CHAPTER II

MATERIALS AND METHODS

2.1 MATERIAL

2.11 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 (Gercules,CA)

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

Merck (Dermstadt, F.R. Germany)

Hydrogen peroxide, sodium chloride, sodium hydrogen carbonate, potassium chloride

Pharmacia LKB, Biotechnology AB (Uppsala, Sweden)

Ammonium persulfate, coomassie blue brilliant R-250, EAH Sepharose 4B, glycine, mercaptoethanol, *N,N'*-methylene bis acrylamide

SERVA (Heidenberg, New York)

Triton X-100

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

Bovine serum albumin, 1-ethyl-3-(-3-dimethylamino-proply) carbodiimine (EDAC), guanidine hydrochloride, hyaluronidase, hyaluronic acid (from human umbilical cord), polyoxyethylene sorbitan monolaurate (Tween 20), trypsin inhibitor type I-S from soybean, *N*-hydroxysuccinimidobiotin, O-phenylenediamine (OPD), dimethyl sulfoxide (DMSO), FITC (fluorescein isothiocyanate)

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2.1.2 Lists of the Antibodies Used in This Study

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

2.1.3 Experimental Instruments

Analytical balance, AC 100	(Metter Instrument A.G., Switzerland)
Fluorescent microplate reader	(Biotek, Germany)
Fluorescent microscope	(OLYMPUS, Deutschland)
Lyophilizer	(CHRIST,Germany)
Magnetic stirrer	(Thermolyne Co., U.S.A.)
Membrane filter	(Gelman Sciences Inc, U.S.A.)
Mini-PROTEIN II	(Bio-Rad,U.S.A.)
Power Supply, Model 200/2.0	(Bio-Rad,U.S.A.)
pH meter	(METTLER TOLEDO Limited, U.S.A.)
Vortex-Genesis2	(Macalaster Bicknell Company, U.S.A.)
Water purifier	(ELGA, England)
96 well microtiter plate,Maxisorp	(Nunc, Denmark)

2.1.4 Study Subjects

Serum from 57 normal subjects and 149 patients with cancer disease from Lampang Cancer Center, Ministry of Public Health, Thailand and Chiang Mai University were collected and stored at -20 °C until used. All samples were run using the same assay method and values were given as means of triplicate determinations.

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2.2 Experimental Procedure

2.2.1 Proteoglycan Preparartion

Chicken cartilage, were washed with 1 % (v/v) Triton X 100 in normal saline solution, dried on filter paper. The cartilage were agitated in 10 times its weight of cold 4 M guanidine hydrochloride (Heinegard, 1977) at pH 5.8 for 24 hour at 4 ^{O}C. The extract was filtered through a

guaze on a beaker. The filtrate was dialyzed in distilled water and lyophilized. The lyophilized powder was designated cartilage extract or proteoglycans (PGs).

2.2.2 Hyaluronan Binding Protein Preparation

2.2.2.1 Preparation of HA-Sepharose Column

Umbilical cord hyaluronan was conjugated to EAH-Sepharose 4B according to the procedure described by Tengblad (Tengblad, 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°C) with 1 mg of hyaluronidase. The mixture was boiled for 3 min to terminate 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, the hyaluronan digested and EAH-Sepharose 4B were dissolved in distilled water to 30 ml and pH was adjusted to 4.5, added 0.575 g of 1-ethyl-3-(-3-dimethylaminoproply) carbodiimide (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 1M NaCl, 0.1 M Tris-HCl (pH 8.1), 1 M NaCl, 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°C for months without loss of binding activity. Diagram of HA-Sepharose column preparation was shown in Figure 16 and carbodiimides reaction was shown in Figure 17.

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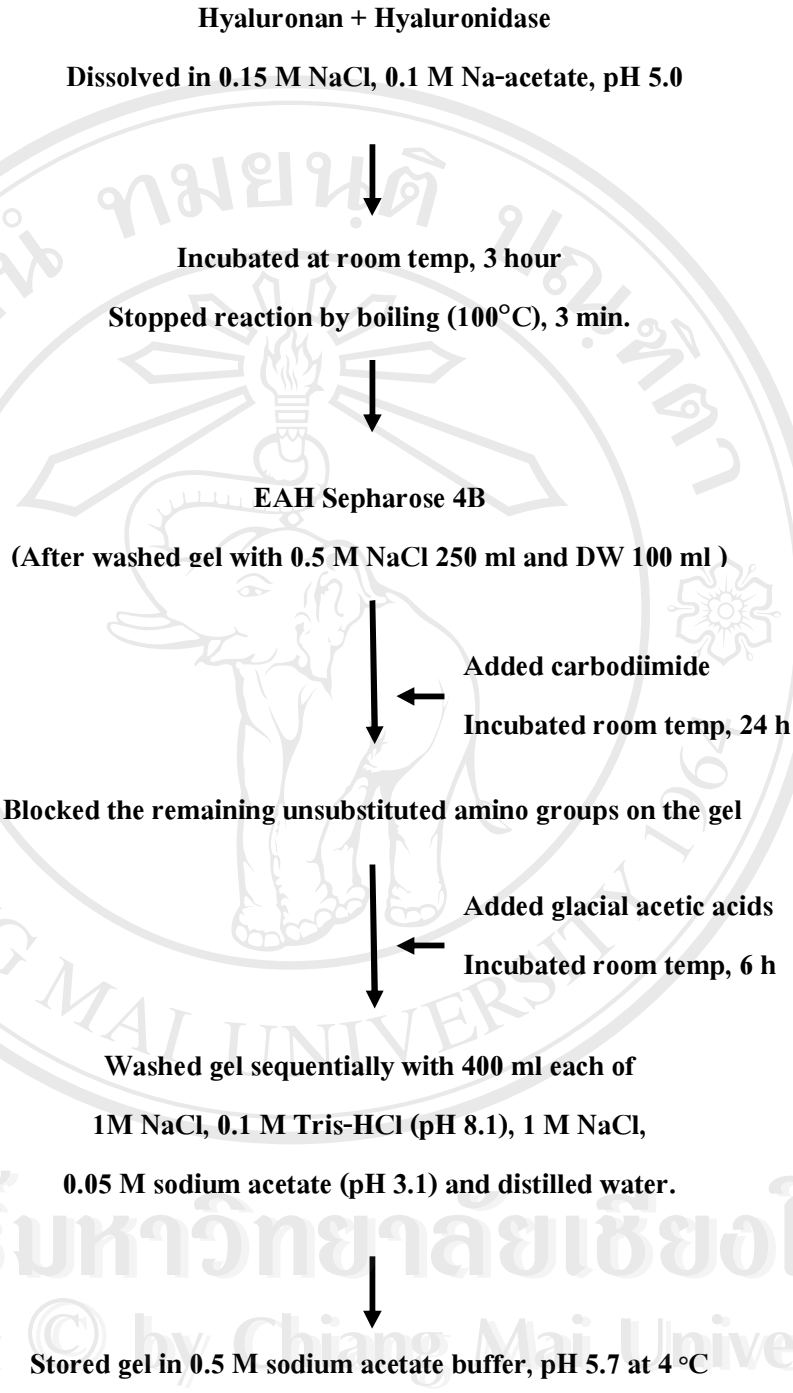

Figure 16. Diagram of HA-Sepharose column preparation.

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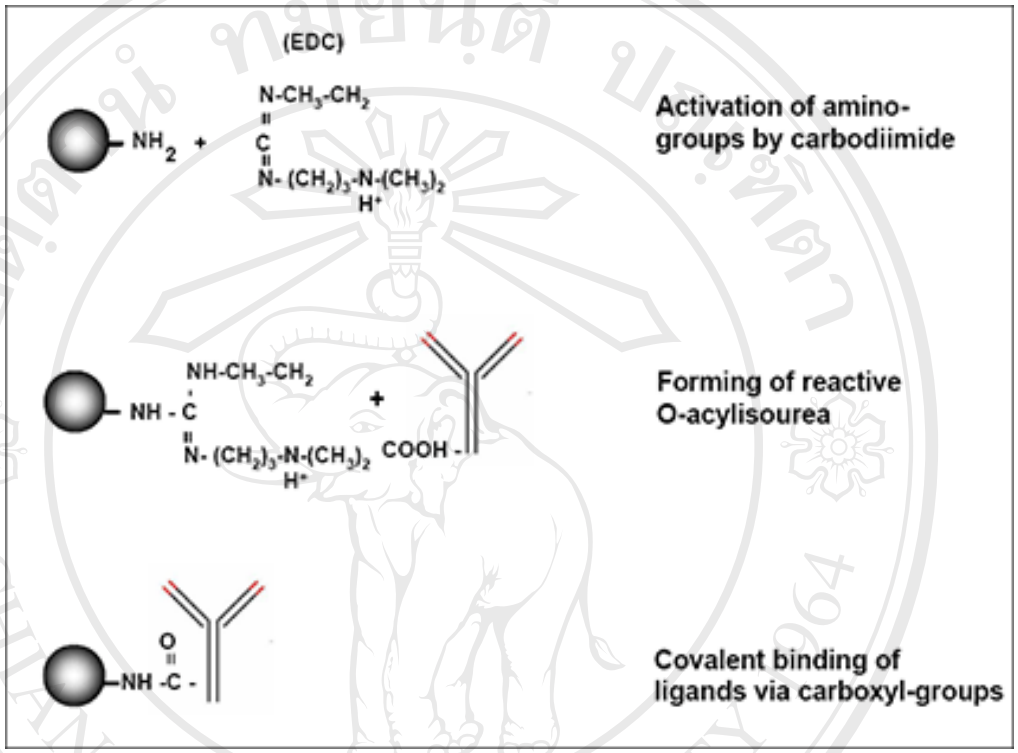

Figure 17. Carbodiimides reaction. EDAC or EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) react with the terminal amino-group from the bead to highly reactive O-acylisourea derivatives and react readily with carboxyl-groups of the ligands. This figure represent antibody as a sample (http:// www.chemicell.com/.../ protocols/carboxyl/).

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2.2.2.2 Isolation and Purification of HABPs

A lyophilized cartilage extract was dissolved with 0.1 M sodium acetate, 0.1 M Tris-HCl, pH 7.3 in 50 ml tubes and incubated at 37°C with 0.8 mg of trypsin. After 2 hour, 1 mg of trypsin inhibitor was added. The mixtures were centrifuged at 2000 rpm, 4°C, 10 min and then used supernatant for dialyzed with distilled water and lyophilized. The lyophilized powder was designated trypsinized cartilage extract or trypsinized PGs

A trypsinized cartilage extract was dissolved with 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 hour at 4°C with 9 parts of distilled water. The gel slurry was packed into a column (10 x 1.5 cm) and the column were washed twice with sodium chloride and then followed by sodium chloride with linear gradient from 1 to 3 M to remove non-specifically adsorbed material. The proteins that bound to HA Sepharose was then eluted with 40 ml 4M GuHCl (Tengblad, 1979). The column operated at room temperature. The eluent collected in 2 ml/fractions was estimated by ultraviolet absorption at 280 nm. The effluent fractions containing HABPs were pooled and dialyzed with Tris-HCl pH 8.4. Then the dialysate was lyophilized and measured protein concentration by Bradford assay (Figure 18, 19 and 20). Principle of affinity column chromatography was shown in Figure 21.

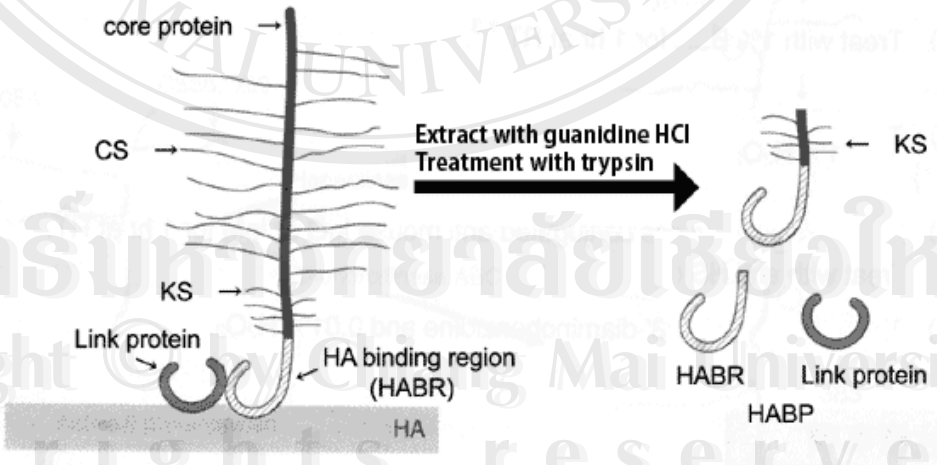

Figure 18. Principles of proteoglycan preparation.
(http://www.seikagaku.co.jp/bio-e/02tech/habp/p01.htm)

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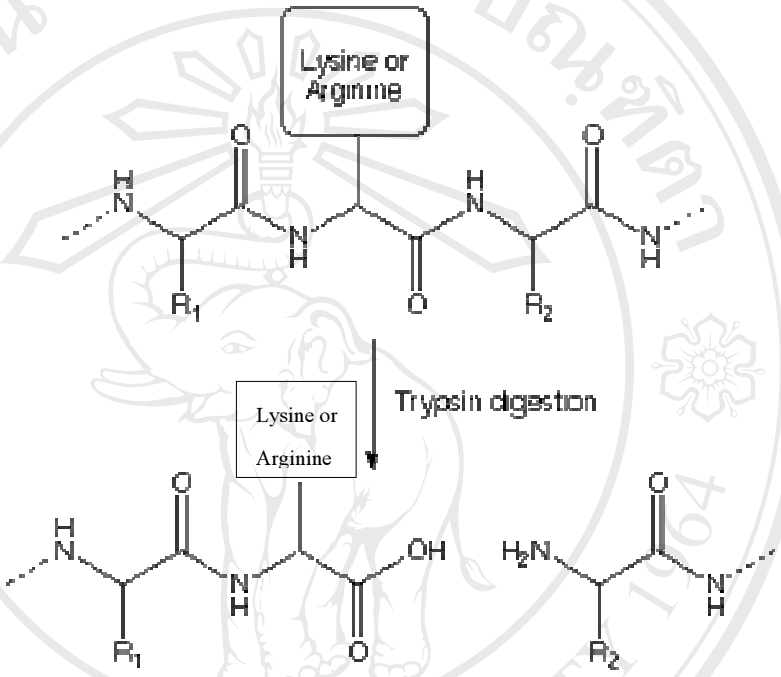

Figure 19. Trypsin digestion. Trypsin are enzymes that cleave proteins at the carboxyl side (or "C-terminus") of the basic amino acids lysine and arginine (http:// tonga.usip.edu/ gmoyna/biochem341/).

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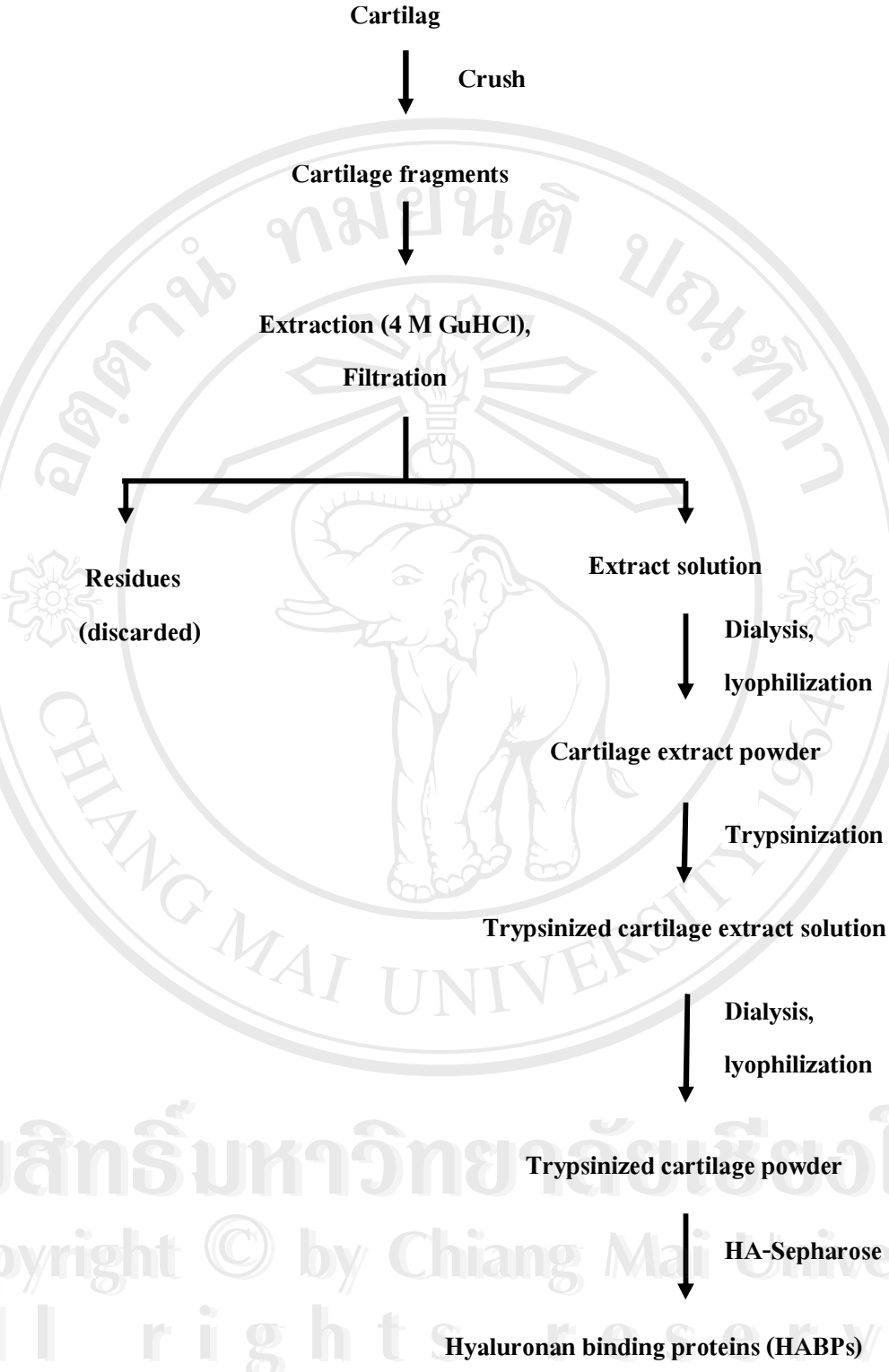

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Figure 20. Procedures for the isolation of HABPs from cartilage (Heinegard, 1977).

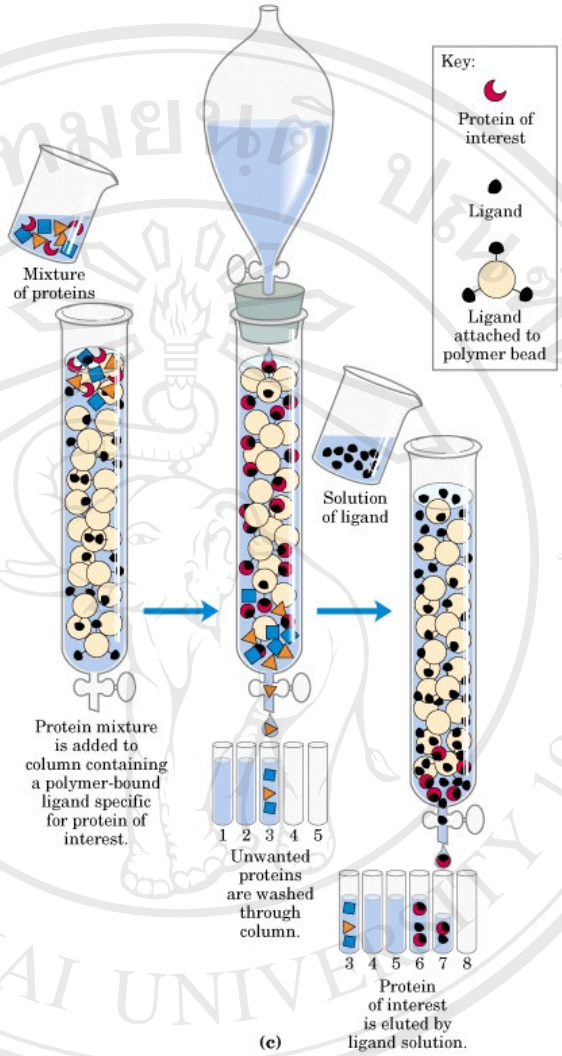

Figure 21. Principle of HABPs purification by affinity column chromatography. (http://courses.cm.utexas.edu/jrobertus/ch339k/overheads-1.htm)

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2.2.3 FITC Conjugation of HABPs

Fluorescein isothiocyanate (FITC) is a small organic molecule, and is typically conjugated to proteins via primary amines (i.e., lysine) (Figure 22). Usually, only a few FITC molecules are conjugated to the protein and higher conjugations may result in solubility problems as well as lower conjugate to reduced brightness. Thus, the experiments should probably try several parallel reactions using different amounts of FITC, and compare the resulting reagents for brightness (and background stickiness) to choose the optimal conjugation ratio. Fluorescein is typically excited by the 488 nm line of an argon laser, and emission is collected at 530 nm.

2.2.4 Preparation of FITC Conjugated HABP (FITC-HABP)

(modified technique by affinity column chromatography (Yingsang, 1996)).

Trypsinized cartilage extract were used in FITC conjugation, modified method from biotinylated-HABP. A trypsinized cartilage extract was dissolved with 4 M GuHCl, 0.5 M sodium acetate pH 5.8. The solution was mixed with 5 ml of hyaluronan-substituted Sepharose 4B and dialyzed for 24 hour at 4°C with 9 parts of distilled water. At this stage HABP reassociated with HA-Sepharose in order to protect the functional binding sites (Heinegard, 1977). The gel slurry was packed into a column (10 x 1.5 cm) and washed the column twice with sodium chloride and then followed by sodium chloride with linear gradient from 1 to 3 M to remove non-specifically adsorbed material. Equilibrated the column 5 times with conjugation buffer and then conjugation buffer was added to the column for conjugation reaction. FITC was dissolved in anhydrous DMSO immediately before use. The optimal conjugation ratio of FITC to trypsinized cartilage extract was 1: 100-300 (W/W) (1-2 mg of FITC to 300-500 mg of trypsinized cartilage extract). Wrapped the column with foil and rotated at 4°C overnight. The proteins that bound to HA-Sepharose column was then eluted 4 times with 4M GuHCl (Tengblad, 1979). Disassociated of FITC-HABP from HA on Sepharose was eluted with 4 M GuHCl. The column operated at room temperature. The eluent collected in 1 ml/fractions was estimated by ultraviolet absorption at 280 nm and 485 nm. The active fraction were pooled and unreacted FITC was removed by dialysis in 0.05 M Tris-HCl, pH 8.6 and stored in aliquots at -20°C, for used as a stock solution.

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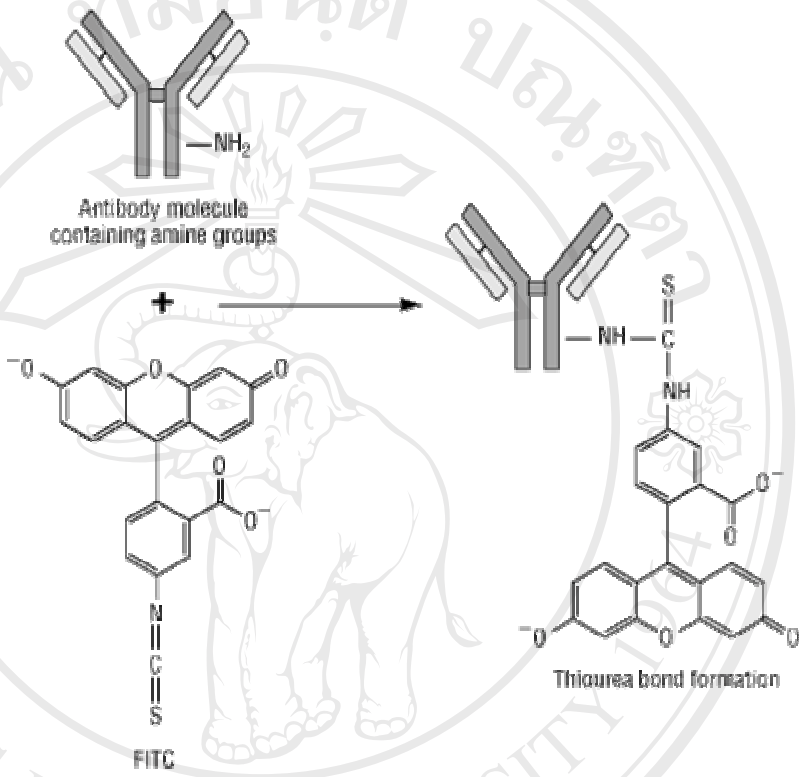

Figure 22. FITC conjugation. FITC reaction with a primary amine (such as lysine). This figure represent antibody as a sample (http:// www.piercenet.com/Objects/ view.cfm?type=Page&..).

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2.2.5 Histochemical Staining

All cells were maintained in DMEM containing 10% fetal bovine serum. For histochemical staining, cells were seeded on slides tissue culture dishes at 2 x 10^5 cells per dish. In certain experiments, cells on the glass slides were incubated in a moist chamber for all steps, fibroblast and chondrocyte cells on the glass slides were rehydrated in ethanol linear concentration step (100%, 90%, 80%, and 70%) and then cells were rinsed three times with PBS buffer. Slides were blocked with blocking buffer at room temperature. After 1 hour, cells were rinsed and stained with FITC-HABP at room temperature for 1 hour in the dark condition. As a control, cells were preincubated with hyaluronidase (340 U/ml in 0.1 M sodium acetate, 0.15 M NaCl, pH 6.0, 37°C, 1 hour). Hyaluronidase degradation was shown in Figure 23. Finally, slides were rinsed and cells were analyzed by fluorescent microscopy.

Frozen slides from skin tissue were rinsed with PBS buffer and then blocked at room temperature for 1 hour. All steps of the method for tissue sections staining were similar to cells staining technique as described above.

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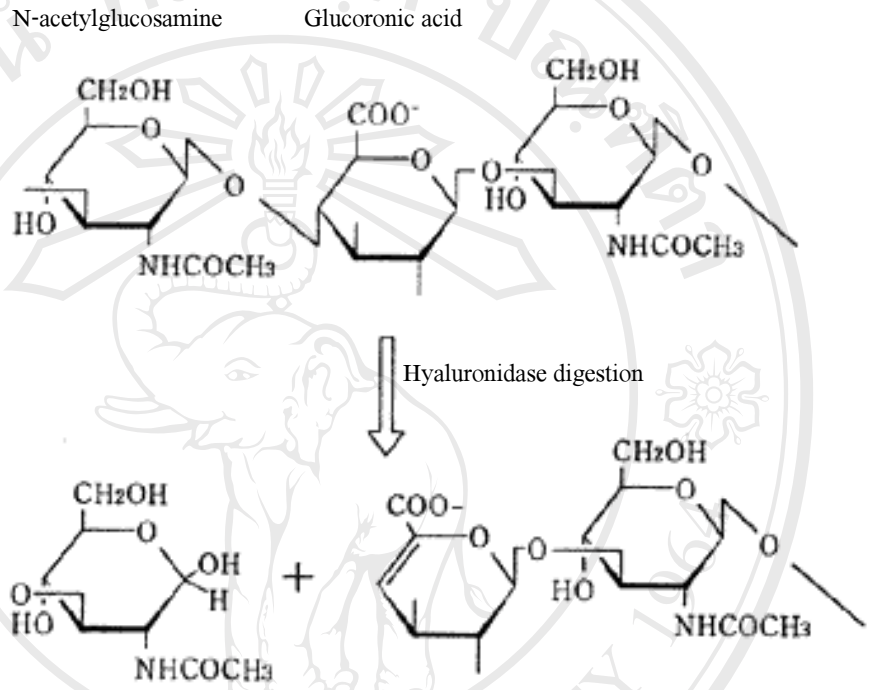

Figure 23. Hyaluronidase digestion. Eliminative cleavage of the substrate at N-acetylglucosamine-D-glucuronic acid linkage, producing unsaturated tetra and hexasaccharides (http://www.seikagaku-hit.com).

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2.2.6 Competitive Fluorescence-ELISA Based Assay

2.2.6.1 Optimization of Competitive ELISA Condition for FITC-HABP

Microtiter plate (Maxisorp ®, Nunc) was coated with optimized HA (10 μg/well) in coating buffer at 4°C, overnight. HA with concentration 1 mg/ml was diluted (v/v) 10 folds with coating buffer, 100 mM Na_2HCO_3, pH 9.6. PBS-Tween as a washing solution for all washing steps in these experiments. Plate was washed and nonspecific adsorption of protein was blocked with 1 % BSA (diluted in PBS buffer) 150 μl/well, the inhibition mixture was prepared by mixing the standard competitor (HA) and the diluted FITC-HABP 1:10, 1:20, 1:40, on rows A to H respectively in eppendorf tubes. The inhibition mixtures were incubated at room temperature, 60 min. Each dilution of the incubation mixtures were then transferred to microtiter plates after washing step and dried plate. Plate was incubated at room temperature. After 60 min, plate was washed and dried. Access FITC-HABP bound to the coating HA were measured for the exciting/emission absorbance 485/530 nm using fluorescent microplate reader (Figure 24 and 25).

2.2.7 Competitive Fluorescence-ELISA Based Assay for HA Determination in Cancer Serum

Microtiter plates were coated with HA in coating buffer at 4°C, overnight. The plates were washed three times with washing buffer, the plates were added with blocking buffer and incubated 1 hour at room temperature. Serum sample from normal subjects and from cancer patients, containing unknown amounts of HA, were mixed with optimized dilution of FITC-HABP in the eppendorf tubes. The mixtures were incubated at room temperature, 1 hour in dark condition. After incubation, the mixtures were added to each well of the microtiter plates after washing step and plate drying. Plates were incubated at room temperature in dark condition. After 1 hour, plates were washed and dried. Finally, the plates were measured for exciting/emission absorbance 485/530 nm using fluorescent microplate reader. Diagram and principle of the fluorescence-ELISA based assay were shown in Figure 24 and 25.

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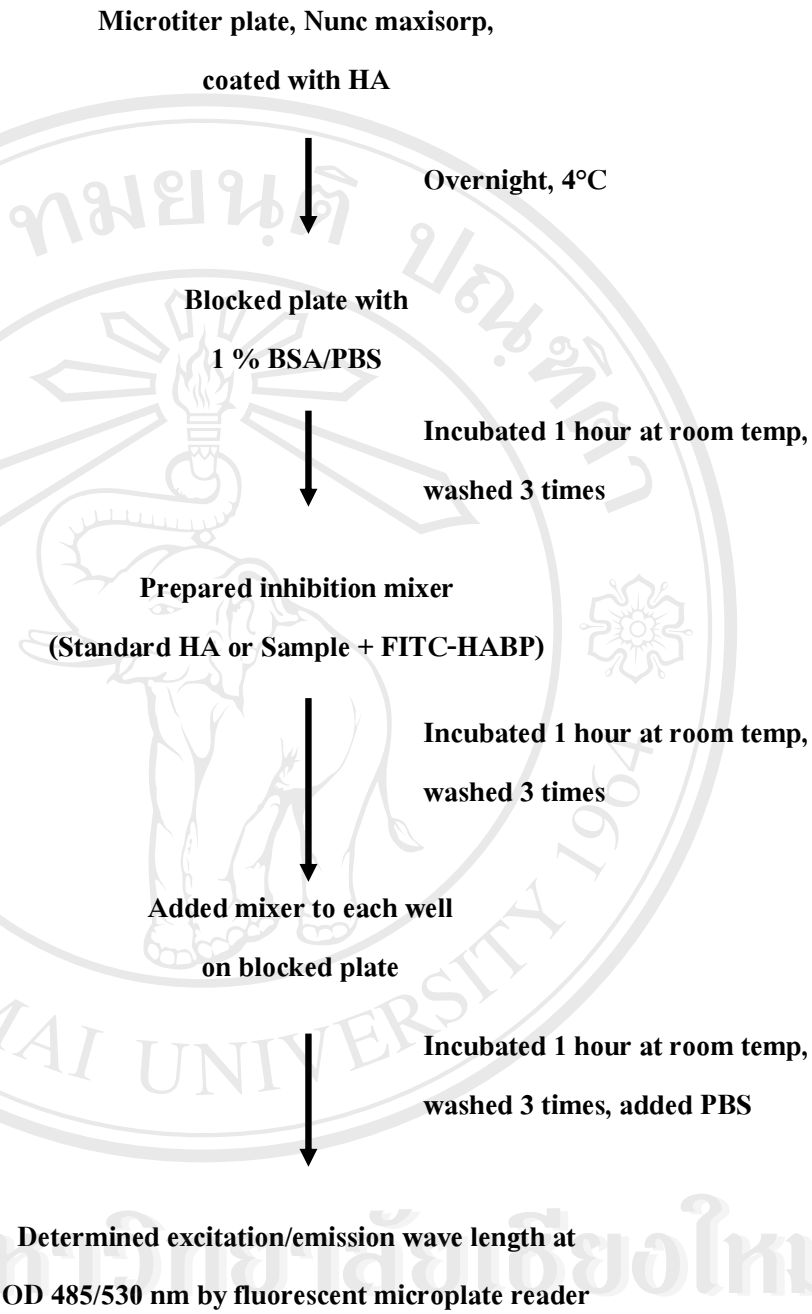

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Figure 24. Diagram of the fluorescence-ELISA method developed for hyaluronan determination.

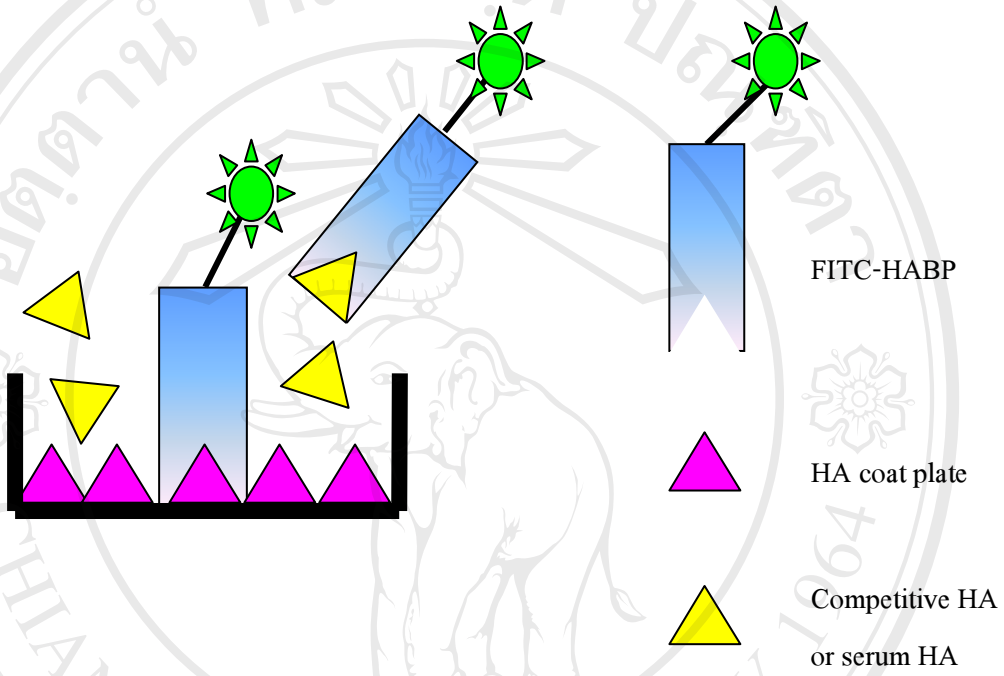

Figure 25. Principle of the fluorescence-ELISA based assay.

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2.2.8 Analytical Procedure

2.2.8.1 Bradford Assay

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

A calibration curve was prepared using bovine serum albumin as a standard. The assay required only a single reagent, an acid solution of coomassie brilliant blue G-250. After addition of dye solution to a protein sample, color development was completed in 2 min and remained 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).

2.2.8.2 Electrophoresis

SDS-PAGE determined the molecular weight of HABPs from column chromatography. Separating gel consisting of 12 % 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, sample were supplemented with blue native sample buffer and applied onto polyacrylamide gradient slab gel. Molecular mass markers were run on both borders of the gel and were visualized by coomassie brilliant blue staining. Electrophoresis was carried out at a constant voltage of 100 Volt until the dye migrated toward the end of the gel. The gels were then removed from the glass plates.

2.2.9 Evaluation for Accuracy and Precision of The Developed Method

2.2.9.1 Intra Assay and Inter Assay Variation

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

Intra assay precision was determined by using 20 aliquots of pooled serum in triplicate analysis within the same plate.

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Inter assay precision was determined by using individual serum of above pooled serum in triplicate measurement different plate.

2.2.9.2 Recovery of Serum HABPs

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.

2.2.10 Statistical Method

2.2.10.1 Percentage Inhibition

The percentage inhibition was calculated by following formula:

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

A standard inhibition curve for HABPs was constructed by using log/linear coordinators. The HABPs levels in the test samples were determined by excitation/emission wave length development at OD 485/530 nm relative to standard curve constructed using computer software.

2.2.10.2 Serum HA Concentration

For serum HA, the healthy and patient groups were expressed as means and SD. Comparisons between subjects with cancer and healthy population were evaluated with ANOVA test. For these analyses, $P < 0.05$ (two-tail test) was considered significant. Statistical calculations were performed by using SPSS program.

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2.2.10.3 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 with standard molecular weight and expressed on a semi-logarithmic scale.

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