CHAPTER IV
DISCUSSION

4.1 Isolation and purification of HABPs

Many hyaluronan-binding proteins contain a common domain, termed a link module, which is involved in ligand binding. The link module has been first described in cartilage link protein, which is comprised of an immunoglobulin domain and two contiguous Link modules. This molecular arrangement is also found in the G1-domains of the chondroitin sulfate proteoglycans aggrecan, versican, neurocan and brevican. Aggrecan forms huge link protein-stabilized complexes with hyaluronan that provide load-bearing function in articular cartilage. Similar aggregates, in which versican, neurocan or brevican substitute for aggrecan, are likely to contribute to the structural integrity of many other tissues such as, skin, blood vessels and brain. The immunoglobulin domains are responsible for the link protein-proteoglycan interaction, while the Link modules mediate binding to hyaluronan (Kohda *et al.*, 1996).

The aims of this study are to develop newly competitive Enzyme Linked Immunosorbent Assay (ELISA) technique by using mAb 1H8 for HABPs determination in human serum. The concentration of HABPs in normal subjects and patients with OA and RA are compared to HA by using newly developed ELISA. HABPs standard was prepare by a modified method of Tengblad (Tengblad, 1978) due to its simplicity, economy and efficiency in obtaining HABPs. HABPs were prepared by a sequence of procedures where proteoglycan aggregates were initially extracted, using 4 M GuHCl with proteinase inhibitor. HABPs was isolated by sequential digestion of purified extract and then extract was dialyzed against water. Extract was lyophilized and digested with trypsin, resulting in fragment of trypsinized proteoglycans. Aggrecan, link protein and complex of HA-binding region (HABR), were isolated from other components by specific affinity chromatography, on HA-Sepharose,by coupling hyaluronan to agarose gel bead. Carboxyl groups of HA were coupled to amino group on the gel. The unbound materials were removed by washing with 1 M sodium chloride. However, there is small amount of additional material was removed when the column was eluted with a sodium chloride gradient. Percent yield

of HABPs from bovine PGs and shark PGs were 46.25% and 25.63% respectively. However, the yield of HABPs obtained by this procedure is low may be the cause of incomplete binding of HABPs to gel bead. Other reason may be the process is performed at room temperature led to the deterioration of the proteinases. Purification procedures at 4 ^{0}C should be give higher yield. From the studied of Yingsang (Yingsang, 1996), the purified HABPs and G1 obtained from the same method were characterized by running SDS-PAGE and immunoblotting. The patterns of HABPs from this process were showed two proteins bands for the bovine preparation and three bands for the shark preparation. It can be concluded that these components may be small G1 or link protein fragments, because these components (on nitrocellulose) were reacted both anti-G1 and anti-link protein.

4.2 Isolation and purification of monoclonal antibody 1H8

The purification of monoclonal antibody 1H8 is base on salt-promoted adsorption chromatography on thiophilic absorbent or T-Gel illustrated by Poarth (Porated *et al.*, 1985).

Purified monoclonal antibody 1H8 was characterized by direct chequerboard ELISA for 1H8 activity and also tested for reactivity by ELISA using shark PGs as the antigen. From the results, It indicated that mAb 1H8 could react with PGs. However, most activity was lost in lyophilized step when compare with step before powder making, because the conformation of mAb may changed or lost some factor that associated conformation of mAb. Its better use culture media is rather than purified powder by using T-Gel.

4.3 Development of ELISA technique for HABPs assay

The specific interaction of HABPs with antibodies, such as mAb HABR-1 (against HABR in aggrecan), has also been used by other to quantitate HABPs level in biological fluids (Poole, *et al.*, 1990, Kobayashi *et al.*, 2000). Competitive ELISA technique was modified and applied to work rang 10 to 10000 ng/ml. Nunc maxisorp microtiter plate were used as a solid phase throughout this experiment. Bovine serum albumin as a blocker for non-specific protein site. Washing and drying were done every step. The effect of some parameters on the competitive ELISA assay was

performed by the optimal concentration of PGs as antigen coating plate. It was found that 10^{-5} mg/ml of PGs is the optimal concentration. Overnight incubation with PGs coating at 4 ^{0}C was sufficient to ensure maximal binding. In addition, the optimal concentration of 1H8 is 1:30.

The optimal inhibitor for HABPs determination which the competitive ELISA is shark A1. There is more activity with optimal 1H8 concentration (1:30) than shark HABPs, Bovine A1 and Bovine HABPs compared in the same condition. It may be cause of the conformation of shark A1 is well interaction with mAb 1H8 or may be cause of shark A1 as antigen for immunization into mouse in mAb 1H8 inducing process.

The optimal diluent for the inhibitor shark A1 in the method is 6% BSA. PBS buffer was compared as diluent with 6%BSA but 6% BSA is the best protein representation used as represented protein in serum for standard dilution. The optimal concentration of Anti-mouse IgM peroxidase conjugate is 1:2,000 and optimal time for color development was 15 min.

In this study, PBS 7.4 had been used for the preparation in the step of optimized concentration of Anti-mouse IgM peroxidase conjugate. PBS-tween as washing solution each steps. All wells at the edge of plates were not used due to the edge effect. These well apparently adsorb more than wells toward the center of the plates (Burt *et al.*, 1979).

From the results, shark A1 was suitable for using as an inhibitor for HABPs concentration determination in sera samples. The absorbance in this assay was converted to concentration of HABPs by Delta soft program building logarithmic concentration of the HABPs in the range 10 to 10,000 ng/ml, which is gave accepted values of precision, recovery. Thus samples containing abnormally high concentrations of HABPs can be used without pre-assay dilution.

4.4 HABPs application in OA and RA sera samples

In this study, the sera HABPs in healthy adults ranges from 5.44 to 134.91 ng/ml. (mean ± SD; 26.58±37.52 ng/ml). The Result shows an increase HABPs concentration only in RA patients (mean ± SD; 668.60±1834.70 ng/ml). The concentration of HABPs is higher level than OA (78.31±58.77ng/ml) and wells above

normal in healthy sera (26.58±37.52ng/ml). It was likely that in cartilage of RA patients had been destroyed because there is complex mechanism of cartilage metabolism by cytokine in RA cause of inflammation. The pathogenesis of RA still unclear and most factors joined in concert of cartilage destruction are immune complex.

Although the earlier HA investigation by Pothacharoen (Pothacharoen, 2000) found that HA were also high in RA patients but there were no correlation between HA and HABPs every pairs in comparison between normal, OA and RA. However, the HABPs concentration trend was opposite HA concentration. It likely that large amount of HA in sera could be re-associated with HABPs.

Earlier studies, the elevated concentration of HABPs in the sera of RA patients appeared to be related accelerated production of HABPs in inflamed joints, such as TSG-6 (Parkar *et al.*, 1998), link protein (McKenna *et al.*, 1998). Resultant increases in the amount of degradation products appearing in the serum (Engstrom-Laurent *et al.*, 1985) and stimulate immune complex production in joint cartilage (Zhang, *et al.*, 1998). Arthritis development is directly related to humoral immunity to PGs (Mikecz *et al.*, 1987, Zhang *et al.*, 1998).

Immunoblotting was shown that biotinyl-HA could reacted some molecules in serum. It could be suggested that these bands might be HABPs and/or other HA binding molecules because mAb 1H8 had no reaction with some protein in this experiment.

4.5 Future studies

Due to the good sensitivity, specificity, and practicability of the developed competitive ELISA assay for HABPs determination in sera, it was suggested that the developed method could be used in local clinical laboratories. It should be compared with existing diagnostic joint disease assessment method, and detected for the other cartilage diseases.

It seemed that different patterns of HABPs existed both in normal and joint diseases. Unfortunately, in this thesis the numbers of sera samples analyzed were limited.

CONCLUSION

The competitive ELISA with monoclonal antibody 1H8 was developed to analyze hyaluronan-binding protein (HABPs) in joint disease and healthy serum. Antigen (shark PGs), inhibitors (shark A1, shark HABPs, bovine A1, and bovine HABPs), monoclonal antibodies 1H8, anti-IgM peroxidase conjugate, and conditions were optimized. It was found that the mAb 1H8 powder had low activity than supernatant, and then 1H8 cell culture medium was used in ELISA assay. Shark A1 was the best inhibitor for interaction with mAb 1H8 in inhibition complex. From experiments, the sensitivity of this method was at 10 to 10,000 ng/ml of HABPs. Using newly developed method for joint disease, It was found that the concentration of HABPs in serum were increased in many RA, and less increased in OA compared with healthy sera. The concentration of HABPs were also compared with HA in the same sample, but no correlation among any paired- group of RA, OA and healthy serum.

It might be concluded that this newly developed method could be useful in the follow up treatment of joint diseases, and used for diagnostic in joint diseases, and should be combined with other biomarkers.