKA), 5 % sucrose, 0.05 % $NaN_3$ in PBS, samples and standard were incubated for 2 h. Detecting antibody in concentration of 3 µg/ml for TNF-$\alpha$, 0.1 µg/ml for IL-6, 0.625 µg/ml for IL-12, 0.05 µg/ml for MIP-1$\alpha$, and 0.1 µg/ml for KC were added for 2 h at RT. After washing,

SA-HRP (Streptavidin-HRP, ELISA-Grade, Biosource) was added for 30 min. Following short incubation with Peroxidase substrate-TMB reaction was stopped with 1 M $H_2SO_4$. Colorimetric reaction was analysed in a microplate reader (SLT, Spectra LabInstruments Deutschland GmbH, Crailsheim, Germany) as absorbance at 450 nm with 540 nm as reference wavelength. Cytokine and chemokine amounts were normalized to the total cell protein as measured using BCA protein test assay (Pierce, Bonn, Germany). The sensitivity of assays is very high: 3 pg/ml for IL-1ß; ≤1.8 pg/ml for IL-6; ≤ 2.5 pg/ml; 25 pg/ml for IL-18; ≤ 5.1 pg/ml for TNFα; ≤1.5 pg/ml for MIP-1α; ≤3 pg/ml for MIP-1ß; ≤ 2 pg/ml for MCP-1.

### 3.8 Nitric oxide release assay

The amount of NO produced by microglia was measured as nitrite accumulation in the cell supernatants using Griess assay. The assay is based on nitrate reductase catalyzed reduction of nitrate into nitrite. In the final step, Griess Reagent converts the nitrite into a purple-colored azo compound, which can be quantified by spectrophotometer as absorbance at 540 nm.

Cell culture supernatants were assayed by the addition 1:1 (v/v) of reaction mixture to each well of a 96-well plate (in triplicate). Reaction mixture consisted of 1:1 freshly mixed Griess Reagent A (0.1% N-1-naphthyl ethylene diamine dehydrochloride 0.1% in $H_2O$) and Griess Reagent B (1% sulphonil amide in 5% H3PO4) (Sigma). After 5-10 min incubation at RT, level of developed colorimetric product was measured in a microplate reader (SLT, Spectra LabInstruments Deutschland GmbH, Crailsheim, Germany). The NO concentrations (nmol/ml) were calculated according to a standard curve obtained in each assay by using $NaNO_2$ (NACALAI TESQUE Inc., Kyoto, Japan; dissolved in 15 mM Hepes, pH 7.5).

### 3.9 Total cell protein measurement

In order to normalize values of the release parameters, the measured values were considered in reference to total protein of treated cells. Following cell treatment amount of cell protein was determined using Micro BCA™ Protein Assay Reagent, Pierce. The Kit enables quantitative colorimetric determination of total protein in dilute aqueous

solutions, utilizing bicinchoninic acid (BCA) as the detection reagent for $Cu^{+1}$, which is formed when $Cu^{+2}$ is reduced by protein in an alkaline environment (Smith et al., 1985). The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion ($Ca^{+1}$). This water-soluble complex then exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations.

Cells plated in 24 WP or 96 WP were washed three times with PBS and dried. 0,005% SDS solution (in PBS) was added and samples were kept for 1 h at -80°C. Subsequently 150 µl of cell lysate was mixed with 150 µM of BCA reagent. The BCA protein assay working reagent consists of reagent A (contains sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1N sodium hydroxide) and reagent B (contains 4% copper sulphate) mixed 50 parts: 1 part, respectively. Samples were well shaken (30 min) on plate shaker at RT, and then incubated for 2 h at 37°C. The absorbance was measured at 562 nm in a plate reader. Absolute values were calculated according to the bovine serum albumin (BSA) standard values.

### 3. 10 Cell proliferation assay

BrdU (5-Brom-2`-deoxy-uridine Labeling and Detection Kit - cell proliferation assay (Roche Molecular Biochemicals) was used for quantification of cell DNA synthesis and cellular proliferation according to manufacturer's instructions. BrdU test is a colorimetric assay based on the incorporation of BrdU in place of thymidine into DNA. Cells, which have incorporated BrdU into DNA, can be easily detected using monoclonal antibody against BrdU. The microglia cells, plated in 96 WP were treated with $\alpha$2M (100 nM, 250 nM) in presence of BrdU labeling mixture, for 18 h, at 37°C under 5% $CO_2$. The colorimetric reaction was quantified in a microplate reader (SLT, Spectra Lab Instruments Deutschland GmbH, Crailsheim, Germany) as absorbance at 405 nm with 490 nm as reference.

### 3.11 Immunoprecipitation

Protein A-sepharose CL4B (Pharmacia, 400 µl, 10% suspension) was placed in the filter reservoir of a MICROCON concentrator (100 kD MW cut off) and centrifuged for 15 min

at max 10,000 x g in order to collect the protein A-sepharose on the filter. Beads were then resuspended in 250 µl of PBS, and 20 µl of anti-α2M antibody (Calbiochem, 3.24 mg/ml, 90% rabbit IgG) was added to filter reservoir and incubated for 60 min at RT in order to form an anti--α2M antibody/ protein complex. After centrifugation and resuspension in 60 µl of PBS, 20µl of α2M (20 µM) was added to anti-α2M antibody/ protein complex and mixture was let for 60 min incubation at RT with occasional mixing of material. Following incubation, samples were centrifuged in order to get sepharose-beads precipitated and supernatant collected. The supernatants were then used for microglial stimulation at final original concentration of 250 nM of α2M. In parallel α2M absorbed only with protein-A-sepharose beads, without antibody, was used as a positive control.

### 3.12 Fast Performance Liquid Chromatography (FPLC)

An α2M (Calbiochem) preaparation was fractionated by FPLC gel chromatography using a Pharamacia LCC501 Plus (Controller), Pump P-500 FPLC system with a Superose 12 HR 10/30 column. The column was equilibrated with the elution buffer, 50 mM $Na_2HPO_4/NaH_2PO_4$, 150 mM NaCl, pH 7.2, before the application of α2M (10 µM). The same buffer was used to elute the sample with a flow rate of 0.5 ml/min. 0.5 ml fractions (after 4 ml) were collected. Protein was recorded as absorbance at 280 nm.

### 3.13. Statistical analysis

Statistical analysis was performed using SPSS Software. The results are expressed as mean values ± standard errors of the mean (SEM). Significant differences have been determined by Mann-Whitney U Test for unpaired samples. Significance was assumed if $P^* < 0.05$ or $P^{**} < 0.01$