CHAPTER III

RESULTS

3.1 Isolation and Purification of Hyaluronan-Binding Proteins

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 with distilled water and lyophilized. The white powder of extract was achieved 17.47 g from 348.91 g chicken cartilage. The globular protein core of proteoglycan was cleaved by trypsin, lyophilized and incubated with HA-Sepharose dialyzed with 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 chicken cartilage extract was shown in Figure 26. The first peak is unbound material observed at 280 nm, and the second peak is HABPs eluted by 4 M GuHCl buffer, pH 5.8. The protein was measured in lyophilized powder by Bradford assay.

The yield of HABPs from 300 mg of trypsinized chicken cartilage extract was 18 mg (6% yields). From this experiment indicated that chicken cartilage extract was isolated efficiently by this method but small amount was achieved (table 1).

The chicken HABPs pattern from this study composed of two protein bands. The molecular weight of two proteins chicken HABPs were approximately 33-34 kDa and 40-45 kDa, respectively (Figure 27 and 28).

Table 1. Isolation and purification of HABPs from chicken cartilage.

Samples	Weight (g)	% Yields		
		% Chicken cartilage	% Chicken cartilage extract	% Trypsinized cartilage extract
Chicken cartilage	348.91	-	-	-
Chicken cartilage extract	17.47	5.007 %	-	-
Trypsinized cartilage extract	8.68	2.488 %	49.685 %	-
HABPs	0.5208	0.149 %	2.981%	6 %

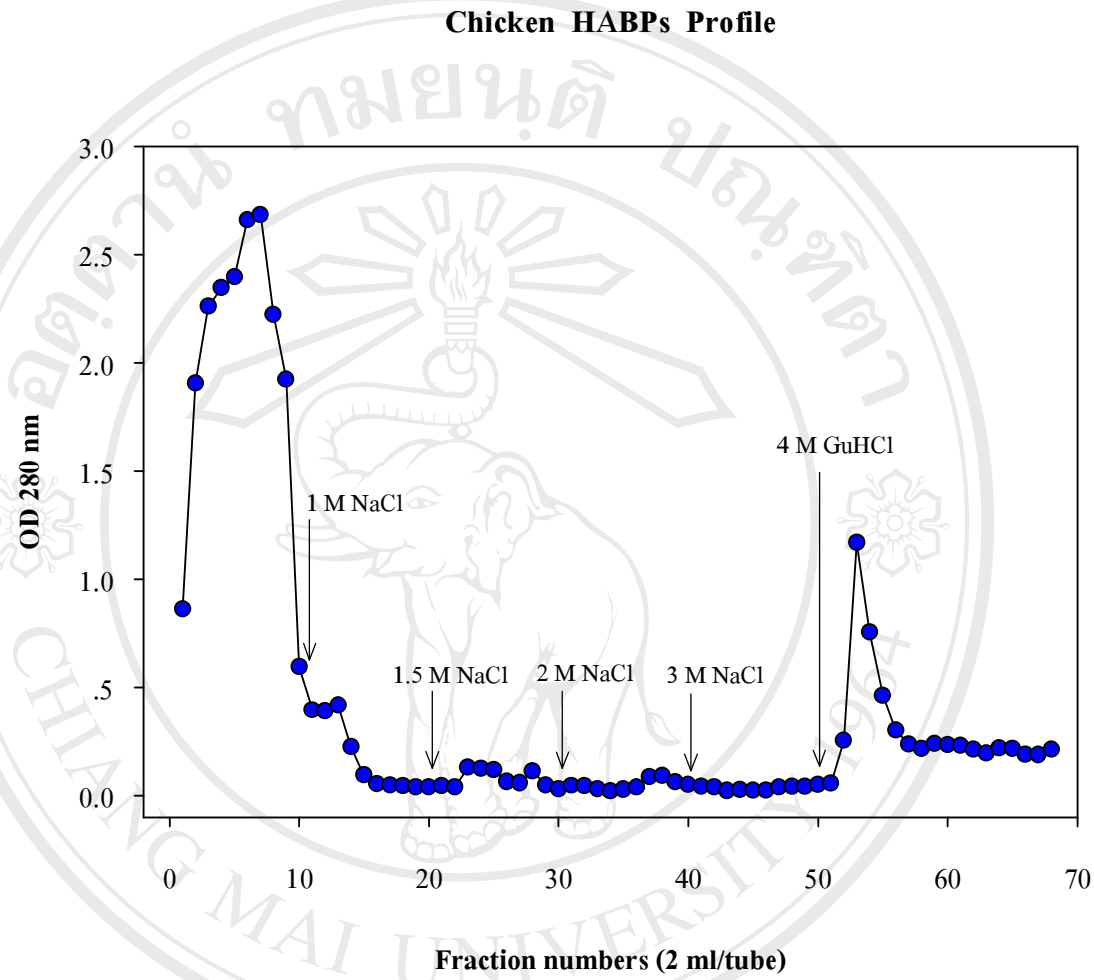


Figure 26. The chromatography purification of HABPs from trypsinized chicken cartilage by affinity HA-Sepharose column chromatography.

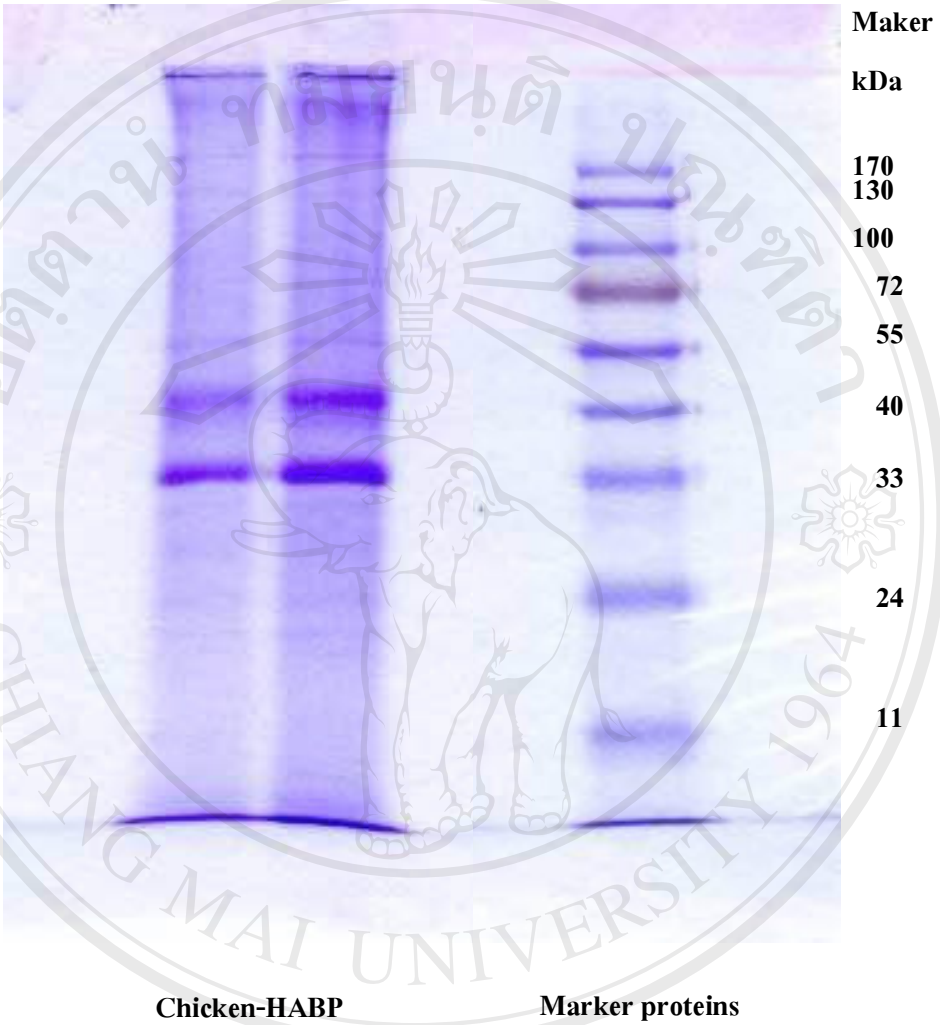


Figure 27. HABPs from chicken and marker proteins were analyzed by SDS-PAGE on 12 % polyacrylamide gel.

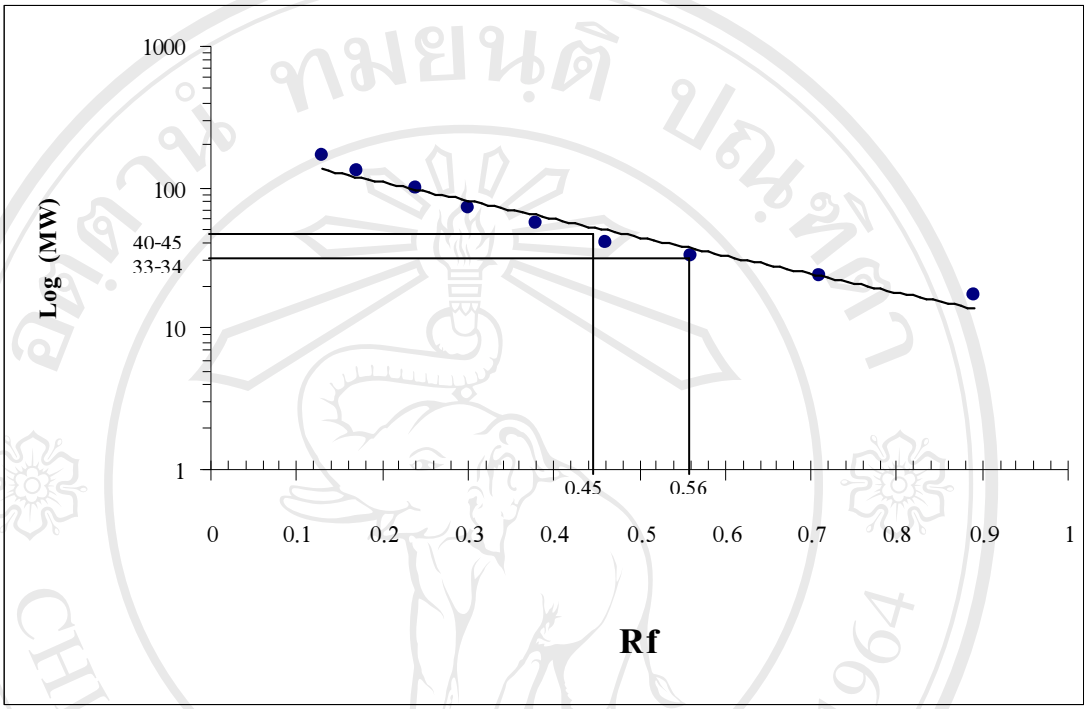


Figure 28. Standard curve of molecular weight protein marker were analyzed by SDS-PAGE on 12 % polyacrylamide gel.

3.2 FITC Conjugation of HABPs

Affinity column chromatography was modified for using to prepare fluorescence conjugated proteins, the procedure was developed from the original method for biotinylated HABPs, this method described by Yingsang (Yingsang, 1996). From the results, FITC conjugated proteins were prepared with preincubated FITC solution with protein bound in column. After incubation, the conjugated proteins were eluted by 4 M GuHCl, collected FITC-HABP and removed excess FITC by applying to dialysis with Tris-HCl, pH 8.6 in dark condition. FITC-HABP profile was shown in Figure 29.

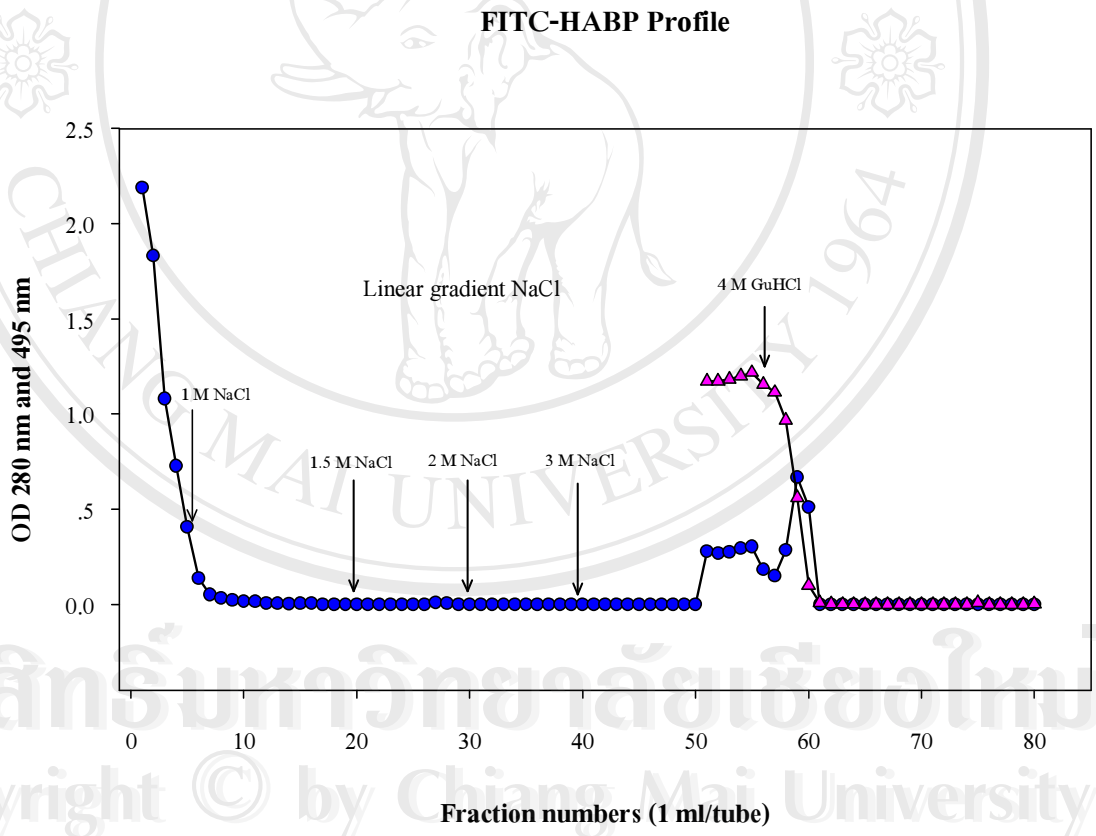


Figure 29. FITC-HABP profile from prepared by modified column chromatography technique.

3.3 Histochemical Study

To study biological function of FITC-HABP, fibroblast, chondrocyte and rat skin tissues were untreated or pretreated with hyaluronidase for 1 hour before stained with FITC-HABP. The results demonstrated that untreated hyaluronidase cells and tissue showed fluorescent signal but control groups, pretreated with hyaluronidase, observed decrease signal detectable. This result indicated that the FITC-HABP probably recognized hyaluronan (Figure 30, 31 and 32).

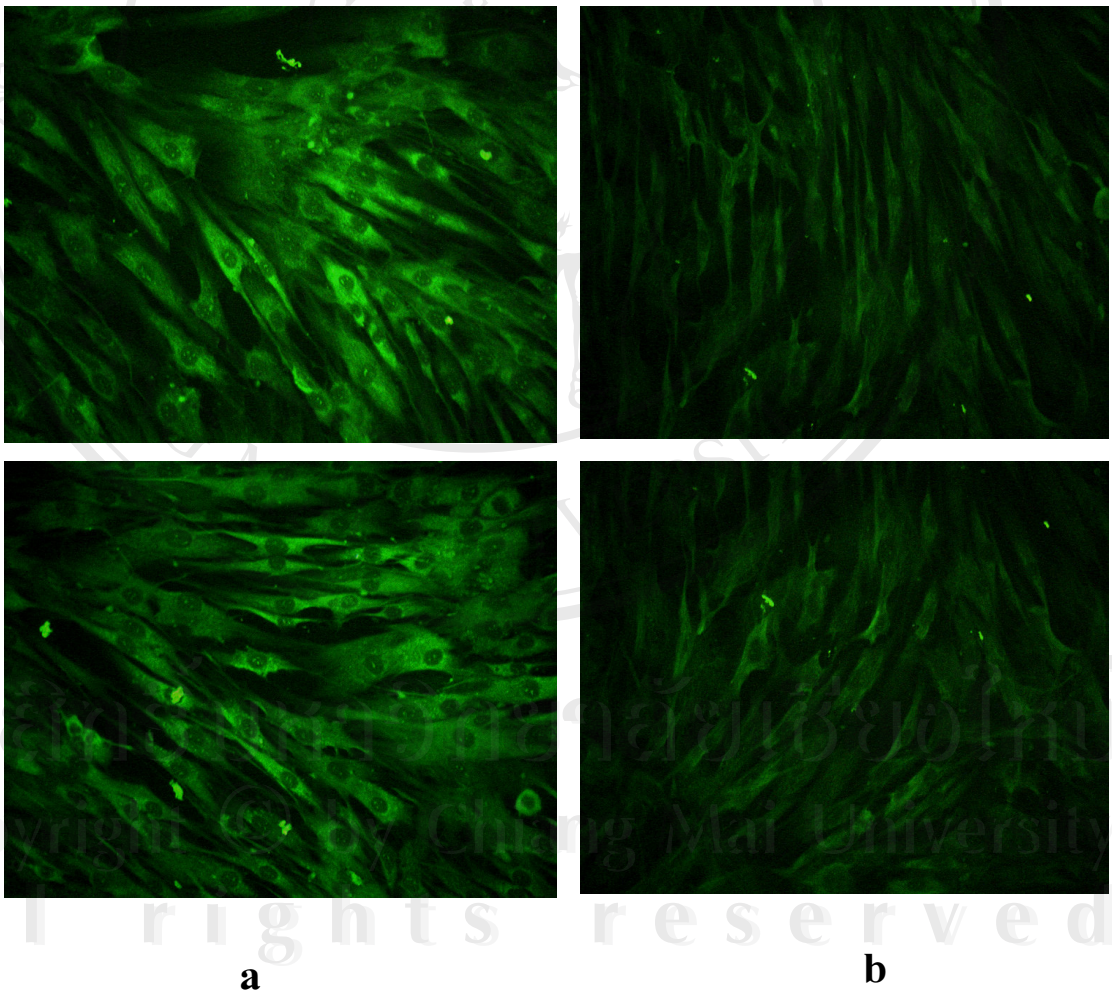


Figure 30. Fibroblast cells (a) and control cells (b) stained with FITC-HABP. Magnification was analyzed at 200X.

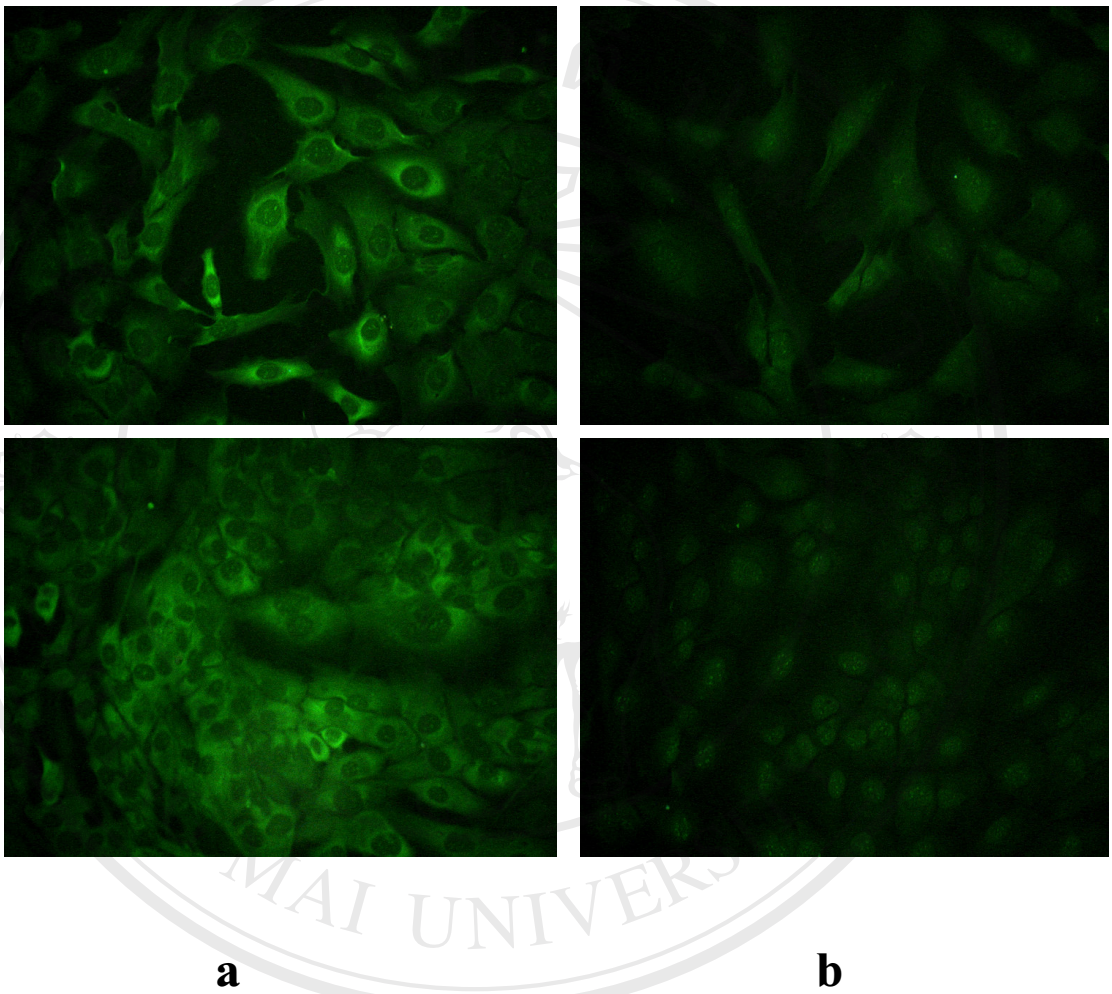
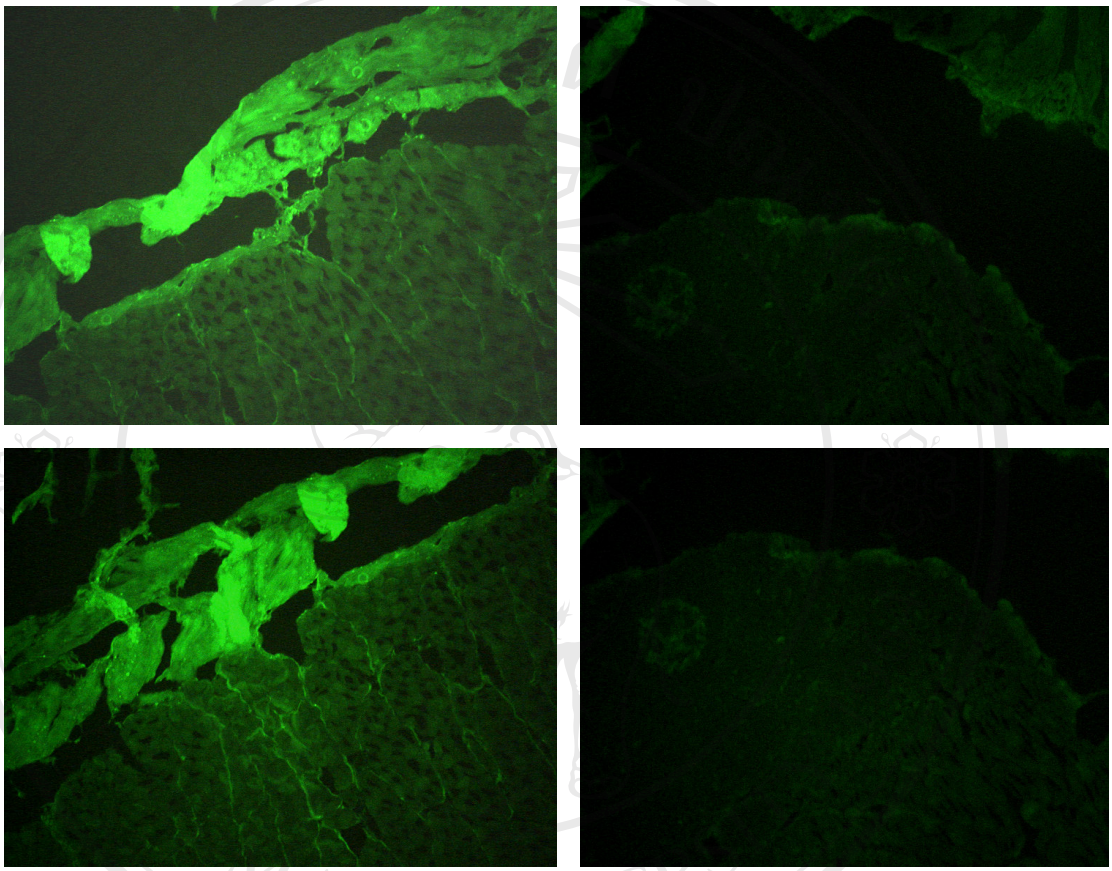


Figure 31. Chondrocyte cells (a) and control cells (b) stained with FITC-HABP. Magnification was analyzed at 200X.



a b

Figure 32. Rat skin tissue sections (a) and control sections (b) stained with FITC-HABP. Magnification was analyzed at 200X.

3.4 Competitive Fluorescence-ELISA

This study was developed fluorescence method based on ELISA assay as described in method and using FITC-HABP prepared for determined HA concentration for set standard curve in rang of 10-10,000 ng/ml (data was shown in Figure 33).

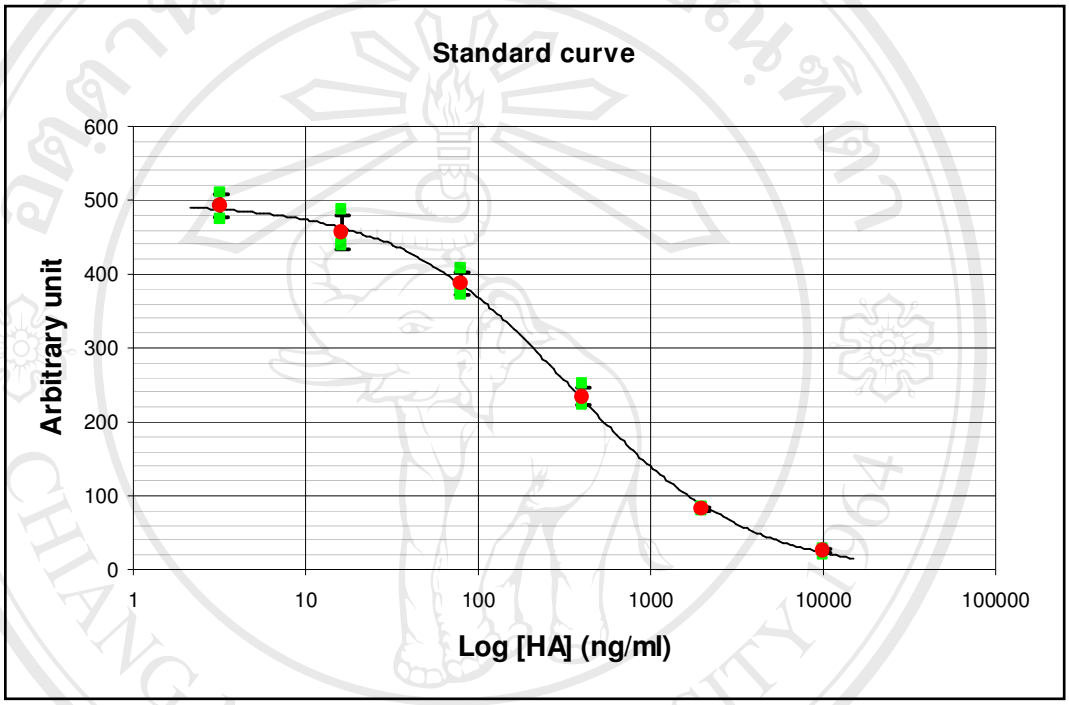


Figure 33. Standard curve of competitive fluorescence-based assay for detection of hyaluronan. Each point is the mean of three determinations. The vertical bars show the standard deviations of the measurements.

3.5 Optimal Dilution of FITC-HABP with Fluorescence-ELISA Based Assay

Plates were coated with HA and performed as described in method. The FITC-HABP was diluted in Tris-HCl buffer, pH 8.6 to 1/10, 1/20, and 1/40 (v/v) dilution. The result was shown in Figure 34. The 1/10 dilution of FITC-HABP was suitable for fluorescence-ELISA based assay, which showed higher sensitivity than other dilutions.

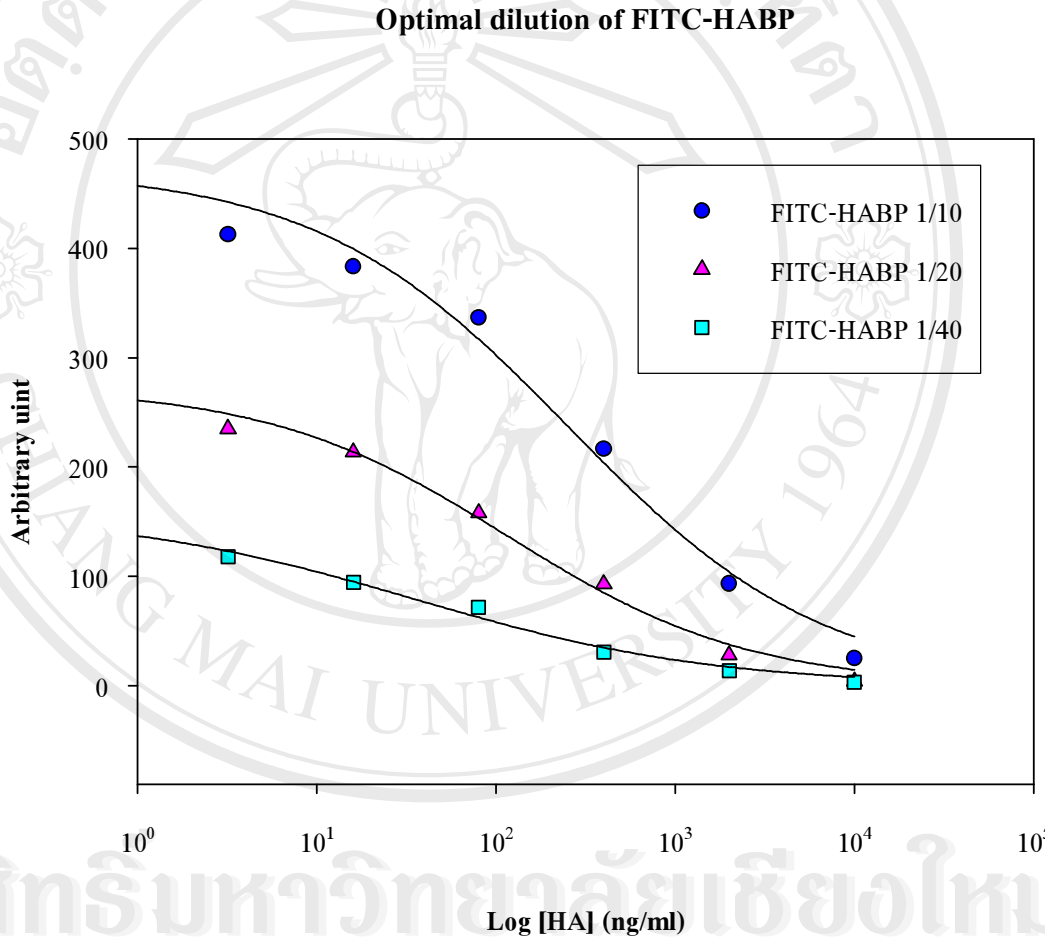


Figure 34. The optimal dilution of FITC-HABP with Fluorescence-ELISA based assay.

3.6 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 control subjects. The result was shown in table 2. The coefficient variations of intra- and inter assay were 6.51 % and 11.01%, respectively. The accuracy of this assay was evaluated by percentage recovery. The analytical recovery was assessed by an addition test in which known average of standard inhibition HA. Known amount of HA was added to serum samples. The percentage recovery of the added standard hyaluronan in serum samples was 119.782 %, (table3).

In addition, fluorescence assay in the present study was modified from original method of conventional colorimetric assay (Yingsang, 1996). Therefore, this assay was compared to the previous method. Colorimetric assay used B-HABP and developed signal with anti-biotin peroxidase to determine HA concentration in samples, result shown in table 4. The comparison relation between the fluorescence assay and colorimetric assay were not significantly difference ($r = 0.87$) to detect the concentration of HA in identical serum samples ($n = 20$). Correlation graph was shown in Figure 35.

Table 2. The intra- and inter assay coefficient of variation for serum hyaluronan (HA) determination

Assay	Number of samples	Mean ± SD	% CV
Intra assay	20	385.73 ± 25.12	6.51
Inter assay	28	388.73 ± 42.80	11.01

SD, standard deviation; CV, coefficient of variation.

Table 3. The recovery of hyaluronan in serum samples.

Sample numbers	Standard added (ng/ml)	Recovered added standard (ng/ml)	% Recovery
1	11892.1	9297.96	78.1862
2	7590.15	8012.26	105.561
3	4427.87	7939.25	179.302
4	3333.07	3913.86	117.425
5	1368.06	1435.62	104.938
6	645.941	860.921	133.282
Mean			119.782

Table 4. The comparison of different methods for hyaluronan determination.

Serum subjects	Conventional assay Peroxidase (B-HABP)	Fluorescence-based assay FITC-HABP	Type of samples
S1	35.758	35.418	Cervix
S2	137.85	172.63	Cervix
S3	42.48	12.958	Cervix
S4	566.82	511.452	Cervix
S5	137.67	37.423	Cervix
S6	384.82	240.801	Other cancer
S7	93.553	53.480	Other cancer
S8	633.4	1302.435	Other cancer
S9	1126.5	1603.795	CEA
S10	133.09	62.797	CEA
S11	274.39	240.588	Cervix
S12	195.11	216.136	Cervix
S13	515.66	364.905	Cervix
S14	130.47	108.725	Cervix
S15	254.62	272.521	Cervix
S16	74.884	18.137	Other cancer
S17	191.35	107.118	Other cancer
S18	350.2	83.609	CEA
S19	421.11	163.437	CEA
S20	766.69	492.143	CEA

Sample subjects (S1–S20), from cancer patients. Conventional assay (peroxidase) was carried out as described by Yingsang (Yingsang, 1996); fluorescence-based assay was carried out as present assay.

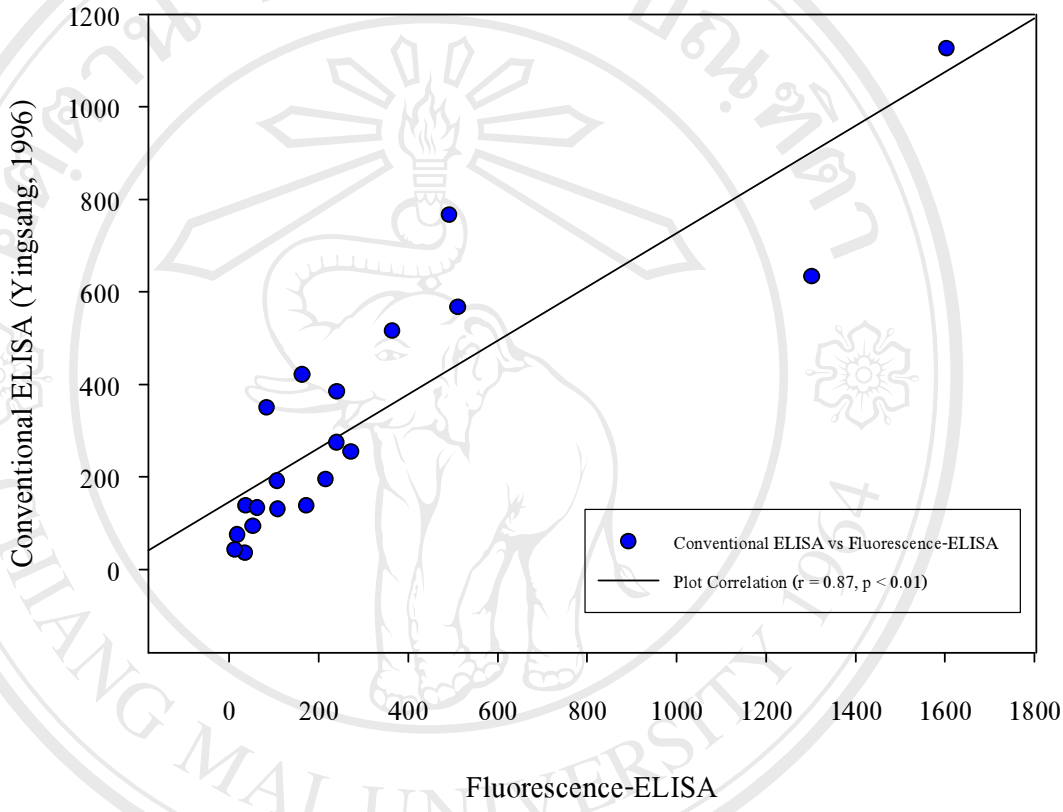


Figure 35. Correlation graph between fluorescence-ELISA and conventional ELISA.

3.7 Application of FITC-HABP for HA Determination in Cancer Serum

Serum from normal subjects (n = 57) were measured the HA concentration by this developed method. The normal range of HA concentration was between 2.14 to 238.02 ng/ml. (mean ± SD = 34.79 ± 49.40 ng/ml). Data was shown in table 5.

In cancer patient's serum, the mean ± SD of HA concentration were 188.99 ± 135.80 ng/ml and 366.09 ± 512.33 ng/ml in cervical cancer and other cancer patient's serum, respectively. In CEA positive patient's serum, rang of HA concentration was 418.90 ± 300.87 ng/ml.

When using fluorescence-ELISA method to detect HA in human serum, normal subjects serum were lower in HA concentration than cervical cancer, CEA positive and other cancer with statistical significantly *($p < 0.01$)*, shown in Figure 36 and 37.

Table 5. The serum hyaluronan concentration in normal subjects and cancer patients.

Subjects	Numbers	Hyaluronan (ng/ml) mean ± SD
Normal	57	34.79 ± 49.40
Cancer	47	366.09 ± 512.33*
Cervical cancer	61	181.99 ± 135.80*
CEA	41	418.90 ± 300.87*
Total cancer	149	305.25 ± 352.90*

* Significant at $p < 0.01$

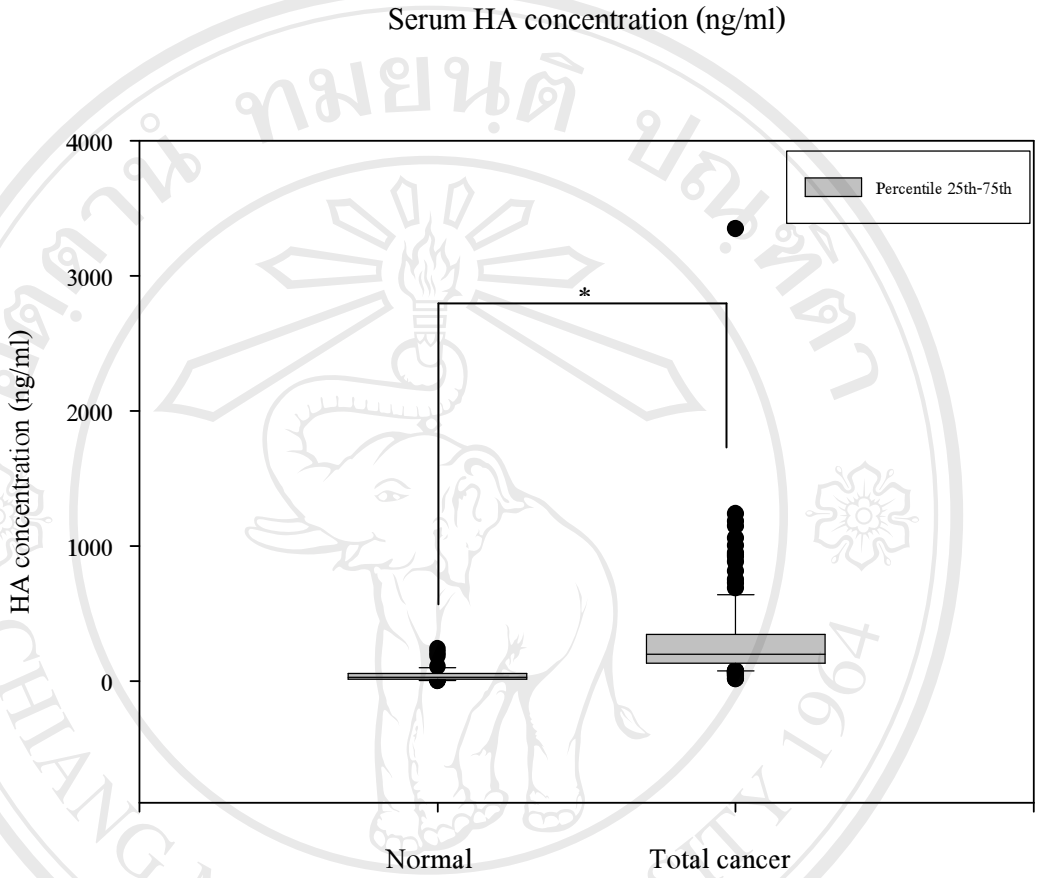


Figure 36. The serum hyaluronan concentration in normal subjects and cancer patients. Comparison of the value obtained from normal subjects (n = 57) and cancer patients (n = 149). Data boxes are presented the median and interpercentile rang, between 25^{th} and 75^{th} percentile with error bar. Statistically significant difference ($p < 0.01$ shown with star symbol *) relative to the median of the normal subjects.

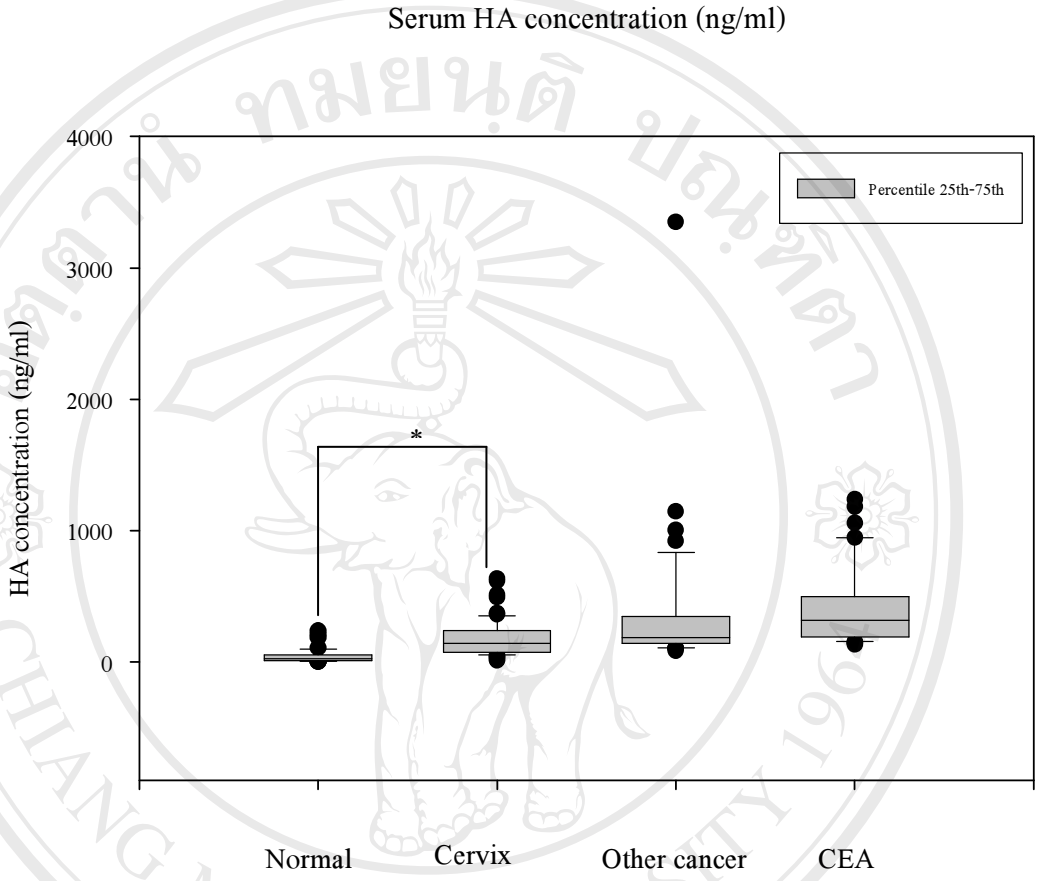


Figure 37. The serum hyaluronan concentration in normal subjects, cervical cancer, CEA positive and other cancer patients. Comparison of the value obtained from normal subjects (n = 57) and separate cancer patients (cancer n = 47, cervix n =61, CEA positive n = 41). Data boxes are presented the median and interpercentile rang, between 25th and 75th percentile with error bar. Statistically significant difference ($p< 0.01$ shown with star symbol *) relative to the median of the normal subjects.