CHAPTER III
RESULTS

3.1 Isolation and purification of Hyaluronan-binding protein

The isolation method of HABPs from cartilage was based on a combination of two major steps. First, the cartilage was extracted with 4M GuHCl. The residues were separated from extract by filtration. Extract then dialyzed against distilled water and lyophilized. The white powder of extract from 20 g cartilage was achieved 1.6 g from bovine cartilage and 1.4 g from shark cartilage. The globular protein core of proteoglycan was cleaved by trypsin, lyophilized and incubated with HA-Sepharose dialyzed against water.

Second step, HA-binding molecules were eluted from HA-Sepharose gel column by washing with 1 M NaCl and followed 1-3 M sodium chloride gradient (Tengblad, 1979). HABPs were released by elute with 4 M GuHCl.

The protein profile obtained from HA-affinity column chromatography of trypsinized bovine cartilage extract was shown in Figure 13. The first peak is unbound material observed at 280 nm, and the second peak is HABPs eluted by 4M GuHCl buffer, pH 5.8. The protein was measured in lyophilized power by Bradford assay.

The protein profiles obtained from HA-affinity column chromatography of trypsinized shark cartilage extract were shown in figure 14. The protocol was done as above.

The yield of HABPs from 1.6 g of trypsinized bovine cartilage extract was 0.74 g, and 0.41 g of HABPs from 1.6 g of trypsinized shark cartilage.

From this experiment indicated that bovine and shark HABPs were isolated efficiently by this method but small amount (46.25% of bovine HABPs and 25.63% of shark HABPs) were achieved.

3.2 Antibody purification

Thiophilic gel was used to purify monoclonal 1H8 from 1H8 cell supernatant. Single peak was observed in this purification method when eluted with low salt

concentration buffer (figure 15). A 0.026 g of monoclonal antibody powder, after dialysis in deionized water and lyophilizing, was obtained from 80 ml of supernatant.

Purified antibody powder was tested by chequerboard of direct ELISA using shark PGs as antigen. Poor activities was observed in mAb 1H8 powder compared to supernatant at the same volume

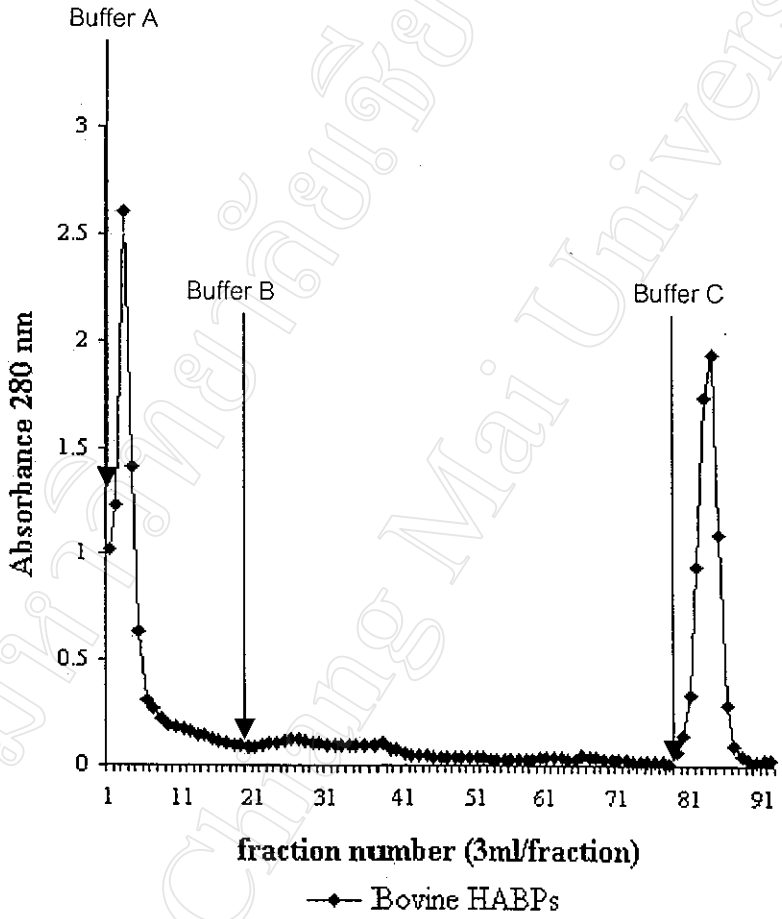

Figure 13 Purification of Bovine HABPs by affinity column chromatography on HA-Sepharose from trypsinized bovine cartilage extract.

Buffer A = 1M NaCl

Buffer B = Linear gradient from 1 to 3 M NaCl

Buffer C = 4 M GuHCl, pH 5.8

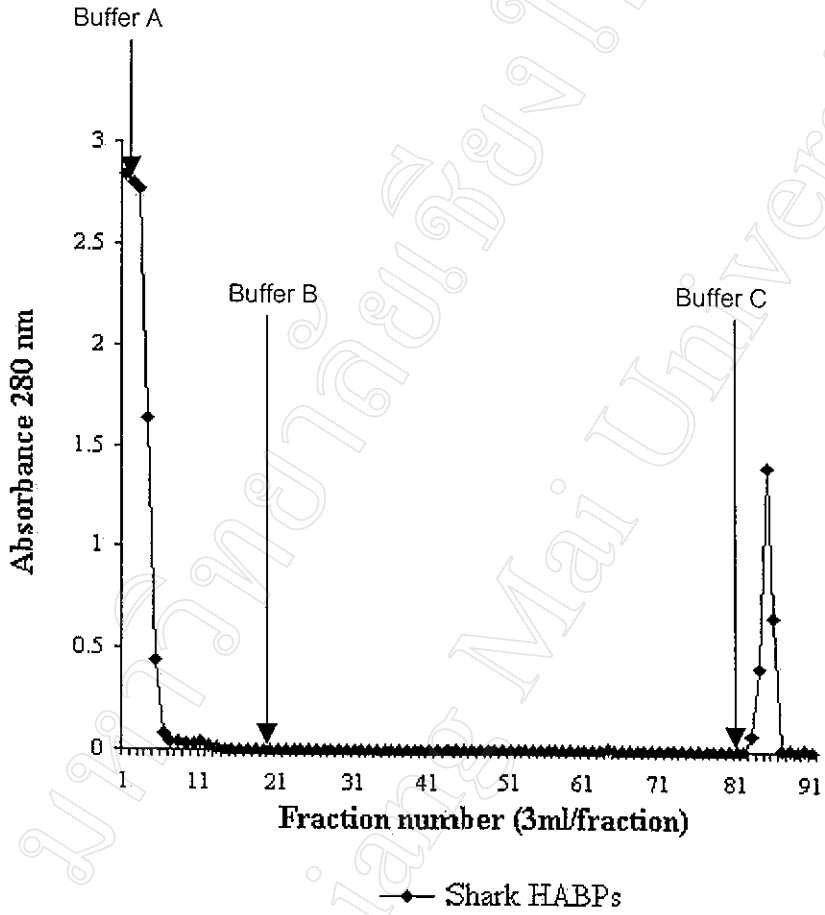

Figure 14 Purification of shark HABPs by affinity column chromatography on HA-Sepharose from trypsinized shark cartilage extract.

Buffer A = 1 M NaCl

Buffer B = Linear gradient from 1 to 3 M NaCl

Buffer C = 4 M GuHCl, pH 5.8

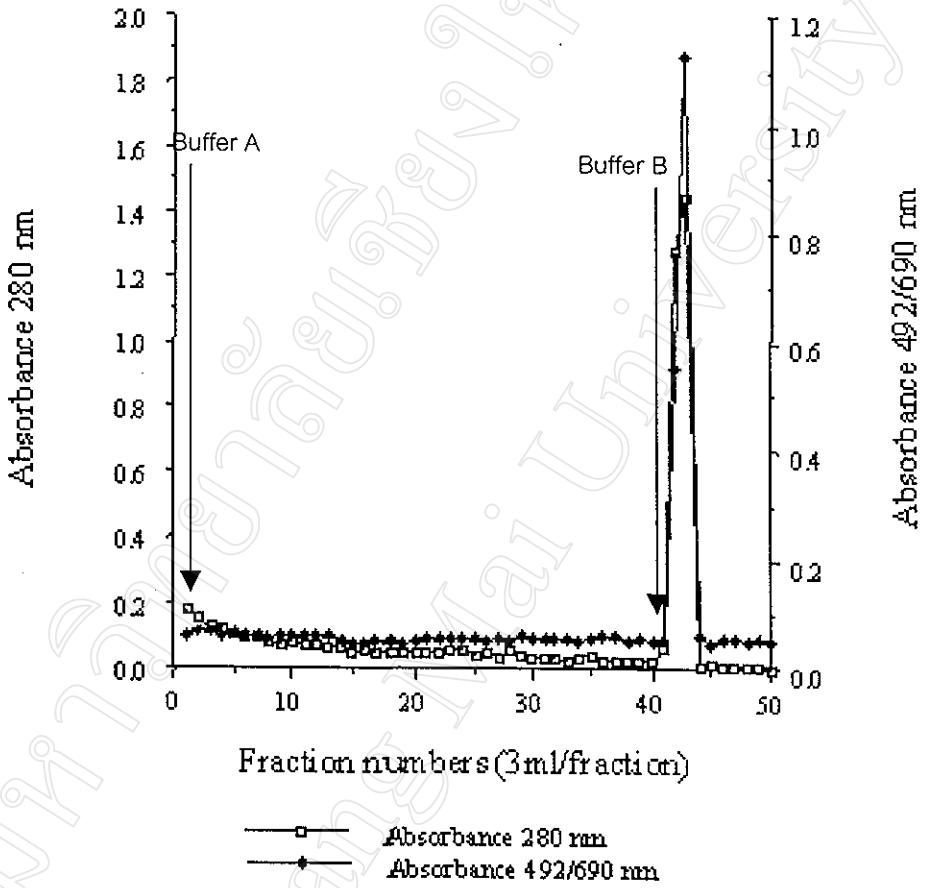

Figure 15 Elution profile obtained from T-Gel chromatography. 80 ml of cell culture supernatant was added with potassium sulfate to final concentration of 0.5 M. The solution was then applied to T-Gel column that was equilibrated with buffer A. Unbound protein was eluted from column with the same buffer. Then bound antibody was eluted with buffer B and 3ml/fraction were collected in tubes by using a fraction collector.

Buffer A: 0.5 M K_2SO_4, 50 mM sodium phosphate, pH 8.0

Buffer B : 50 mM sodium phosphate, pH 8.0

3.3 Optimal concentration of PGs for microtiter plate coating

Shark PGs (1 mg/ml) was serially diluted 10 folds with $NaHCO_3$ Buffer, pH 9.6. A 100 µl of each dilution was incubated on well plate and performed as described.

The effect of various dilutions of PGs on microtiter plate coating was shown in figure 16. Shark PGs at concentration 10^{-3} to 10^{-5} mg/ml gave a similar inhibition curve. Due to the sensitivity and economy concentration 10^{-5} mg/ml was more suitable and chosen for plate coating.

3.4 Optimization for mAb 1H8 concentration in inhibition mixtures

Plate was coated with 10^{-5} mg/ml of PGs and performed as described. The mAb 1H8 was diluted in PBS at concentration 1:30, 1:40, 1:50, 1:60, and 1:70 (v/v). Result was illustrated in Figured 17. The 1:30 dilution of mAb is suitable for HABPs assay. It shows increase sensitivity higher than the other dilutions but near the accepted OD (absorbance = 1.00).

3.5 Optimization for standard Inhibitor condition

Plate was performed as described. But in inhibition mixture, shark A1, shark HABPs, Bovine A1, and Bovine HABPs were competitive inhibitors, and were incubated with 1:30 dilution of mAb 1H8. Results were illustrated in figure 18. Shark A1 is suitable for HABPs assay. It shows increase sensitivity higher than the other dilutions with lower background color but near the accepted OD.

3.6 Optimization for Anti-mouse IgM peroxidase conjugate concentration

Various dilutions (1:1000, 1:2000, 1:3000 and 1:4000 (v/v)) of conjugated anti-mouse IgM peroxidase conjugated were tested with other fixed conditions as previously described. Results were illustrated in Figured 19. The 1:2000 dilution of the conjugate was found to be suitable for HABPs assay at concentration range 10 to 10,000 ng/ml.

3.7 Optimization for PBS and 6% BSA condition

As the same condition, PBS and 6% BSA were used as diluents for standard inhibitor shark A1. The result was shown in figure 20. From the result, 6% BSA and PBS were shown no difference standard curve, but 6% BSA was used as diluent of shark A1 standard, therefor avoided interference from other protein.

3.8 The precision and accuracy of HABPs measurement

The precision of the assay was performed by percentage coefficient of variation (% CV) of the intra- and inter assay, using pooled serum from 12 normal controls for the intra- and inter assay. The result was shown in Table 7. According to results, the coefficient variation of intra- and inter-assay were 9.69% and 17.58% respectively (table 8). In general, the acceptable variations of any assay should not over than 10 % but the precision values of these developed method for HA assay were in acceptable range because its concentration was in nanogram/ml.

The accuracy of the assay was evaluated by percentage recovery. The analytical recovery was assessed by an addition test in which known amounts of standard inhibitor shark A1. Known amount of sharks A1 was added to normal serum. The standard was recovered and found average percentage concentration about 119.20%. Results of the analytical recovery of the assay are shown in table 9.

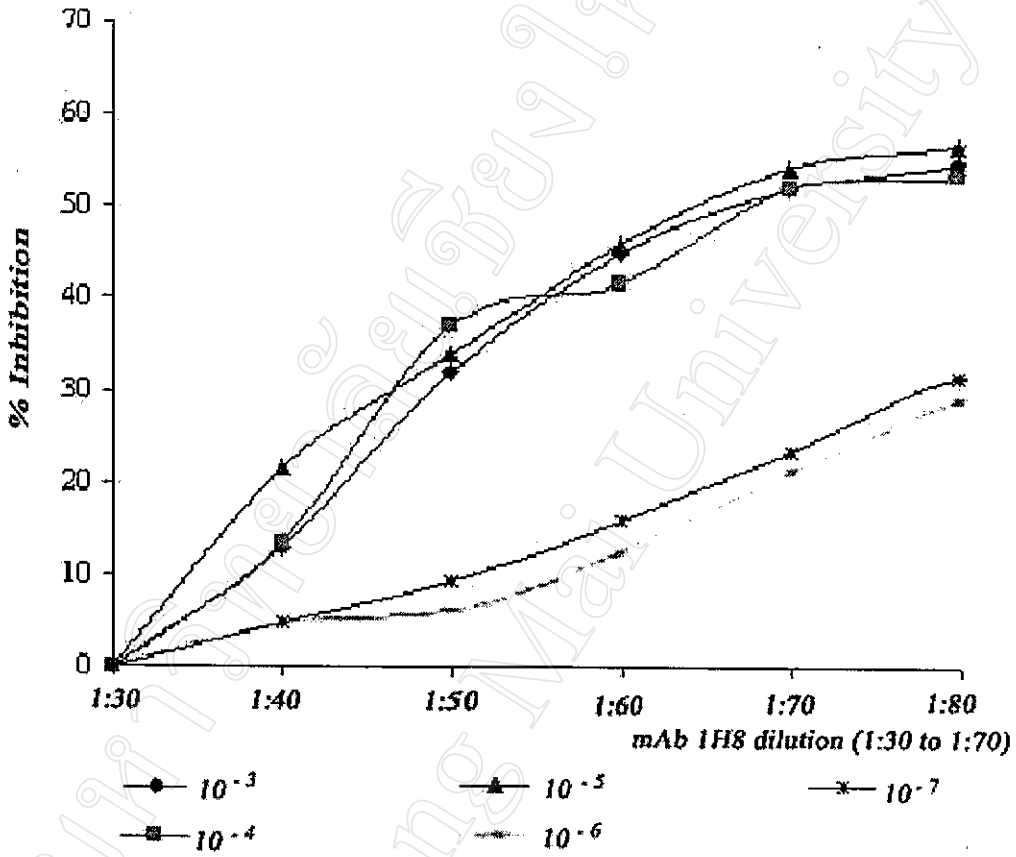

Figure 16 Direct chequerboard using various dilution of shark PGs as antigen plate coating. Various dilutions of 1H8 were added. Excess mAb 1H8 was removed by washing and then the anti-IgM peroxidase conjugated were added to detect remaining IgM on well plate. The color was developed by using peroxidase substrate. The absorbance ratio at 492/690 nm was measured.

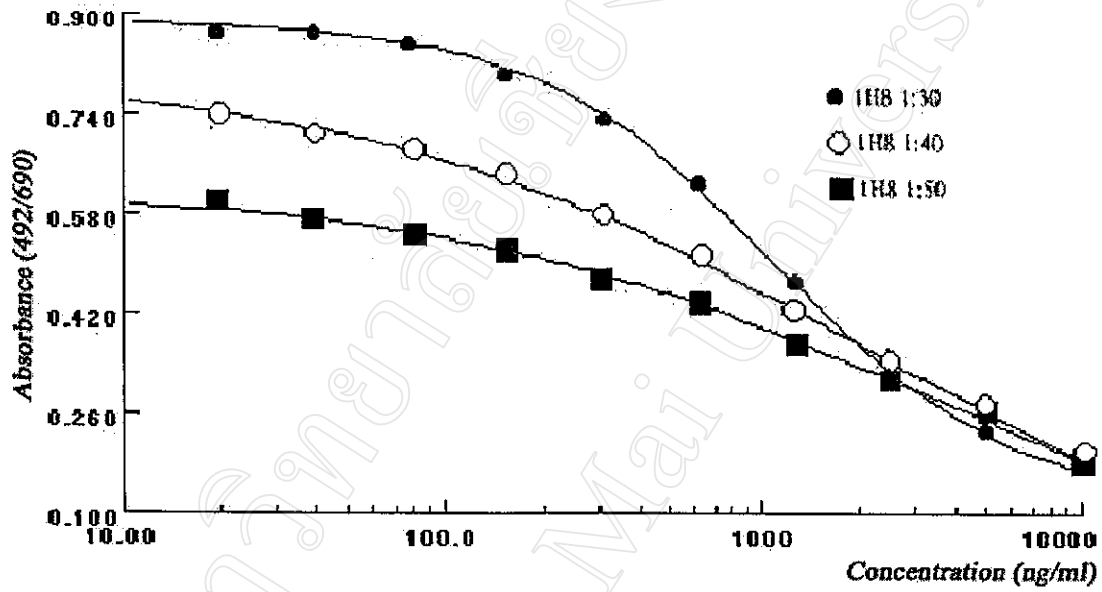

Figure 17 Optimal concentration of monoclonal antibody 1H8. Microtiter plate was coated with shark PGs. Shark A1 was used as the standard inhibitor, and incubated with various concentration of monoclonal antibody 1H8 (1:30, 1:40, 1:50 (v/v)). Excess IgM was removed by washing. Anti-IgM peroxidase conjugated was added and developed color as described

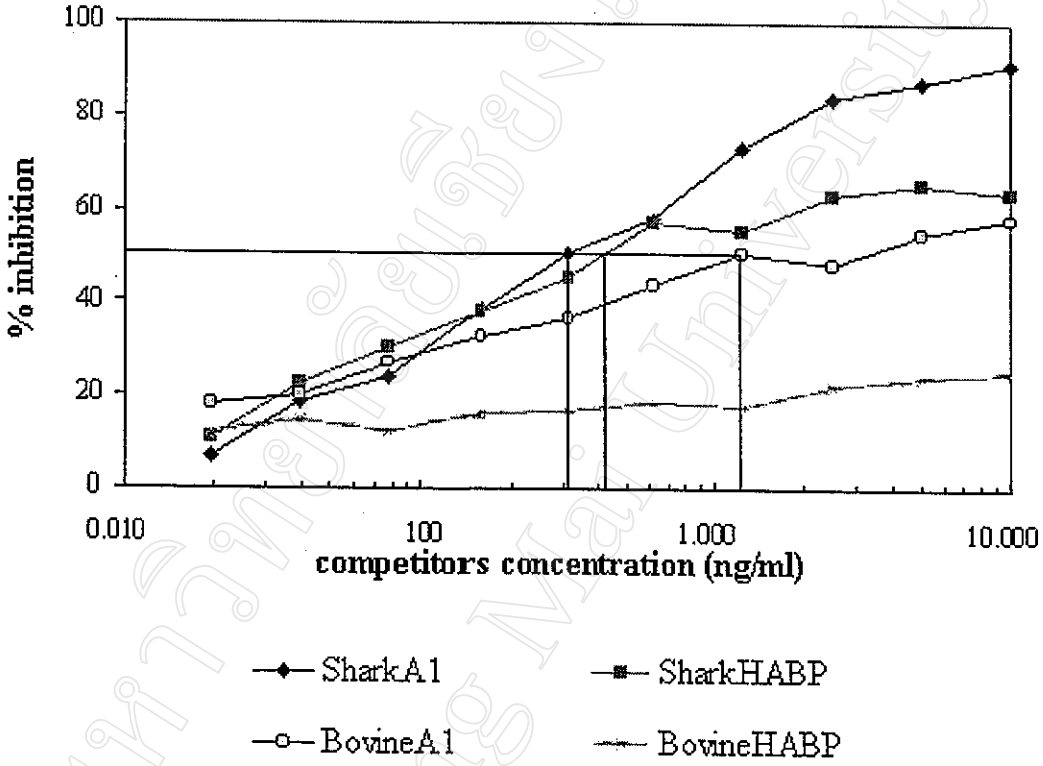

Figure 18 Enzyme-linked immunosorbent inhibition assay for mAb 1H8 epitope. Microtiter plate was coated with shark PGs. Antigens used as competitive inhibitors were shark A1, shark HABPs, bovine A1, and bovine HABPs. The following method was done as above.

Shark A1 = associated form of shark proteoglycan

Bovine A1 = associated form of bovine proteoglycan

Shark HABPs = shark hyaluronan binding proteins

Bovine HABPs = Bovine hyaluronan binding proteins

Table 7. The demonstration of concentration at 50% inhibition (IC 50) of various inhibitors to react with mAb 1H8.

Antigens	IC 50 (ng/ml)
Shark A1	310
Shark HABPs	420
Bovine A1	1,300
Bovine HABPs	Less than 50 %

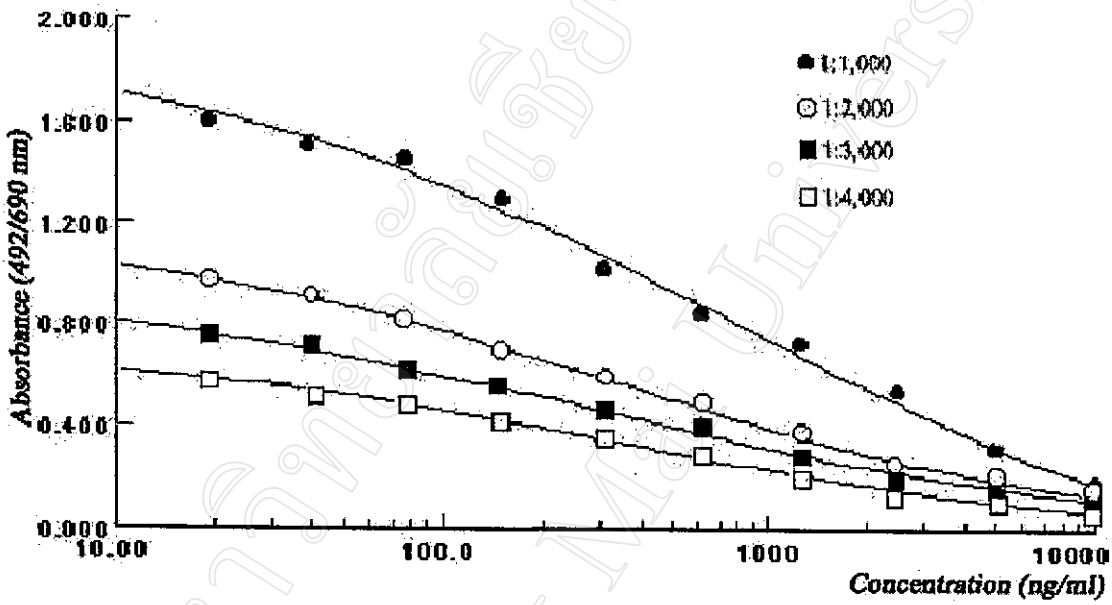

Figure 19 Optimal concentration of Anti-mouse IgM peroxidase conjugated. Microtiter plate was coated with shark PGs. Antigen used as competitive inhibitor is shark A1. Excess IgM was removed by washing. Various dilutions of Anti-mouse IgM peroxidase conjugate (1:1,000, 1:2,000, 1:3,000, 1:4,000 (v/v)) were added to the inhibition mixture. Peroxidase substrate was added to develop color as described.

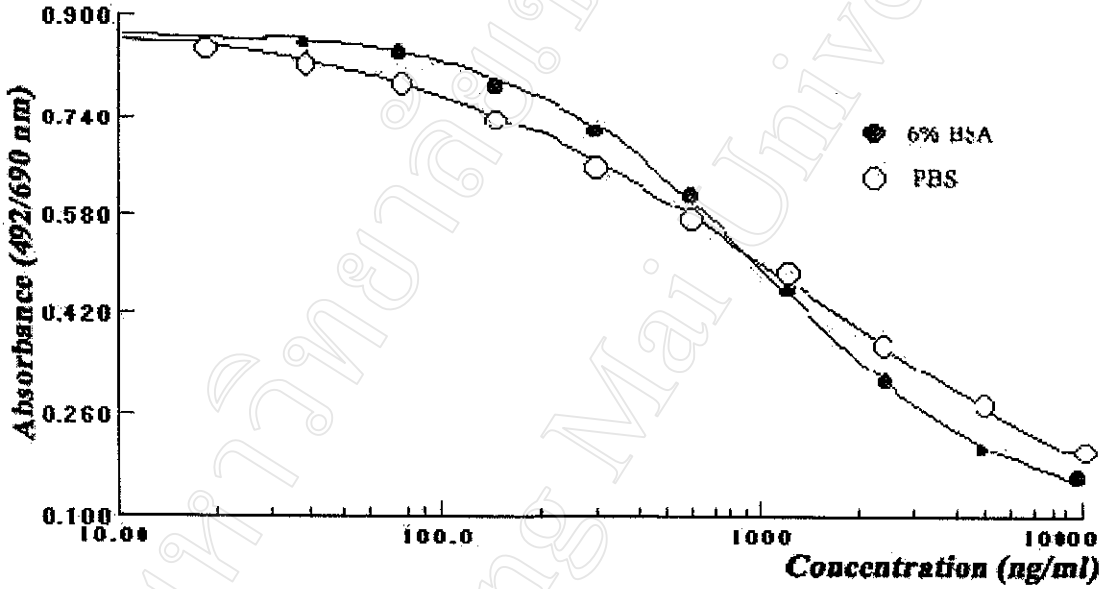

Figure 20 The assessment of diluent effect for standard inhibitor (shark A1) in inhibition mixture. Microtiter plate was coated with shark PGs. The mAb 1H8 was incubated with inhibitor shark A1 in 6%BSA or PBS. Excess IgM was removed by washing. Anti-IgM peroxidase conjugated was added and developed color as described

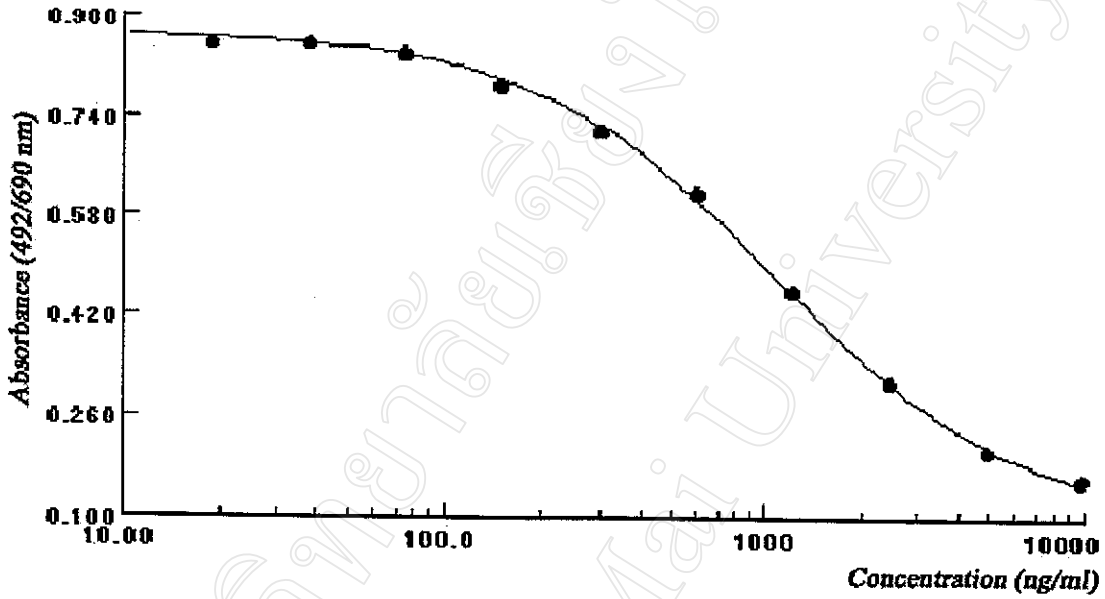

Figure 21 A typical standard curve for newly competitive ELISA assay by using mAb 1H8. Plate was coated with Shark PGs at concentration 10^{-5} mg/ml. Shark A1 was used as the standard competitor (range 0.019 to 10^4 ng/ml), and then incubated with mAb 1H8 at concentration 1:30 (v/v). Excess non-binding molecules was removed by washing and then added anti-IgM peroxidase conjugated (1:2,000) each well. The reaction was stopped (by using 4 M H_2SO_4, 50 µl/well)when the color was developed. Absorbance at 492/690 nm was obtained at the end of the assay and generated typical standard curve by using Deltasoft II program computer.

Table 8 Evaluation of immunoassays

Assay	Number of samples	Mean ± SD	%CV
Intra-assay	12	39.89±3.79	9.69
Inter-assay	20	38.96±6.85	17.58

Table 9 Recovery of shark A1 added to serum

Sample numbers	Standard added (ng/ml)	Recovered added standard (ng/ml)	% Recovery
1	1303.54	1596.13	122.44
2	759.50	977.88	128.75
3	416.58	494.74	118.76
4	203.12	258.16	127.10
5	99.71	104.70	105.00
6	43.06	52.27	121.39
7	30.25	33.58	111.00
Mean			119.20

3.9 Application of newly developed method for HABPs determination

Sera from normal healthy subjects (n = 23) were measured for HABPs concentration by these developed method. The normal range of HABPs concentration was found between 5.44 to 136.91 ng/ml. (mean ± SD = 26.58 ± 37.52 ng/ml). Data was shown in table 10.

In OA and RA patients serum, HABPs were measured. It was found that mean ± SD of HABPs concentration in OA were 78.31±58.77 and 668.02 ± 1834.70 in RA. Ranges of HABPs concentration in OA were 19.54 to 137.08 ng/ml and in RA were 0.247 to 10541.18 ng/ml.

Using this modified method for HABPs in human serum, it was found that in the OA sera, concentration of HABPs were significantly higher than normal ($p=0.001$). In RA serum, the concentration of HABPs were also significantly higher than normal ($p=0.049$).

Comparison relation between concentration of HABPs and HA (Data from Pothacharoen, 2000) in same people sample was found that HABPs had no correlation with HA in any case.

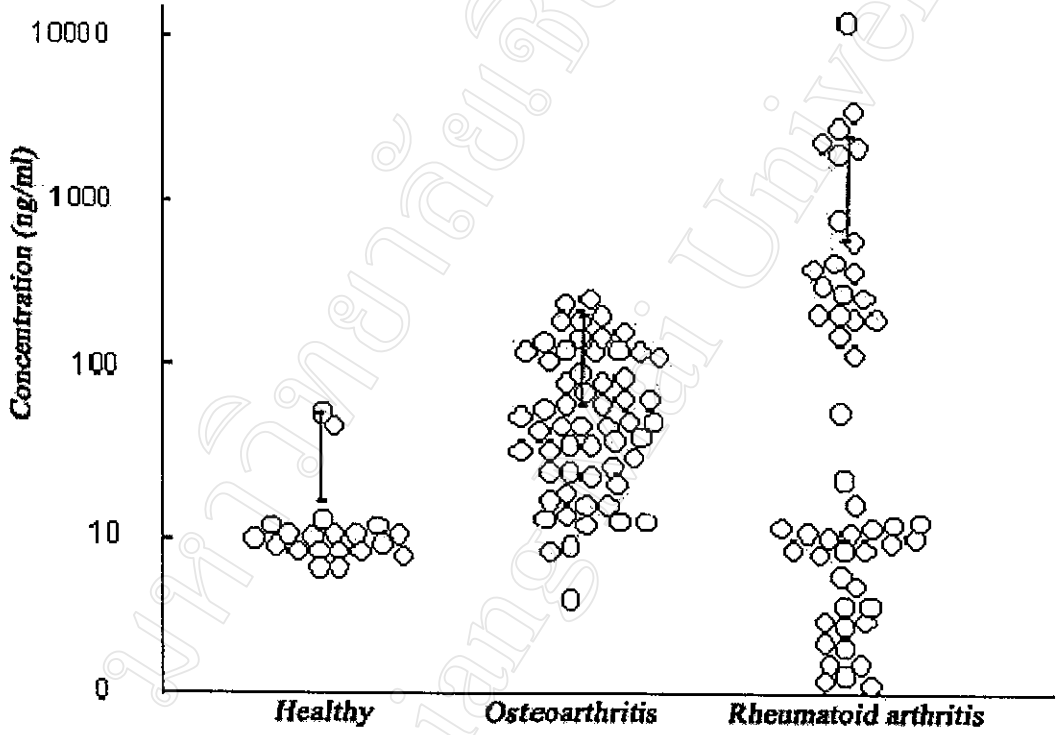

Figure 22 Concentration of HABPs in healthy human sera compared with osteoarthritic sera and rheumatoid arthritis sera. Data are presented for the individual subject and as the mean + SD.

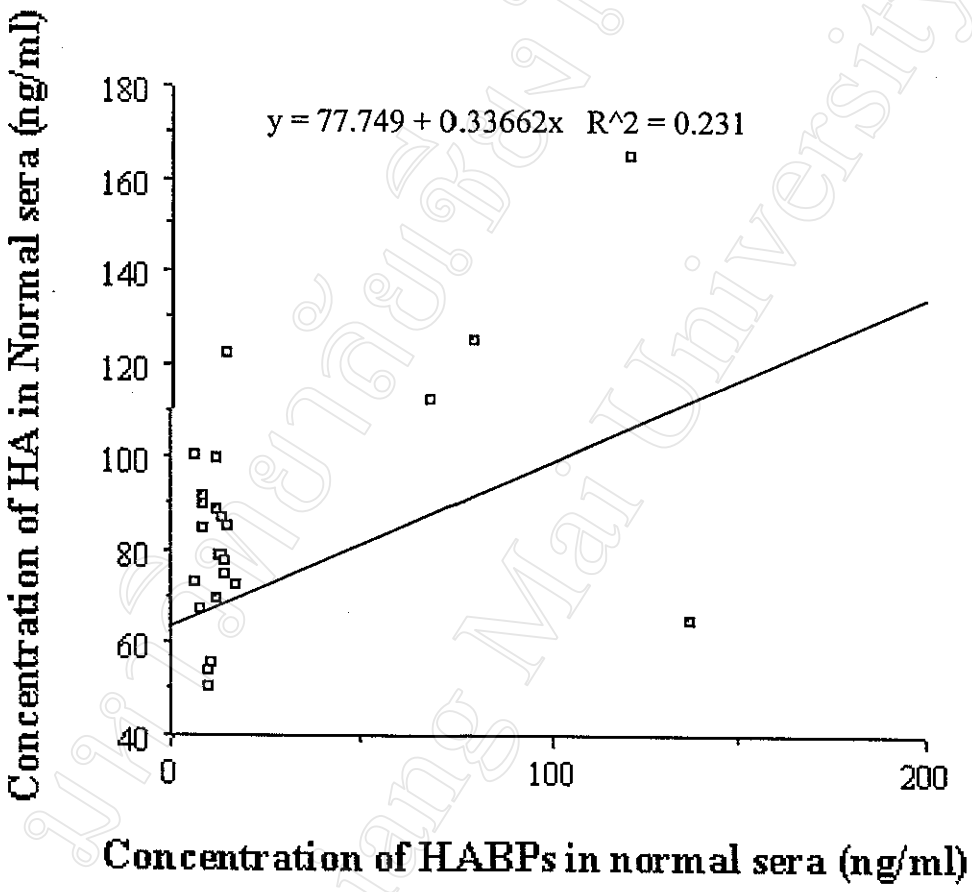

Figure 23 Correlation between concentration of HA and HABPs in healthy serum samples ($r = 0.231$, $n = 23$, $p > 0.05$).

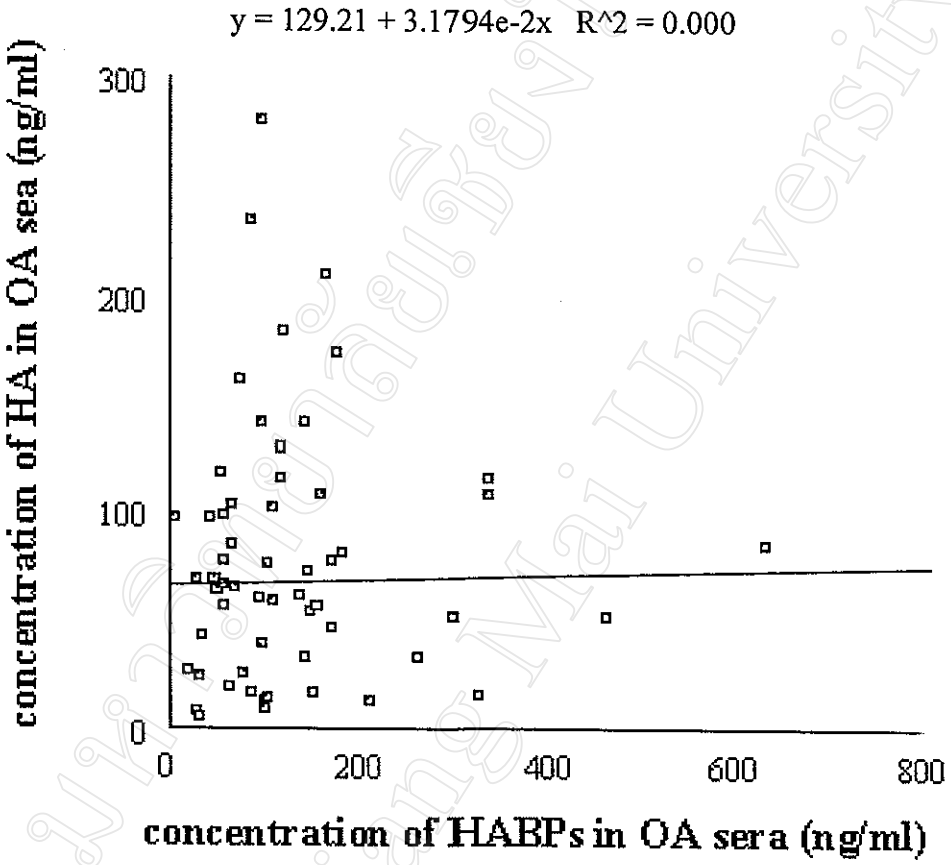

Figure 24 Correlation between concentration of HA and HABPs in OA sera sample ($r = 0.000$, $n = 50$, $p > 0.05$).

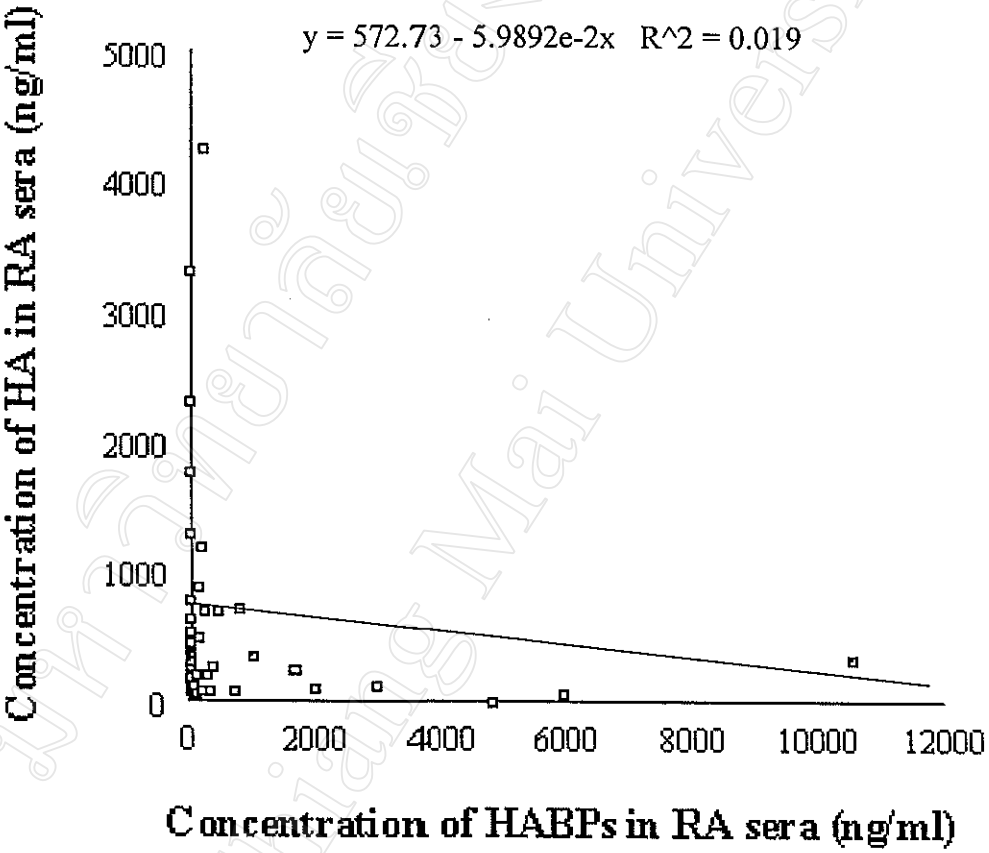

Figure 25 Correlation between concentration of HA and HABPs in RA sera sample ($r = 0.019$, $n = 57$, $p > 0.05$)

Table 10 The amount of HABPs and HA in serum samples of healthy people, osteoarthritic, and rheumatoid arthritic patients.

	HABPs (ng/ml)	HA(ng/ml)
Healthy sera	26.58 ± 37.52^{a}	86.70 ± 26.29^{d}
Osteoarthritis	78.31 ± 58.77^{b}	131.70 ± 112.46^{e}
Rheumatoid arthritis	668.02 ± 1834.7^{c}	532.68 ± 806.13^{f}

Significant value :

a;b, $p = 0.001$, a;c, $p = 0.049$

d;e, $p = 0.031$, d;f, $p = 0.005$

Correlation coefficiency (against healthy sera):

a;d, $r = 0.231$

b;e, $r = 0.000$

c;f, $r = 0.019$

3.10 Assessment of HABPs in serum

Hyaluronan binding proteins were analyzed by using native PAGE method. Healthy, OA and RA serum were serially diluted 1:2 with sample buffer and transferred to 7.5 % acrylamide gel (figure 26). Protein was transferred to nitrocellulose membrane by protein transfer gel method. Biotinyl-HA and mAb 1H8 were used to probe observed protein.

B-HA but not mAb 1H8 appeared board band of hyaluronan binding molecules (figure 27). It likely that these bands might be not HABPs because HA can bind with many positive molecules such as drug or the other covalent binding protein such as ITI. In addition, mAb 1H8 could not detected HABPs at small amount of serum (data not show). The protein serum should be fractionated by filtration before HABPs analysis. It might help increasing of sensitivity of this technique.

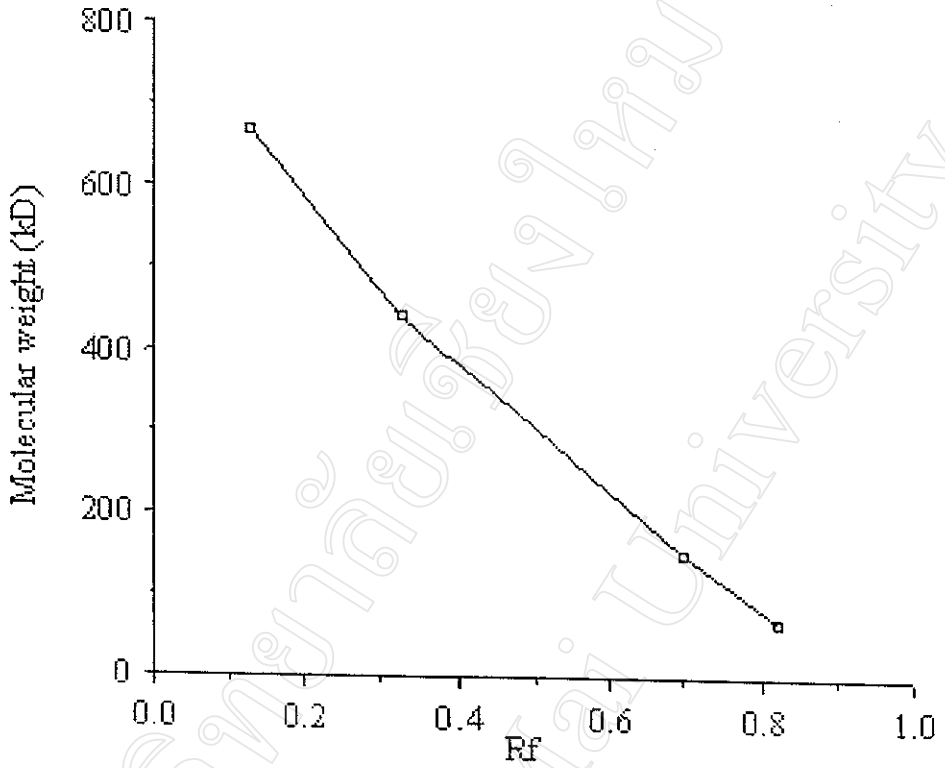

Figure 26 Standard curve of high molecular weight of different protein marker (7.5% polyacylamide gel)

	MW. (kD)
BSA	66
Alcohol dehydrogenase	150
Apoferritin	443
Tryroglobulin	669

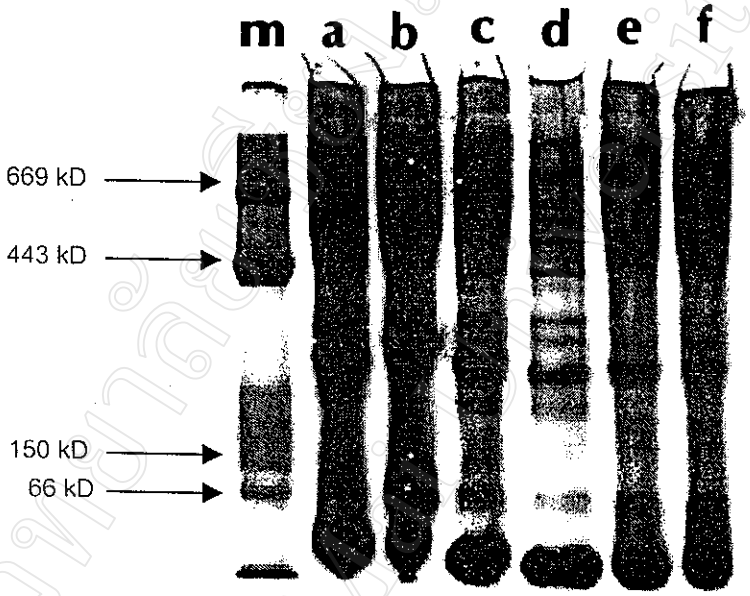

Figure 27 Serum from healthy, osteoarthritis and rheumatoid arthritis were analyzed by native PAGE (Coomassie blue stain) on 7.5 % polyacrlamide gel.

Marker (m) : bovine serum albumin (66 kD), alcohol dehydrogenase (150 kD), apoferritin (443 kD), thyroglobulin (669 kD)

Lane a and b : Healthy serum

Lane c and d : Osteoarthritic serum

Lane e and f : Rheumatoid arthritic serum

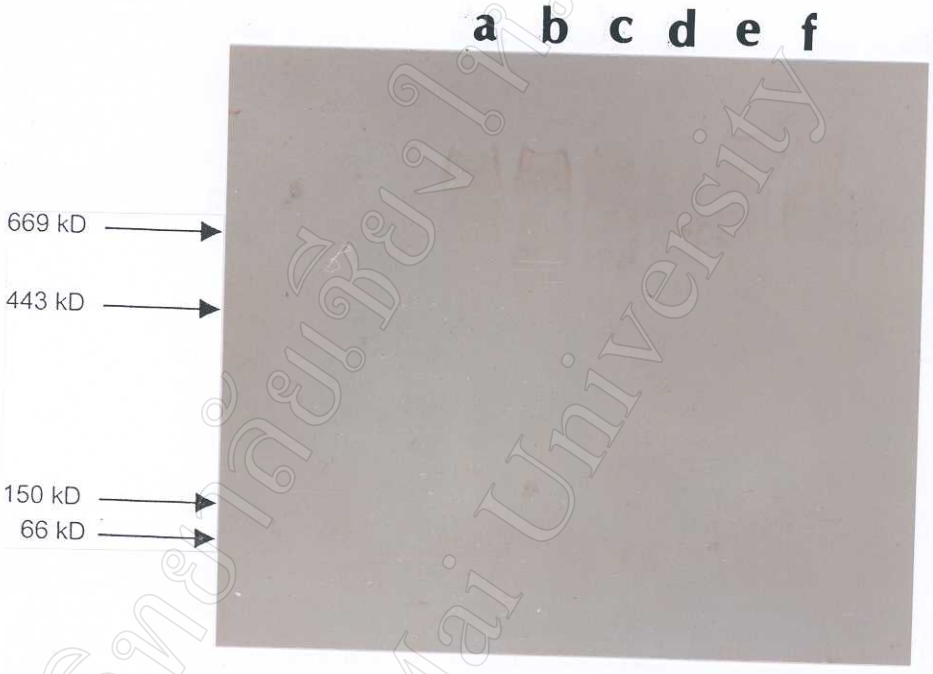

Figure 28 Serum from healthy, osteoarthritis and rheumatoid arthritis were analyzed by using western blotted technique. Proteins were transferred to nitrocellulose membrane and probed with biotinyl-HA.

Lane a and b : Healthy serum

Lane c and d : Osteoarthritic serum

Lane e and f : Rheumatoid arthritic serum