CHAPTER IV

DISCUSSION

The objectives of this study were to develop and apply fluorescence-ELISA technique for HA determination in cancer serum by using prepared FITC-HABP. The HA concentration in normal subjects and cancer patients were compared by using applied fluorescence-ELISA based assay. HABPs were prepared from chicken cartilage by a modified method as described by Tengblad (Tengblad 1978). HABPs were isolated and purified, dialyzed with water and lyophilized. Extracted powder from chicken cartilage was digested with trypsin, resulting to the fragment of trypsinized proteoglycans. Aggrecan, link protein and complex of HA-binding region, were isolated from other components by specific affinity column chromatography, HA-Sepharose, and coupled hyaluronan to agarose gel bead. Carboxyl groups of HA were coupled to amino group on the gel by carbodiimide method. The unbound materials and small amount of additional materials were removed when the column was eluted with a sodium chloride gradient. The 18 mg of HABPs were extracted from 300 mg of chicken trypsinized cartilage extract. However, the yield of HABPs obtained from this procedure was low, it might be the effect of incomplete binding of HABPs to gel bead. The process was performed at room temperature, resulting to the deterioration of the proteinases. Purification procedure at 4°C should be give higher yield. The pattern of chicken HABPs from this process composed of two protein bands. The molecular weight of two proteins chicken HABPs were 33-34, 40-45 kDa, respectively. From Yingsang study, bovine HABPs were analyzed and showed that it had two sizes: 43, 39 kDa. The shark HABPs showed three sizes: 65, 45.5, 14.4 kDa (Yingsang, 1996). In addition, bovine HABPs were purified by Hardingham and colleagues, gave 2-3 sizes: 70, 45, 40-45 kDa (Heinegard and Hascall, 1974; Hardingham, 1981). It might be suggested that these components might be small G1 or link protein fragments.

FITC-HABP was prepared by the modified method using biotinylated HABPs as described by Yingsang (Yingsang, 1996). Prepared HABPs were conjugated with fluorescein isothiocyanate (FITC) by affinity bioconjugation. The trypsinized cartilage extract was loaded to specific HA-Sepharose affinity column chromatography. The unbound components were eluted with sodium chloride gradient. The bound glycoproteins, HABP, were conjugated FITC with

adding FTIC/DMSO solution. The column was rotated to perform conjugated reaction of FITC and HABPs in dark condition.

FITC-HABP was eluted with 4 M GuHCl. The active fractions were pooled. The excess FITC was removed by dialyzing with Tris-HCl buffer, pH8.6. In this study, FITC was used indirect conjugation to HABPs that associated on HA-bead gel in column and the optimal ratio of FITC to HABP conjugation was approximately 1:100-300 (w/w). In general, the ratio of FITC to antibody (IgM MW 150 kDa) was 1:12.5-25 and FITC to Annexin V (MW 40 kDa) was 1:50-100. Therefore, this method was sufficient for FITC-HABP preparation.

From histological study, the cells and tissues were stained with FITC-HABP for elucidation the biological function. The untreated hyaluronidase cells and tissues showed the fluorescent signal when stained with FITC-HABP, because of the present of hyaluronan in the extracellular matrix. On the other hand, the pretreated hyaluronidase cells and tissues showed decrease dectectable, because the hyaluronidase lysed hyaluronan in the pericellular and extracellular matrix. Therefore, the results suggested that the FITC-HABP probably recognized hyaluronan in the pericellular and extracellular matrix.

From the previous study, the measurement of HA was performed by the method that separated HA from other polysaccharides by vary techniques, followed by colorimetric analysis, such as the carbazole method for determination of uronic acid (Scott, 1960; Bitter and Muir, 1962; Galambos, 1967; Hallen, 1972). The discovery that HA can bind specifically to proteins found in cartilage (HABP), led to the development of the techniques for measure the HA concentration. Several ELISA technique which based on the affinity of specific proteins for HA, has been used to measure the HA concentration in biological fluids. Tengblad developed the radiometric assay based on competition of free HA in solution and HA-substituted Sepharose gel with HA-binding cartilage protein labeled with ^{125}I (Tengblad, 1980). Subsequently, this method has been modified for HA determination in biological samples and able to measure HA in nanogram amounts (Laurent and Tengblad, 1980; Engstrom-Laurent *et al.*, 1985; Brandt *et al.*, 1987).

In 1998, Rossler developed a new method for HA determination. This method was performed as an alternative to the conventional radiometric assay and to ELISA, and employed the principle of time-resolved fluoroimmunoassays (TRFIA). The solid-phase immunoassay was

based on the competition between aggregation of hyaluronan with the cartilage proteoglycan monomer (aggrecan), followed by binding of a monoclonal antibody to keratan sulfate of the proteoglycan and a biotinylated anti-mouse IgG. Fluorescence can be measured by a time-resolved fluorometer after binding of europium–labelled streptavidin to the biotinylated IgG. The time-resolved immunoassay for HA can be more sensitive than a radioimmunoassay. The assay has a lower detection limit (95% confidence interval) of 0.24 μg/ l, which is about 10 times more sensitive than the results from the radioimmunoassay (RIA), which makes the assay suitable for measuring HA in several biological fluids. This high sensitivity of TRFIA makes the assay particularly suitable for studying low levels of HA without a prior protein precipitation step with trichloroacetic acid (Rossler, 1998).

Consequently, Martins and colleagues described the new fluorescence-based assay which modified fluoroimmunoassay method using the sandwich technique for determination of HA in biological fluids. This assay employed HABP that isolated from bovine cartilage either immobilized on microwell plates (solid phase), and biotin conjugated (biotinylated probe). This technique is simple, fast, high precision and high sensitivity similar to TRFIA assay, and it able to measure HA in amounts as small as 0.2 μg/l or less. In addition, this noncompetitive assay avoids preincubation and consumes less time (less than 5 h) than the competitive fluorescence-based assay (more than 72 h), and avoids the use of radioactive materials (Martins *et al.*, 2003).

This study was modified the conventional ELISA method that reported by Yingsang. From the principle, the enzyme-linked anti-biotinylated HABP (B-HABP) assay which based on the inhibition system that carried out in eppendorf tubes. This method depended on the specific binding of HA to the B-HABP. Biotinylated HABP was determined by addition of anti-biotin conjugated peroxidase. The concentration of HA was examined by the occurring color product of peroxidase substrate. The HA concentration in samples were calculated by comparing their inhibition capacity in the assay with a standard inhibition curve that obtained by using highly purified HA. The obtained calibration curve was suitable for determine the HA concentration between 10 to 10,000 ng/ml (diagram and principle of conventional ELISA method were shown in appendix) (Yingsang, 1996). In the present study, fluorescence-ELISA technique was modified and applied to detect the HA concentration in the range 10 to 10,000 ng/ml.

Most parameters on the fluorescence-ELISA assay were performed as described by Yingsang (Yingsang, 1996). However, the optimal dilution of FITC-HABP in this experiment was 1/10 (v/v). The intra-(6.51%) and the inter assay (11.01%) coefficients of variation were similar as shown in table 2 and the assay was accurate (recovery 119.78%), data was shown in table 3. This assay was suitable for measuring HA concentration in biological fluids.

In the study, fluorescence-ELISA technique were applied to determine the HA concentration in cancer and normal serum. The serum HA concentration in normal subjects were ranged from 2.14 to 238.02 ng/ml (mean ± SD = 34.79 ± 49.40 ng/ml). HA levels in cancer patients were 2.14 - 3349.22 ng/ml (mean ± SD = 305.25 ± 352.90 ng/ml). The result showed higher level of HA concentration in cancer patients than normal subjects *($p< 0.01$)*. When the cancer patients were divided to three groups, the HA concentration in cervix cancer, CEA positive and other cancer patients (mean ± SD; 181.99 ± 135.80 ng/ml, 418.90 ± 300.87 ng/ml and 366.09 ± 512.33 ng/ml, respectively) were significant higher than normal subjects serum *($p< 0.01$)*.