CHAPTER II
MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents used in this study and their sources

All chemicals used were analytical grade or equivalent. The chemicals below are listed in-groups according to supplier.

BDH Laboratory Reagent (Poole, England)

Sodium acetate trihydrate

Bio-Rad Laboratories (Hercules, CA)

Protein assay (Dye reagent concentrate), N,N,N',N'-tetra-methyl-ethylenediamine (TEMED)

Carlo Erba reagenti (Rodano (Mi), Italy)

Di-sodium hydrogen phosphate anhydrous, potassium sulfate, sodium chloride, sodium-dihydrogen phosphate

Fluka (Buchs, Swizerland)

Chondoitin sulfate A, glacial acetic acid, methanol, sodium hydroxide, Tri-sodium citrate dihydrate

Merck (Darmstadt, F.R. Germany)

Hydrogen peroxide, sodium acetate trihydrate, sodium chloride, sodium hydrogen carbonate, sodium carbonate anhydrous, potassium chloride

Pharmacia LKB, Biotechnology AB (Uppsala, Sweden)

Ammonium persulfate, coomassie blue brillant R-250, CNBr-Sepharose 4B, EAH Sepharose 4B, glycine, mercaptoethanol, Hybond ^{TM}-C super nitrocellulose, N,N'-methylenebis acrylamide

SERVA (Heidenberg, New York)

Triton X-100

Sigma (Sigma Aldrich, St. Louis, MO, USA)

Biotinamidocaproate-N-hydroxysuccinimide ester, bovine serum albumin, cesium chloride, citric acid anhydrous, 3,3'-diaminobenzidine (DAB), 1-ethyl-3-(-3-dimethylamino-proply) carbodiimine (EDAC) guanidine hydrochloride, hyaluronidase, hyaluronic acid (from human umbilical cord), p-nitrophenyl phosphate, polyoxyethylene sorbitan monolaurate (Tween 20), potassium chloride, sodium phosphate, (Tris[hydroxymethyl]-aminomethane hydrochloride), trypsin type XIII from bovine pancrease, trypsin inhibitor type I-S from soybean, N-hydroxysuccinimidobiotin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide,4,4-dicarboxy-2,2-biquinoline, *o*-phenyl enediamine

2.1.2 Lists of the antibodies used in this study

IgM-specific peroxidase conjugated anti-mouse immunoglobulin (μ-chain specific, Sigma Aldrich, U.S.A.)

Peroxidase conjugated anti-biotin monoclonal antibody (Zymed Laboratory, Inc. Ca, U.S.A.)

1H8 monoclonal antibody-obtained from hybridoma cell line derived from Balb/c mice spleen cells after immunized with embryonic shark A1 fraction (Tiengburanathum, 1996)

2.1.3 Experimental instruments

Analytical balance, AC 100 (Metter Instrument A.G., Switzerland)

Fraction collector, 2112 Redirac (LKB, Sweden)

Incubator, B 5050 (Heraeus, Germany)

Lyophilizer (Martin Christ Gefriertrock nugsanlagen GmbH, Germany)

Magnetic stirrer (Thermolyne Co., U.S.A.)

Membrane filter, 0.2 mm (Gelman Sciences Inc, U.S.A.)

Mini-PROTEIN II Electrophoresis Cell (Bio-Rad, U.S.A.)

Mini Trans-blot Electrophoresis Transfer Cell (Bio-Rad, U.S.A.)

Multiple pipette, 852 (Calibra, Switzerland)

pH meter, 71 pH meter (Beckman, U.S.A.)

Pipette tip 10, 100, 1000 µl (Treff lab, Switzerland)

Power Supply, Model 200/2.0 (Bio-Rad, U.S.A.)

Refrigerator –20 ^{0}C (Sanyo, Thailand)

Safeguard centrifuge, Clay Adams (Becton, Dickenson and Company, U.S.A)

UV-Visible spectrophotometer, Spectronic Genesys 5 (Milton Roy, U.S.A.)

Vortex-Genie, K-550-GE (Scientific Industry Springfield, U.S.A.)

96 well microtiter plate, Maxisorp (Nunc, Denmark)

2.1.4 Study subjects

All subjects gave their informed consent before participation.

Healthy subjects. Serum samples were collected from 23 apparently healthy individuals, 13 women and 10 men, age 41-75 years. Serum was stored at –20 ^{0}C before use. All subjects were checked to be free from articular, bone, liver, endocrine, or other chronic disorders. None was currently taking any medication know to modify arthritic disease or influence joint metabolism.

Joint disease subjects. Serum samples were collected from individuals who had been previously diagnosed with OA and RA according to Rheumatology criteria; suffering from unilateral or bilateral of knee according to criteria of the American College of Rheumatology (Hochberg *et al.*, 1995). Samples were obtained from consecutive individual visit to the physician during the collection interval. Serum was store at –20 ^{0}C before use. The OA group consisted of 50 patients, ages 41-82 years. The RA group consisted of 57 patients, age 24-78 years.

2.2 Experimental procedure

2.2.1 Proteoglycans preparation

Shark and bovine cartilage, obtained fresh from slaughterhouse, were washed with 1% (v/v) Triton X-100 in normal saline solution, dried on filter paper, transferred to plastic bags and frozen until required. Frozen tissues were dissected free of adhering tissue and perichondrium and finely shaved with sterile stainless steel surgical blades. The cartilage were agitate in 10 times its weight of cold 4 M-guanidine hydrochloride containing enzyme inhibitors (Heinegard *et al.*, 1977) at pH 5.8 for 24 h at 4 ^{0}C. The extract was filtered through two layers of lint on Buchner funnel. The filtrate was dialyzed against distilled water and lyophilized. The lyophilized powder was designated *cartilage extract* or proteoglycans (PGs).

2.2.2 hyaluronan binding proteins preparation

2.2.2.1 Isolation and purification of A1

Following the extraction steps of proteoglycan, after cartilage were agitate in 10 times its weight of cold 4 M-guanidine hydrochloride containing enzyme inhibitors, the density of dialysate was adjusted to 1.6 g/ml by adding CsCl powder. Associated form and disassociated form of centrifugation were prepared in the ultracentrifuge operating at 38,000 rpm for 48 hours. Tubes were sliced into 5 approximately equal segments denoted A1 to A5 for the associated fraction and A1D1-A1D5 for the disassociated fraction (bottom to top), respectively (Caterson *et al.*, 1979).

The A1 proteoglycan fraction is the partially purified from cartilage. From the highly concentrated guanidine hydrochloride (GuHCl) extraction, proteoglycans are eluted from the collagen network of cartilage and disperse in the form of separated or disassociated molecules (hyaluronan, link protein, proteoglycan monomer, small proteins, and collagen fragment). GuHCl is removed from solution by dialysis leading to re-association of (nearly) all proteoglycan components (Hardingham, 1981). This highly associated form of proteoglycan molecules is separated from other small ones by Cesium chloride gradient centrifugation with the largest molecules (major component in cartilage) situated at bottom of centrifugation tube. The contents of the centrifuge tube are separated into several fractions: 3,4 or 5 according to purpose, that

the lowest bottom fraction is the A1 [associated form] (Vilim *et al.*, 1994). The A1 fraction thus contains the highest concentration of associated proteoglycans.

2.2.2.2 Isolation and purification of HABPs

A 1.6 g. of lyophilized cartilage extract was dissolved in 50 ml of 0.1 M sodium acetate, 0.1 M Tris-base, pH 7.3 and incubated at 37 ^{0}C with 0.8 mg of trypsin. After 2 hours, 1 mg of soybean trypsin inhibitor was added and then dialyzed against distilled water and lyophilized. The lyophilized powder was designated *trypsinized cartilage extract* or *trypsinized PGs*.

A 0.2 g of trypsinized PGs was dissolved in 11.5 ml of 4 M GuHCl, 0.5 M sodium acetate pH 5.8. The solution was mixed with 10 ml of hyaluronan-substituted Sepharose 4B and dialyzed for 24 hours at 4 ^{0}C against 9 parts of distilled water. The gel slurry was packed into a column (10 x 1.5 cm) and washed with 50 ml of 1 M sodium chloride followed by 150 ml sodium chloride with linear gradient from 1 to 3 M to remove non-specifically adsorbed materials. The proteins that bound to HA-Sepharose was then eluted with 50 ml of 4 M GuHCl (Tengblad, 1979). The column was operated at room temperature with a flow rate of 0.5 ml/min. The eluent collected in 3 ml/fractions was estimated by ultraviolet absorbtion at 280 nm. The effluent fractions containing HABPs were pooled and dialyzed against distilled water. Then the dialysate was lyophilized and measured protein concentration by Bradford assay.

Umbilical cord hyaluronan was conjugated to EAH-Sepharose 4B according to the procedure described by Tengblad (Tangblad, 1979). The carbodiimide technique was used to couple carboxyl groups in hyaluronan to amino groups on a substituted agarose.

Thirty mg of hyaluronan were dissolved in 10 ml of 0.15 M sodium chloride and 0.1 M sodium acetate pH 5.0 and incubated for 3 hours at room temperature (25 ^{0}C) with 1 mg of bovine testicular hyaluronidase. Boiling the mixture for 3 min terminated the reaction.

Ten ml of EAH-Sepharose 4B were washed with 25 ml of 0.5 M sodium chloride and 100 ml of distilled water.

For coupling of hyaluronan digested and EAH-Sepharose 4B were mixed with distilled to 30 ml and adjusts pH to 4.5, Add 0.575 g of 1-ethyl-3-(-3-dimethylamino-

proply) carbodiimine (EDAC) guanidine hydrochloride to the mixture and kept pH at 4.5-6.0 for 24 hours at room temperature. To block the remaining unsubstituted amino groups on the gel, 1 ml of glacial acetic acids was added and the solution was allowed to stand for 6 hours at room temperature. The gel was washed sequentially with 400 ml each of (a) 1 M NaCl, (b) 0.1 M Tris-HCl, pH 8.1, 1 M NaCl, (c) 0.05 M sodium acetate pH 3.1 and, finally, distilled water. It was then transferred to 0.5 M sodium acetate, pH 5.7. In this solution the gel could be stored at 4 ^{0}C for months without loss of binding activity.

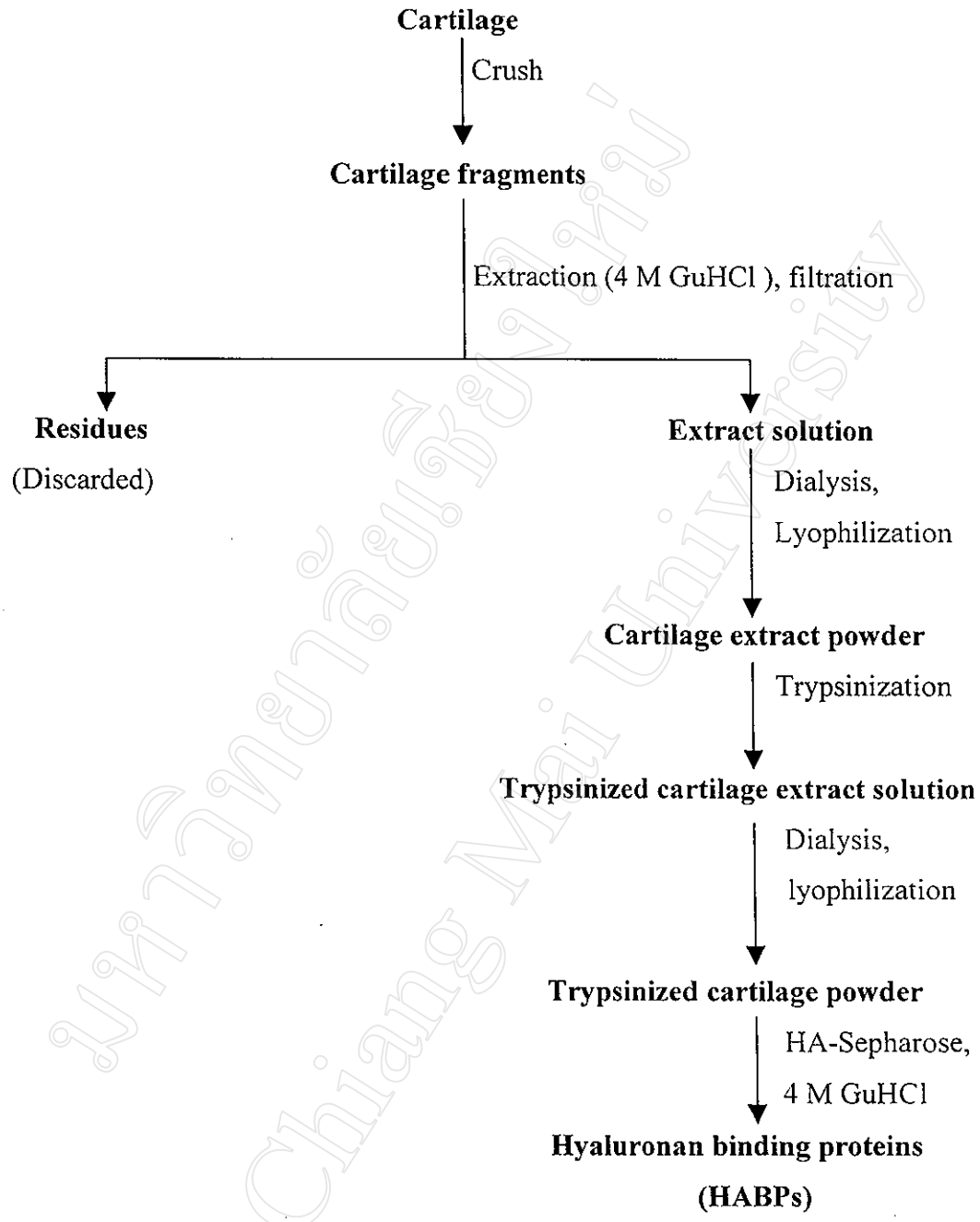

Figure 12 Procedures for the isolation of HABPs from cartilage (Heinegard, 1977)

2.2.3 Monoclonal antibody 1H8 preparation

2.2.3.1 Cell culture procedure

Cell culture was performed under aseptic condition in sterile equipment. Hybridoma cells line used in this study was 1H8. The cells were cultured in IMDM medium supplemented with 10% fetal calf serum at 37 ^{0}C, humidified and 5 % CO_2 condition. Hybridoma cell line was checked for its sterility and transferred to non-fetal serum medium.

2.2.3.2 Monoclonal antibody 1H8 purification

Hybridoma cell line culture medium was centrifuged to get rid off cells or residues and add 87 mg of potassium sulfate per ml of sample then mix well. The final concentration was 0.5 M K_2SO_4 in the sample. The mixture was mixed without foaming to avoid denaturation of the immunoglobulin. When the potassium salt fully dissolved, centrifuge the sample at 10,000 x g for 20 minutes. Carefully remove the clear supernatant and applied 1 ml of mixture to 3 ml columns of T-Gel adsorbent. At larger sample volumes, it was expected that most of the not-bound (NB) fractions contain some immunoglobulin. These NB fractions were pooled and treated as the sample for a subsequent running if it desirable to recover all or most of the immunoglobulin from the original sample.

Then the mixture was equilibrated with T-Gel binding buffer and eluted unbound protein from column with the same buffer. Bound fractions were eluted with T-Gel elution buffer and continue to collect the column, 3 ml per fractions. The protein content in each fraction was determined by spectrophotometer at wavelength 280 nm and determined the mAb 1H8 activity by direct ELISA technique. Fractions containing proteins were pooled and dialyzed against deionized water at 4 ^{0}C overnight and lyophilized. Purified antibody powder was stored at 4 ^{0}C until used.

2.2.4 Optimization of competitive ELISA condition for mAb 1H8

2.2.4.1 Optimization for coating microwell plates PGs concentration

Shark proteoglycans (shark PGs) as antigen on microtitre plate (Maxisorp®, Nunc) coating. Shark PGs with concentration 10 μg/ml was diluted (v/v) 10 fold with coating buffer, 20 mM Na_2CO_3 pH 9.6. Only columns 2 to 12 were coated with each dilution (100 μl/ml) and incubate mixture in microwell plates at 4 ^{0}C, overnight. PBS-tween as a washing solution for all washing steps in these experiments. After washing excess PGs from plates with PBS-tween 100 μl/well 3 times, plates were dried and block nonspecific adsorption of protein with 1% BSA diluted in distilled water. Plate was incubated at 37 ^{0}C. After 60 min, plate was washed and incubates with mAb 1H8 diluted 1:30, 1:40, 1:50, 1:60, 1:70, 1:80, 1:90, 1:100 on rows A to H respectively. After incubation, washing, and drying steps, plate was incubated with anti-mouse IgM (μ-chain specific) peroxidase conjugate diluted 1:2,000 in PBS each wells at 37 ^{0}C, 1 hour. Plate was washed and incubated with peroxidase substrate to develop color. After 15 min, 1 M H_2SO_4 50 μl was used to stop reaction and the absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

2.2.4.2 Optimization for mAb 1H8 concentration in inhibition mixtures

Microtiter plate was coated with optimized shark PGs in coating buffer at 4 ^{0}C, overnight. Plate was washed and blocked with 1% BSA (diluted in distilled water) 150 μl/well, and prepared *inhibition mixture* by mixed dilution of standard competitor (shark A1) and dilution of mAb 1H8 together in eppendrof. The inhibition mixtures were incubated at 37 ^{0}C, 60 min. Each dilution of the incubation mixtures were then transferred to microtiter plates coated with shark PGs. Access antibodies bound to the coating antigen were quantified by incubation with anti-mouse IgM (μ-chain specific) peroxidase conjugated diluted 1:2,000 in PBS (100μl/well) each wells at 37 ^{0}C, 60 min, The following method was also done as above.

2.2.4.3 Optimization for standard Inhibitor condition

Microtiter plate was coated with optimized shark PGs in coating buffer at 4 ^{0}C, overnight. Plate was blocked with 1% BSA (150 µl/well), and prepared *inhibition mixture* by mixed dilutions of competitor inhibitors; Bovine HABPs, Bovine A1, shark HABPs, and shark A1 with optimized concentration of mAb 1H8 together in eppendrof. The following method was done as above.

2.2.4.4. Optimization for Anti-mouse IgM peroxidase conjugate concentration

Microtiter plate was coated with optimized shark PGs in coating buffer at 4 ^{0}C, overnight. Plate was blocked (after washed) with 1% BSA(150 µl /well), and prepared *inhibition mixture* by mixed dilution of competitor (shark A1) and optimized concentration of mAb 1H8 together in eppendrof. The inhibition mixtures were incubated at 37 ^{0}C, 60 min. Each dilutions were then transferred to microtiter plates coated with shark PGs. Access antibodies bound to the coating antigen were quantified by incubated with anti-mouse IgM (µ-chain specific) peroxidase conjugated and diluted at concentration 1:2,000 (row A to C), 1:3,000 (row D to F) and 1:4,000 (G to H) in PBS (100µl/well) at 37 ^{0}C, 60 min. The following method was done as above.

2.2.4.5. Optimization for PBS and 6% BSA condition

After optimized shark PGs plate coating, mAb 1H8, standard HABPs and anti-mouse IgM peroxidase conjugate, in the step of competitor (shark A1) standard dilution, 6%BSA (w/v) was used in order to maintain the protein content of the solution. It was compared with PBS in order to study influence of 6%BSA solvent and non-protein solvent (PBS) for ELISA condition. Plates were done as the same condition. In rows A to D, 6%BSA were used to be as solvent for competitor and PBS as solvent for the same competitor in rows E to H. The following method was done as above.

2.3 Analytical procedure

2.3.1 Bradford assay

Bradford assay is the best methods for quantitation of protein amount. Its based on protein binding of coomassie brilliant blue dye. The binding of dye at wavelength absorption at 595 nm is directly related to the concentration of protein

A calibrate curve was prepared using bovine serum albumin as a standard. The assay required only a single reagent, an acidic solution of coomassie brilliant blue G-250. After addition of dye solution to a protein sample, color development was completed in 2 min and the color remains stable for up to 60 min. The Bradford assay could be used to determine proteins in the range of 1 to 20 μg (Bradford, 1976).

2.3.2 Electrophoresis

Native-PAGE determined the HABPs in serum according to the method of Ornstein and Davis (Ornstein *et al.*, 1964). Separating gel consisting of 7.5% polyacrylamide, 1.5 M Tris-HCl (pH 8.8) was prepared between glass plates of Casting frame. Stacking gel composed of 4% polyacrylamide, 0.5 M Tris-HCl (pH 6.8) was overlaid to the separating gel. Just before gel loading, serum sample were supplemented with blue native sample buffer to ratio 1:3 and applied onto polyacrylamide gradient slab gels. Molecular mass markers were run on both borders of the gel and were visualized by Coomassie Blue staining. Electrophoresis was carried out at a constant voltage of 120 V until the dye migrated toward the end of the gel. The gels were then removed from the glass plates.

2.3.3 Immunoblotting

The sample was separated by SDS-PAGE under non-reducing conditions and was electrotransferred onto (Hybond™-C super) nitrocellulose for 45 min at 4 °C. The membrane was blocked with 1% BSA in PBS, 0.05% Tween 20 and agitated (every steps) for 60 min at room temperature. The membrane was washed and incubated with primary mAb 1H8 in PBS, 0.05% Tween 20 for 60 min at room temperature, Unbound proteins were removed by rinsing (3 x 5 min) in PBS, 0.05% Tween 20. The bound proteins reacted with IgM-specific peroxidase conjugated anti-mouse immunoglobulin

1:2,000 in PBS, 0.05% Tween 20, and treated with peroxidase substrate to detect proteins that bound to mAb 1H8.

For the HA-Transblot assay, the membrane was incubated with biotinylated-hyaluronan (b-HA) and then reacted with Peroxidase conjugated anti-biotin monoclonal antibody 1:1,000 in PBS, 0.05% Tween 20 for 60 min at room temperature, and treated with DMB to detect proteins that bound to b-HA (Austin *et al.*, 1988).

2.4 Evaluation for accuracy and precision of developed method

2.4.1 Intra assay and inter assay variation

Both intra- and inter-assay using competitive ELISA procedure. Serum was diluted in 6% BSA in order to maintain the protein content of the solution.

Intra-assay precision: determined by using 12 identical aliquots of 20 pooled serum in triplicate analysis within the same plate.

Inter-assay precision: determined by using 20 individual sera of above pooled serum in triplicate measurement on different plates.

2.4.2 Recovery

The analytical recovery of serum HABPs was determined by using pooled serum which has been spiked with known amount of standard HABPs. The HABPs concentration in combined samples was measured and compared with the theoretical concentration.

2.5 Application of newly developed method for mAb 1H8 epitopes

Microtiter plates was coated with optimized shark PGs in coating buffer at 4 ^{0}C overnight. Non-binding active site on plates was blocked with 1%BSA and incubated at 37 ^{0}C, 60 min. after washing with PBS, 0.05% Tween 20 (3 x 5 min), 100 µl of mixture, sample or standard competitor were added. After incubation at 37 ^{0}C for 60 min, plates were washed and then IgM-specific peroxidase conjugated anti-mouse immunoglobulin 1:2,000 (100 µl/well) in PBS was added and incubated at the same condition. The plates were washed and then added peroxidase substrate (100 µl/well) and allowed the color to develop 15 min. The reaction was stopped by addition 4M H_2SO_4 (50 µl/well). The

absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

2.6 Statistical method

2.6.1 Percentage inhibition

The percentage inhibition was calculated by following formula:

$$\% \text{ Inhibition} = 100 - \left[\frac{(\text{OD control} - \text{OD blank}) - (\text{OD sample} - \text{OD blank})}{[\ \text{OD control} - \text{OD blank}\ ]} \right] \times 100$$

A standard inhibition curve for HABPs was instructed by using log/linear co-ordinators. The HABPs levels in the test samples were determined by compairing their capacity to inhibit color delvelopment at OD 492/690 nm relative to standard curve constructed using a computer software (Deltasoft II).

2.6.2 Serum HABPs concentration

For serum HABPs epitopes, the healthy and patient groups were expressed as means and SD. Comparisons between subjects with untreated arthritis and healthy population were evaluated with unpair t-test. For these analyses, $P < 0.05$ (two-tail test) was considered significant. Statistical calculations were performed by using StatView 512+ software on a Macintosh computer.

2.6.3 Rf value of protein and marker dye

The Rf value of protein and marker dye was calculated by following formula:

$$\text{Mobility (Rf)} = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

The Rf values were plotted against standard molecular weight and expressed on a semi-logarithmic scale.