# CHAPTER I

# INTRODUCTION

## 1.1 Statement of the problems

Degenerative joint disease (degenerative arthritis) is a non-infectious, progressive disorder of the weight bearing joints. Arthritis is a common cause of morbidity and disability in the elderly. It was found mostly in persons over the age of 65 and is expected to double between 1990 and 2020. It is estimated to affect more than 20 million American adults (1)

Joint tissue, particularly articular cartilage undergoes various degrees of structure degradation in osteoarthritis (OA) and rheumatoid arthritis (RA). Both diseases are involved with inflammation but the degree of inflammation in RA is much greater than OA. Inflammtion is accompanied by destruction of the connective tissue of the joint, particulary to the layer of articular cartilage covering the ends of bone in diarthrodial joints. The destruction of connective tissue and cartilage matrix in both OA and RA is currently believed to be due mainly to the action of matrix enzymes (metalloproteinases), which include collagenases and stromelysins (1). The articular cartilage is important to the joint function. So damage of the articular cartilage leads to total joint dysfunction. To prevent the destruction of articular cartilage has long been a goal in the treatment of arthritic diseases.

There are many different pharmacological agents used in the treatment of arthritis. The aim of the treatment is to stop or slow down the progress of inflammation, thus relieving symptoms, improving function, and preventing joint damage and other complications. Steroid and nonsteroidal anti-inflammatory drugs (NSAIDs) represent the mainstay of treatment which can reduce inflammation but can not inhibit cartilage destruction. A group of pharmacological agents has been used to treat the disease is the disease modifying antirheumatic drugs (DMARDs) which can slow or stop the progression of arthritis and thus joint damage and disability. However, it has many side effects such as gastrointestinal discomfort, nausea, vomiting, loss of appetite, abdominal pain, skin rashes and allergic manifestations, and headaches (2;3) .

Recently, alternative treatments to relieve pain, symptom other than pain and less side effects than prescribed medications have been studied. Previous study showed that active compounds from some herbs could relieve and inhibit these problems. Preliminary study revealed

the anti-inflammatory effect of Plai extracts which could inhibit edema in carrageenan-induced rat paw (4) . Plai has long been regarded by Thai massage therapists as one of those oils necessary to have in their kit to combat joint and muscle problems. The plant has been proven to be extremely useful for human health and then developed into several products such as creams and massage oils for relieving muscle pain. Thus, the aims of this study were to investigate the effects of Zingiber cassumunar (Roxb.) or Plai cxtracts on cartilage degradation induced by interleukin-1β (IL-1β) or retinoic acid (RetA). The results of this study will bring about alterative pharmacological agents for arthritis disease.

## 1.2 Literature reviews

### 1.2.1 The articular joints

The articular joints is an important structure that gives movement and mobility to an otherwise rigid bony skeleton (5) . Under normal healthy conditions, they function in a nearly frictionless and almost entirely wear resistant manner throughout our lives. Diarthrodial joints have some common structural features (6) . First, all diarthrodial joint are enclosed in a strong fibrous capsule. Second, the inner surfaces of the joint capsules are lined with a metabolically active tissue, the synovium, which secretes the synovial fluid to provide the nutrients required by the tissues within the joint. Failure of the bearing surfaces of the joint (the articular cartilage), as with engineering bearing, means a failure of these bearings to provide their essential biomechanical functions. In biomedical terms, failure of the artilcular cartilage of diarthrodial joints often leads to osteoarthritis (OA) .The picture of articular joint is shown in Figureure 1.

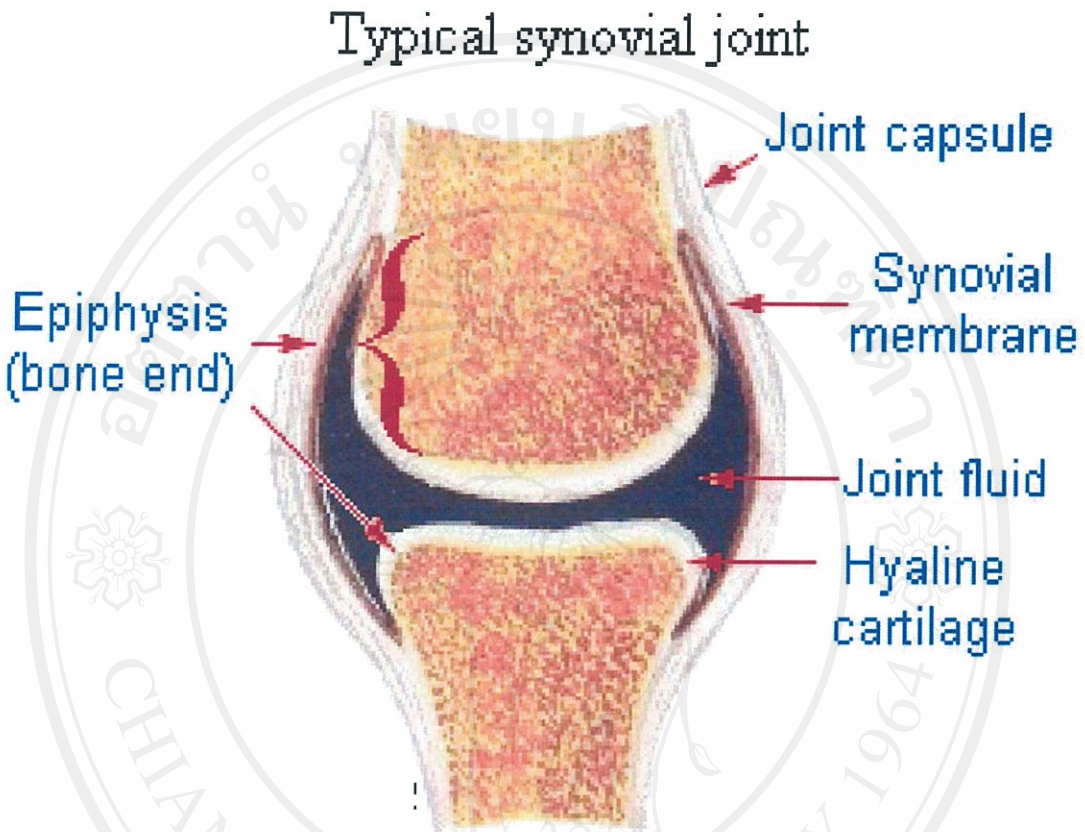


**Figure 1.** Features of a typical articular (synovial) joint. The extent of the synovial joint cavity is exaggerated to show features more clearly.
(http://www.webschoolsolutions.com/patts/systems/skeleton.html)

### 1.2.2 Articular cartilage

Articular cartilage, in its young, normal and healthy state appears glassy, smooth, glistening and bluish white to the naked eye (6) . Articular cartilage is organized in a manner that reflects the tensile and compressive forces and shear stresses acting on this tissue (7) . It covers the articulating ends of bones and serves as a lubricated, wear-resistant, friction-reducing surface that is slightly compressible to evenly distribute forces onto the bone.

Cartilage is divided, according to its minute structure, into elastic cartilage (outer ear), fibrous cartilage(knee meniscus, vertebral disk), and hyaline cartilage(covers joint surfaces). Articular cartilage can be regarded as a multiphasic material, with two major phases : a fluid phase composed of water and electrolytes ($Na^{+}$, $Ca2^{+}$, $Cl^{-}$, etc), and a solid phase composed primarily of type II collagen, proteoglycans, hyaluronan, other collagens, noncollagenous proteins, and the chondrocyte cells . Each phase of the tissue contributes significantly to its known mechanical and physicochemical properties. Water is the major component of cartilage, contributing up to 85% of the wet mass. Of the organic components, the collagens provide the quantitatively major component, followed by proteoglycans and hyaluronan (Table 1). Quantitatively minor components include link protein, the smaller proteoglycans, versican, biglycan, decorin, perlican, fibromodulin, thrombospondin, and cartilage oligomeric matrix protein (COMP).

**Table 1.** Summary of the composition of articular cartilage (6) .

| Component | Wet Weight |
|---|---|
| **Quantitatively major** | |
| Water | 60-85% |
| Collagen, type II | 15-22% |
| Aggrecan | 4-7% |
| **Quantitatively minor** | (<5%) |
| Link protein, Hyaluronan, Biglycan,Collagen typeI, Collagen typeV, Collagen type VI, Collagen typeIX, Collagen typeXI,COMP, Decorin, Fibromodulin, Perlican,Thrombospondin | |

### 1.2.3 Structure of Proteoglycan

Proteoglycans are a class of heterogeneous molecules consisting of a specific type of polysaccharide chain attached covalently to a core protein. The polysaccharides found in proteoglycans typically contain acetylated aminosugars and are referred to as glycosaminoglycans (GAGs). GAGs composition in proteoglycan are chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, keratan sulfate. The protein component of proteoglycans is a core protein which directs the biosynthesis of proteoglycans to different molecular constructions and functions. Models of proteoglycans are shown in Figureure 2.

The GAG chains are linear polymers of repeating disaccharide units containing an amino sugar consisting of N-acetylgalactosamine(GalNAc) or N-acetylglucosamine(GlaNAc) or-N-sulfonylglucosamine($GlcNSO_3$) residues links with glycosidic bonds in alternating with glucuronic acid(GlcUA), iduronic acid (IdUA) or galactose (Gal) residues to form the unbranched polysaccharide chain attached to the core protein through a specific oligosaccharide linkage (Figure 3) (8) . They are variably substituted with sulfate, leading to the generation of high degree of negative charge. In fact, the core proteins differ greatly, and the GAG chains vary widely in number, length and structural complexity. Many of the PG are prominent in extracellular matrix (ECM), which a range of structural and metabolic function have been established for proteoglycan in cartilage, bone, ligaments, tendon, skin, and blood vessel (Table 2).

The PG constitute the second largest portion of the non-water (dry-weight) component of articular cartilage (Table 1). The major PG in articular cartilage is aggrecan , and it has been extensively studied because of its role in skeleton growth, joint function, and development of arthritis. Other more recently described proteoglycans in articular cartilage include versican, biglycan and decorin .In term of function, the large aggregating PGs (aggrecan and versican) contribute 50-85% of the PGs, with the large non-aggregating type contributing 10-40%. The small PGs (e.g., biglycan and decorin) probably contribute less than 10% of the total PGs in mature cartilage. As aggregate, the aggrecan molecules can form macromolecular complexes of $(300\text{-}400)\times10^6$ Da, and make major contributions to the mechanical and physicochemical properties of articular cartilage (6) .

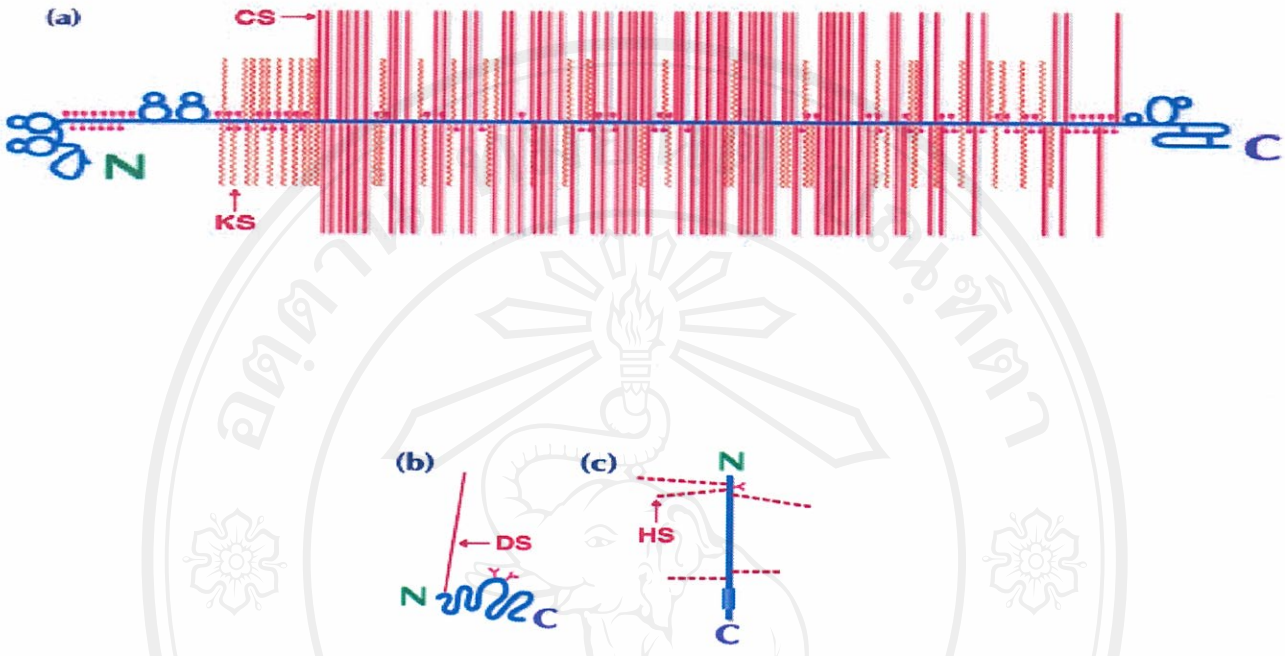


**Figure 2.** Models of typical proteoglycans; (a) aggrecan, (b) decorin and (c) syndecan 1. Amino and carboxy termini of core proteins are indicated by N and C, respectively. Glycosaminoglycans are depicted by red lines (i.e., solid line, chondroitin sulfate / dermatan sulfate CS / DS; broken line, heparan sulfate HS; wavy line, keratan sulfate KS). Models show approximate, comparable sizes of molecules when they are stretched. A block in the syndecan 1 core protein illustrates the transmembrane hydrophobic domain.
(http://www.glycoforum.gr.jp/science/word/proteoglycan/PGA00E.html)

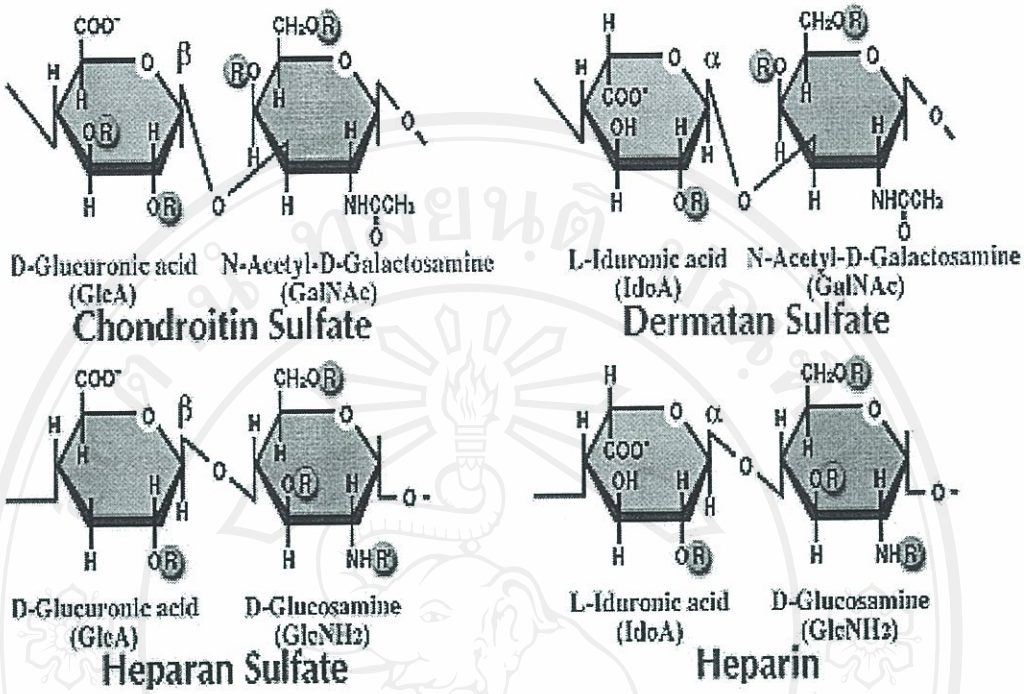


**Figure 3.** Schematic representation of molecular structure of glycosaminoglycans, including: chondroitin sulfate; dermatan sulfate; heparan sulfate and heparin. (http://www.glycoforum.gr.jp/science/word/proteoglycan/PGA06E.html)

**Table 2.** The structural diversity of glycosaminoglycans correlates with various biological functions.

(www.glycoforum.gr.jp/science/word/proteoglycan/PGA12E.html)

| Glycosaminoglycans | Function |
|---|---|
| Chondroitin Sulfate | |
| GlcAβ1-3GalNAc(4S)β1-4 | Receptor for malaria-infected erythrocytes,Receptor for CD44 |
| GlcAβ1-3GalNAc(6S)β1-4 | Binding of pleiotrophin to 6B4 proteoglycan, Binding of granules in mast cell, macrophage receptor for lipoprotein lipase. |
| GlcAβ1-3GalNAc(4S,6S)β1-4 | Anticoagulant activity |
| GlcA(2S)β1-3GalNAc(6S)β1-4 | Neutrient growth promoting activity |
| Dermatan Sulfate | |
| IdoAα1-3GalNAc(4S)β1-4 | Binding to HGF |
| IdoA(2S)α1-3GalNAc(4S)β1-4 | Binding domain for heparin cofactor II |
| Heparan Sulfate, Heparin | |
| (IdoA(2S)α1-4GlcNSα1-4)3 | Binding domain for FGF2 |
| (IdoA(2S)α1-4GlcNS(6S)α1-4)3 | Binding domain for FGF1 |
| (IdoA(2S)α1-4GlcNSα1-4IdoA(2s)α1-4GlcNS(6S)α1-4IdoA(2S)α1-4GlcNSα1-4IdoA(2S)α1-4GlcNS(6S) | Binding domain for HGF |
| GlcNAcα1-4GlcAβ1-4GlcNS(3S)α1-4IdoA(2S)α1-4GlcNS(6S) | Binding domain for antithrombin III |
| Keratan Sulfate | |
| GlcNAc(6S)β1-3Galβ1-4 | Transparency of cornea, Mark of immunogenicity of G1 domain of aggrecan |
| GlcNAc(6S)β1-3Gal(6S)β1-4 | |

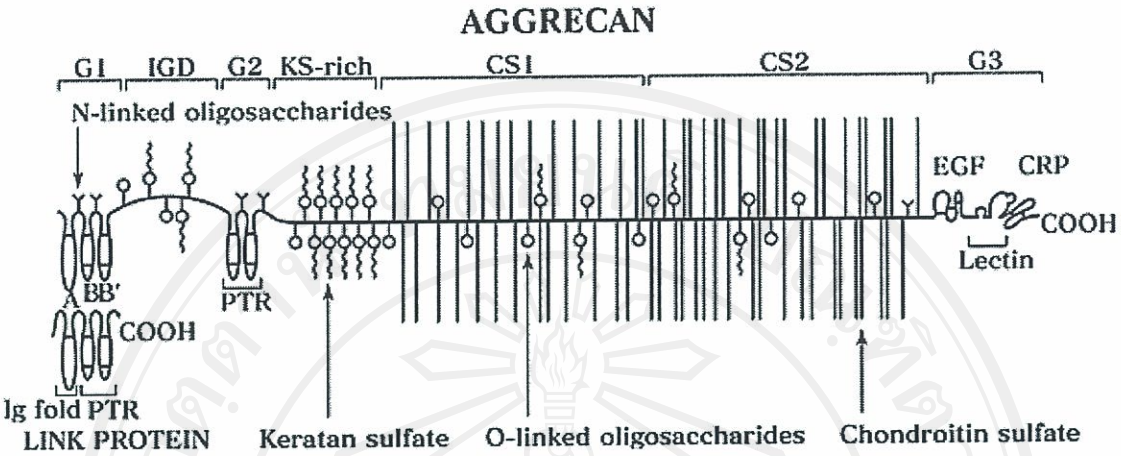


**Figure 4.** Structure of aggrecan - Aggrecan contains 3 globular domains (G1, G2, G3) and 2 extended regions, which form the interglobular region between G1 and G2, and the main glycosaminoglycan attachment region. The GAG attachment region is composed of a variable keratan sulfate region and 2 chondroitin sulfate regions (CS-1 and CS-2) distinguished by their sequence patterns. The domain structure of link protein is also shown, which is similar to the aggrecan G1 domain. In aggregates the G1 domain of aggrecan binds to hyaluronan and this binding is stabilized by link protein. PTR,proteoglycantandemrepeat.

(http://www.glycoforum.gr.jp/science/hyaluronan/HA05/HA05E.html)

### 1.2.4 Metabolism of proteoglycans (aggrecan)

During the process of normal maintenance of cartilage, in its synthesis, repair and degradation, the proteoglycans of cartilage are continually being broken down, and released from the matrix (9). Proteoglycans (aggrecan) are the specialized type of glycoproteins, and are synthesized by the chondrocytes along the intracellular pathway in common with other secreated proteins. The synthes is process begins with translation of the core protein and its transport into the lumen of the rough endoplasmic reticulum (RER). Subsequently to the activation of sugar and formation and translocation of the precursor sugar nucleotides, xylose (Xyl) is added to serine residues of the core protein, and a tetrasaccharide linkage region is completed by sequential addition of two galactose residues, followed by a glucuronic acid (GlcA) residue to produce Glc-Gal-Gal-Xyl-Ser (Figureure 5). Finally, the nascent PG is transported through the Golgi, the repeating disaccharides of the GAG chain are added individually and sulfated (Figureure 6). The enzymes responsible for each of these sugar transfer are organized within the membranes of these secretory pathway and function in different compartments of the ER and Golgi apparatus. The specific structure of both the core protein and the attached GAG chain are improtant for the function of the mature PG, the outcome of this regulated biosynthesis has significant consequences.

The catabolic pathway of aggrecan via by proteolytic cleavage site on aggrecan is between the G1 and G2 domain (10), at amino acid 273-274 (11) . This action separetes the part of the proteoglycan involved in aggregation (the G1 domain) from the glycosaminoglycan containing regions. Then the non-aggregating glycosaminoglycan containing fragment pass through the matrix and is lost to the synovial fluid. Further degradation of the G1 domain and link protein results in their release. These fragment are taken up by the synovium, pass through the lymphatic system, to the blood, and are cleared by the liver and kidneys (Figureure 7).

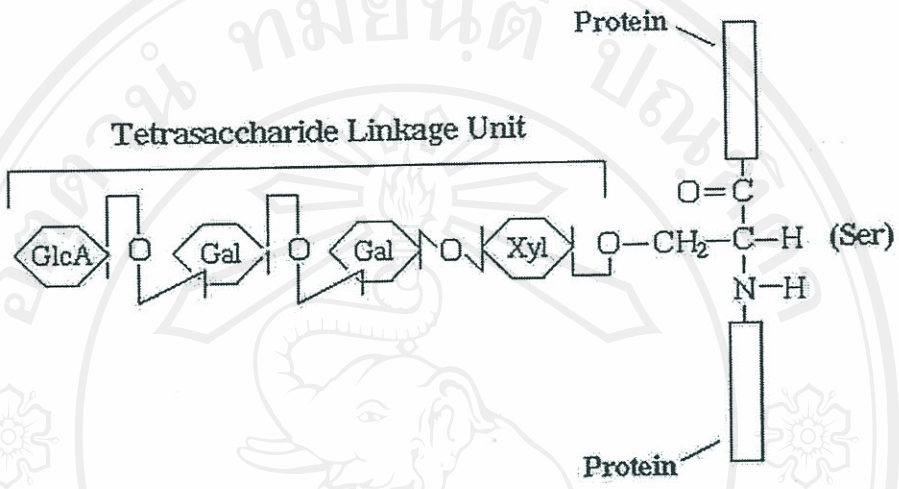


**Figure 5.** Attachment of glycosaminoglycans to protein. The sugar are linked to serine or threonine residue of the protein (12) .

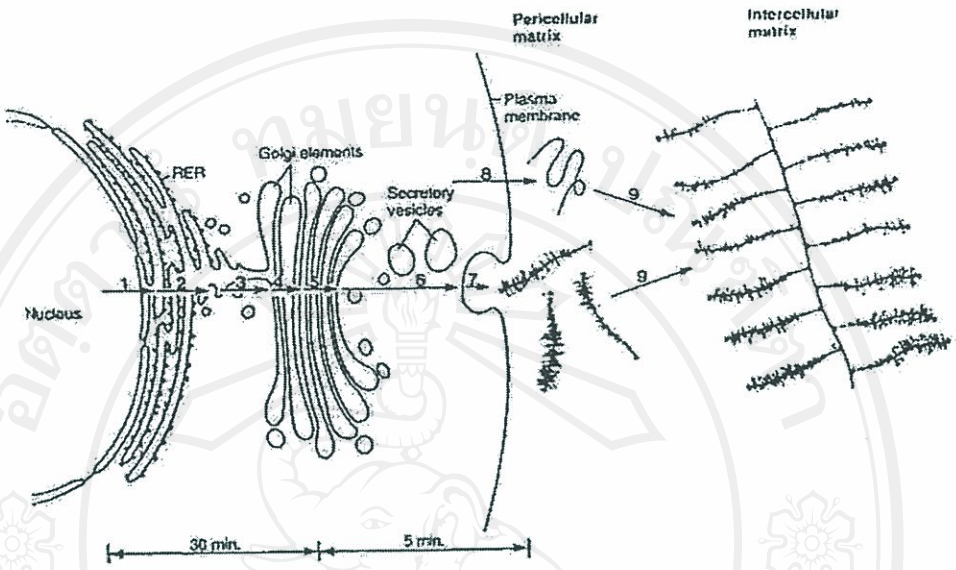


**Figure 6.** Diagram showing the various stages involving in the synthesis and secretion of aggrecan, the protein and hyaluronan by chondrocyte (10) .

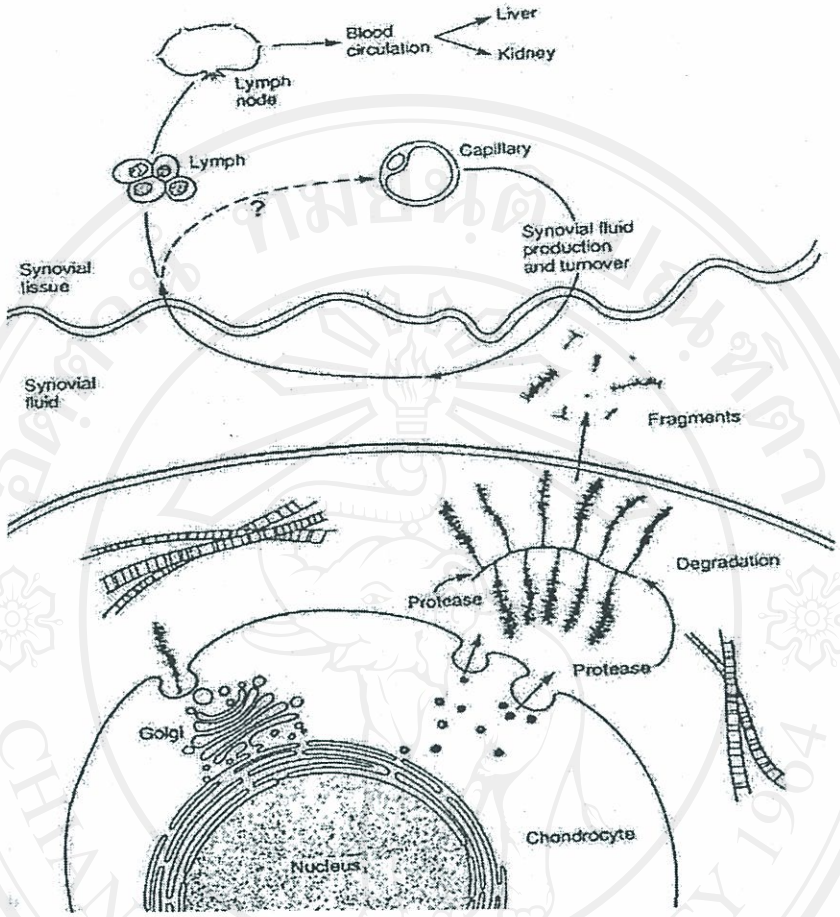


**Figure 7**. A schematic representation of the metabolic events controlling the proteoglycans in cartilage. The chondrocytes synthesize and secrete proteoglycan, link protein and HA, and they become incorporated into the matrix as functional aggregate, the fragments are released from the matrix into the synovial fluid, and from there the fragments are taken up by the lymphatic and are moved to the circulating blood (10) .

### 1.2.5 Interleukin-1β and retinoic acid

Joint diseases that affect the integrity of articular cartilage and eventually bring about its destruction are the largest cause of disability in the elderly and present a major health problem. Interleukin-1β (IL-1β), a pro-inflammatory factor, has been shown to contribute *in vitro* to the accelerated damage of articular tissue and to amplify the inflammatory response. The ability of IL-1β to promote tissue degradation appears to be intimately associated with its ability to stimulate the synthesis of matrix metalloproteinases (MMPs) such as collagenase and stromelysin (13) . Biological activity of IL-1β requires the processing of its inactive precursor, a function generally attributed to IL-1β converting enzyme (ICE or caspase-1) (14) . It is mediated by binding to a specific plasma membrane receptor of high affinity. Two types of IL-1 receptor (IL-1R) have been identified and characterized: 80 kDa type I and 60-kDa type II. However, chondrocyte synthesizes only the type I IL-1R and the intensity of the response to stimulation by IL-1 appears to relate to the number of cell receptors (15) . The action of IL-1 can be inhibited by a protein named interleukin receptor antagonist (IL-1ra). The amount of IL-1ra synthesized in OA cartilage may not be sufficient to inhibit the effects of the produced IL-1. It has been shown that the increased level of IL-1R in OA chondrocytes may be a part of the mechanism leading to the over-stimulation of these cells by IL-1 during the disease process (15) . The up-regulation of IL-1R in these cells may explain the increased synthesis of proteolytic enzymes found to be part of the OA pathophysiology. The signal transduction pathways for IL-1β are not yet completely understood but, taken together, the data strongly argue against involvement of protein kinase C in its action (16). In the cartilage cells, the Fos family of tran-114 scription factors has been shown to play role in IL-1β induction of MMP-13, as well as of MMP-1 and MMP-3, by binding to the AP-1 site of these MMPs (17) .

Retinoic acid (RetA) is an important alternative stimulus of cartilage degradation in vitro because it mediates its effects primarily through binding to RetA receptors, nuclear receptors that are members of the steroid/thyroid hormone receptor superfamily (18) . Preliminary study using retinoids in one of the first explant culture studies showed that retinoids (RetA) promoted proteoglycan degradation in chick limb-bud rudiments. Pharmacologic doses of RetA were used to promote the release of both proteoglycan and collagen from porcine articular cartilage explants (19) . The in vivo injection of retinoid into animal joints can also result in

changes that mimic human OA, including loss of proteoglycan and fibrillation (20). In chondrocyte cultures, RetA can inhibit cell proliferation and protein synthesis, including inhibition of type II collagen synthesis (21) .

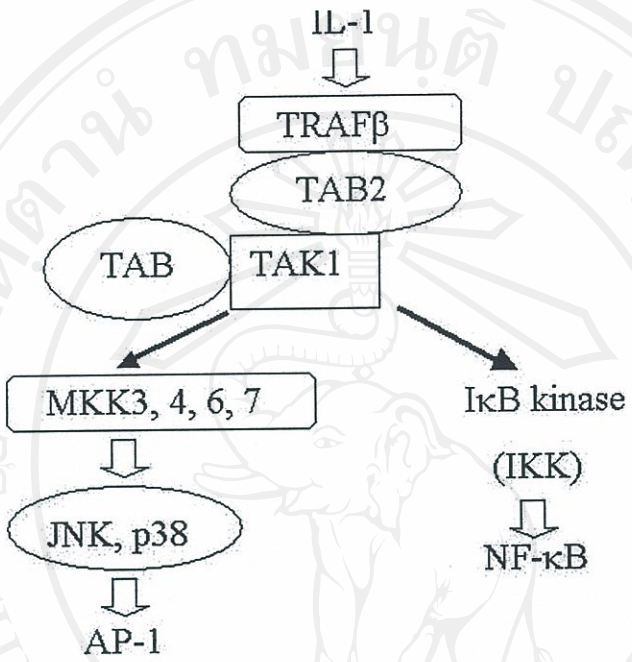


**Figure 8.** Signal transduction of IL-1, involve of ECM degradation.

### 1.2.6 Matrix metalloproteinases

A major event in many forms of arthritic, including osteoarthritis (OA) and rheumatoid arthritis (RA), is the destruction of the major articular tissues, the cartilage. Controlling degradation of the extracellular matrix is crucial in arthritic diseases, as conventional treatments do not positively affect the structural properties of the articular tissue degeneration.The matrix metalloproteinases (MMPs) constitute a multigene family of zinc- and calcium-dependent endopeptidases with extensive sequence homology (22) . To date, at least 25 different MMPs have been identified that share significant sequence homology and a common multi-domain organization. According to their structural and functional properties, the MMP family can be subdivided into five major groups (I) the collagenases (MMP-1, -8, -13), (II) the gelatinases (MMP-2, -9), (III) the stromelysins (MMP-3, -10, -11), (IV) a heterogeneous subgroup including matrilysin (MMP-7), enamelysin (MMP-20), macrophage metalloelastase (MMP-12) and MMP-19, and (V) the membrane-type MMPs (MMP-14 to –17 and –24, -25 or MTI-6-MMP) (17) .

All MMP enzymes are composed of three distinct domains: an amino-terminal propeptide domain that is involved in the maintenance of enzyme latency; a catalytic domain that binds zinc and calcium ions which are required for the stability and expression of enzymatic activity and a haemopexin-like domain at the carboxy terminus (Figure 9).

All MMP are synthesized as inactive zymogens (or pro MMPs) and must be activated by proteolytic clevage of the propeptide domain from the N- terminus of the enzyme. Generally, they are present as soluble forms, but some are membrane bound. Turnover of MMPs are controlled by both physiological and pathological factors such as pro-inflammatory cytokines, hormones, growth factors and proteases (23) .The MMPs are capable of degrading a variety of ECM biomolecules including collagens, proteoglycans, fibronectin and laminin (24) . The members of the MMPs family are distinguished by the substrates that they degrade (Table 3).

Although the link between single MMPs and individual substrates is not as direct as once thought, it is clear that as a family, the MMPs are capable of breaking down any extracellular matrix component. In normal physiology, MMPs produced by connective tissue are thought to contribute to tissue remodeling in development, in the menstrual cycle, and as part of repair processes following tissue damage. The obvious destructive capability of MMPs initially focused most researchs onto diseases that involve breakdown of the connective tissues (e.g.,

rheumatoid arthritis, cancer and periodontal disease). Leukocytes, particularly macrophages, are major sources of MMP production. MMPs released by leukocytes play vital roles in allowing leukocytes to extravasate and penetrate tissues, a key event in inflammatory disease. The MMP action not only permits leukocyte emigration into tissues and causes tissue damage, it also generates immunogenic fragments of normal proteins that may escalate autoimmune disease. In an analogous way, metastatic cancer cells also use MMPs to get in and out of tissues and to establish a blood supply. Drug companies have synthesized low molecular weight MMP inhibitors that have shown efficacy in models of these diseases, reinforcing their central role in pathology (22;23) .

The MMP axis is highly regulated to avoid excessive tissue damage. Most MMPs, with the exception of 72 kDa gelatinase and the MT-MMPs, are not constitutively expressed in normal tissues. Inflammatory cytokines such as IL-1 and TNF, growth factors such as TGF-β and noxious stimuli are required to initiate transcription. MMPs are also expressed as inactive zymogens (the pro-piece must be dissociated from the catalytic domain before the enzyme is activated). This dissociation can be achieved by autocatalysis or by the action of enzymes such as furin, plasmin or even other MMPs. For example, the activation of pro-MMP-2 occurs at the surface of many cells and is mediated by MT-MMPs. Once activated, MMPs are subject to inactivation by TIMPs and by binding to plasma proteins such as alpha-2 macroglobulin. It is thought that the local balance of MMP expression and activation versus the level of TIMP governs the level of destruction mediated by MMPs. This is of great significance when studying MMP involvement in disease processes (22;23) .

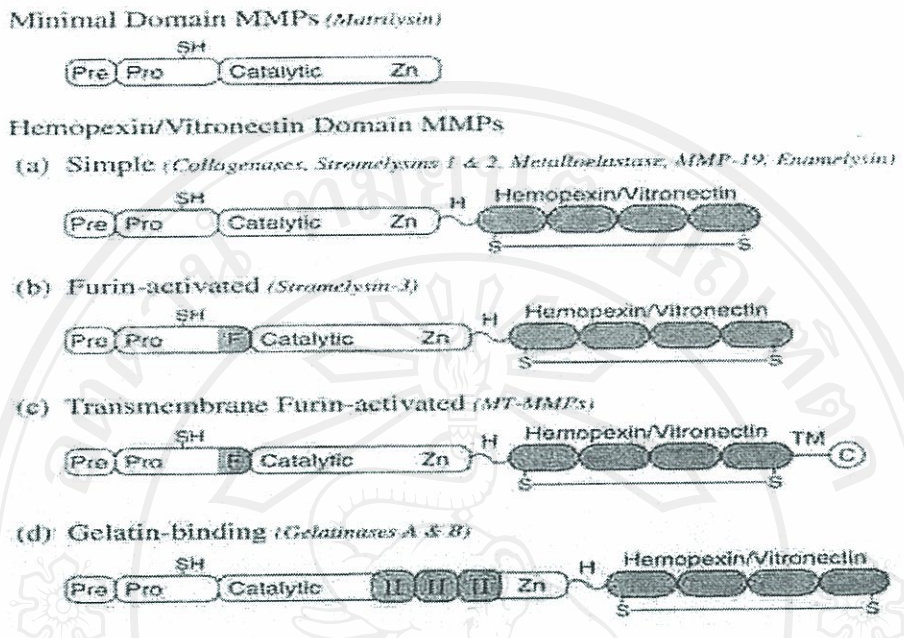


**Figure 9.** Domain structure of the MMPs. Pre, signal sequence; Pro, propeptide with a free zinc-ligating thiol group; F, furin-like enzyme-recognition motif; Zn, zinc-binding site; II, collagen-binding fibronectin type II inserts; H, hinge region; TM, transmembrane domain; C, cytoplasmic tail. The haemopexin/vitronectin-like c-terminal domain contains four repeats, the first and fourth being connected by a disulfide bridge (22) .

**Table 3.** Matrix metalloproteinases (MMP) and their substrates (25) .

**Matrix metalloproteinases (MMPs) and their substrates**

| MMP | Enzyme | $M_r$ latent | $M_r$ active | Known substrates |
|---|---|---|---|---|
| MMP-1 | Interstitial collagenase (collagenase-1) | 55,000 | 45,000 | Collagens I, II, III, VII, VIII and X, gelatin, aggrecan, versican, proteoglycan link protein, casein, $\alpha_1$-proteinase inhibitor, $\alpha_2$-M, pregnancy zone protein, ovostatin, nidogen, MBP, proTNF, L-selectin, proMMP-2, proMMP-9 |
| MMP-2 | Gelatinase A | 72,000 | 66,000 | Collagens I, IV, V, VII, X, XI and XIV, gelatin, elastin, fibronectin, aggrecan, versican, proteoglycan link protein, MBP, proTNF, $\alpha_1$-proteinase inhibitor, proMMP-9, proMMP-13 |
| MMP-3 | Stromelysin-1 | 57,000 | 45,000 | Collagens III, IV, IX and X, gelatin, aggrecan, versican, perlecan, nidogen, proteoglycan link protein, fibronectin, laminin, elastin, casein, fibrinogen, antithrombin-III, $\alpha_2$M, ovostatin, $\alpha_1$-proteinase inhibitor, MBP, proTNF, proMMP-1, proMMP-7, proMMP-8, proMMP-9, proMMP-13 |
| MMP-7 | Matrilysin-1 (PUMP-1) | 28,000 | 19,000 | Collagens IV and X, gelatin, aggrecan, proteoglycan link protein, fibronectin, laminin, entactin, elastin, casein, transferrin, MBP, $\alpha_1$-proteinase inhibitor, proTNF, proMMP-1, proMMP-2, proMMP-9 |
| MMP-8 | Neutrophil collagenase (collagenase-2) | 75,000 | 58,000 | Collagens I, II, III, V, VII, VIII and X, gelatin, aggrecan, $\alpha_1$-proteinase inhibitor, $\alpha_2$-antiplasmin, fibronectin |
| MMP-9 | Gelatinase B | 92,000 | 86,000 | Collagens IV, V, VII, X and XIV, gelatin, elastin, aggrecan, versican, proteoglycan link protein, fibronectin, nidogen, $\alpha_1$-proteinase inhibitor, MBP, proTNF |
| MMP-10 | Stromelysin-2 | 57,000 | 44,000 | Collagens III, IV and V, gelatin, casein, aggrecan, elastin, proteoglycan link protein, fibronectin, proMMP-1, proMMP-8 |
| MMP-11 | Stromelysin-3 | 51,000 | 44,000 | $\alpha_1$-proteinase inhibitor |
| MMP-12 | Macrophage metalloelastase | 54,000 | 45,000/ 22,000 | Collagen IV, gelatin, elastin, $\alpha_1$-proteinase inhibitor, fibronectin, vitronectin, laminin, proTNF, MBP |
| MMP-13 | Collagenase-3 | 60,000 | 48,000 | Collagens I, II, III and IV, gelatin, plasminogen activator inhibitor 2, aggrecan, perlecan, tenascin |
| MMP-14 | MT1-MMP | 66,000 | 56,000 | Collagens I, II and III, gelatin, casein, elastin, fibronectin, laminin B chain, vitronectin, aggrecan, dermatan sulfate proteoglycan, MMP-2, MMP-13, proTNF |
| MMP-15 | MT2-MMP | 72,000 | 60,000 | proMMP-2, gelatin, fibronectin, tenascin, nidogen, laminin |
| MMP-16 | MT3-MMP | 64,000 | 52,000 | proMMP-2 |
| MMP-17 | MT4-MMP | 57,000 | 53,000 | |
| MMP-18 | Xenopus collagenase | 55,000 | 42,000 | |
| MMP-19 | | 54,000 | 45,000 | Collagen IV, gelatin, laminin, nidogen, tenascin, fibronectin, aggrecan, COMP |
| MMP-20 | Enamelysin | 54,000 | 22,000 | Amelogenin |
| MMP-21 | XMMP (xenopus) | 70,000 | 53,000 | |
| MMP-22 (MMP-27) | CMMP (chicken) | 52,000 | 43,000 | Gelatin, casein |
| MMP-23 | CA-MMP | ? | ? | |
| MMP-24 | MT5-MMP | 63,000 | 45,000 | proMMP-2, proMMP-9, gelatin |
| MMP-25 | MT6-MMP, leukolysin | | 56,000 | Collagen IV, gelatin, fibronectin, fibrin |
| MMP-26 | Matrilysin-2, endometase | 28,000 | | Collagen IV, fibronectin, fibrinogen, gelatin, $\alpha_1$-proteinase inhibitor, proMMP-9 |
| MMP-28 | Epilysin | 59,000 (55,000) | | Casein |

$\alpha_2$-M, $\alpha_2$-macroglobulin; COMP, cartilage oligomeric matrix protein; MBP, myelin basic protein; $M_r$, relative molecular mass; TNF, tumour necrosis factor.

### 1.2.7 Proteoglycan turnover

The normal turnover of proteoglycan in healthy cartilage appears to involve the proteolytic cleavage in the region close to the G1 domain. This is the most important site of attack, as it release a large, glycosaminoglycan-bearing fragment and separates it from its site for aggregation. This is thus an efficient mechanism for mobilizing the proteoglycan because it involves the minimum of enzyme action. It is important that turnover is an essentially conservative process; cleavage and release of only a small fraction of tissue content is required at any one time because the overall tissue content must be conserved in order to retain the biomechanical properties (26) .

### 1.2.8 Joint diseases

**Osteoarthritis**

Osteoarthritis (OA) is a result of both mechanical and biologic events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix, and subchondral bone (27) . The exact cause of OA is unknown, there are known to be several possible causes including: injury, age, congenital predisposition and obesity. Osteoarthritis commonly affects the hands, feet, spine, and large weight-bearing joints, such as the hips and knees. The main symptoms of osteoarthritis are pain, stiffness and swelling of the joints. The joint may have restricted movement, and there may be tenderness or deformity. The joint may also crack or creak (called crepitation). When the joint becomes severely damaged, it may become misshapen, with bony swellings, and unstable. This puts stress on the ligaments and tissues surrounding the joints, and can lead to deformity.

As the disease progresses, the cartilage that protects the bone becomes roughened, then thins and wears away. The body tries to compensate for this, which causes the outer edges of the bones to thicken and change shape so that "outgrowths", known as osteophytes, form at the outer edges. At the same time, the membranes lining the joints can become inflamed. With severe osteoarthritis, chalky deposits of calcium crystals can form in the cartilage. This is called calcification. These calcium crystals can come loose from the cartilage, and cause the joint to become hot, red and swollen (called pseudogout). The changes in articular cartilage seen in OA are the results of complex interplay between the macromolecules of the cartilage matrix

(glycosaminoglycans, proteoglycans, and collagen) and the chondrocytes that produce the matrix and are in turn supported by it. Chondrocytes play a fundamental role in the pathogenesis of OA by producing interleukin-1, releasing a cascade of cytokines including TNF-alpha (tumor necrosis factor), TGF-beta (transforming growth factor), interleukin-1 (IL-1) and various prostaglandin derivatives. These cytokines in turn induce chondrocytes to release lytic enzymes, including metalloproteinases, which degrade collagen II and proteoglycans. Simultaneously, normal matrix synthesis by chondrocytes is inhibited. On the molecular level, these events result in a reduced amount of glycosaminoglycans in the matrix, decreased binding between glycosaminoglycans and collagen II, and an increase in the amount of water in the matrix. These biochemical changes in the cartilage matrix decrease its tensile strength and resiliency, preventing it from functioning normally in transmitting forces, supporting chondrocytes, and protecting subchondral bone.

**Rheumatoid arthritis**

Rheumatoid arthritis (RA) is a chronic destructive inflammation process involving tissue derived from the embryonic mesenchyme, but particularly focusing on diarthridial (synovial) joints (28) . Rheumatoid arthritis tends to persist over prolonged periods of time, the inflamed joints eventually can become damageing. Some other forms of arthritis come in shorter of episodes and are less likely to cause permanent damage. In RA the joints tend to be involved in a symmetrical pattern. That is, if knuckles on the right knee are inflamed, it is likely that knuckles on the left knee will be inflamed as well. This symmetry is not found as often in most other types of arthritis.

In RA the white blood cells move from bloodstream into joint tissue. Joint fluid may increase, and the white cells are found in the fluid as well. The white cells in the joint tissue and fluid produce may substances, including antibodies and other molecules, that lead to the joint damage and the sick feeling that occurs in people with rheumatoid arthritis. Inflamed joints may be warm, swollen, tender, often red, and painful or difficult to move. These physical signs of arthritis are due to inflammation of the lining of joints and tendons in a layer of tissue that is call synovium. The cells of the immune system within the synovium appear active and capable of causing tissue damage. If this inflammation persists or does not respond well to treatment,

destruction of nearby cartilage, bone, tendons, and ligament can follow. This leads to deformity and disability that can be permanent.

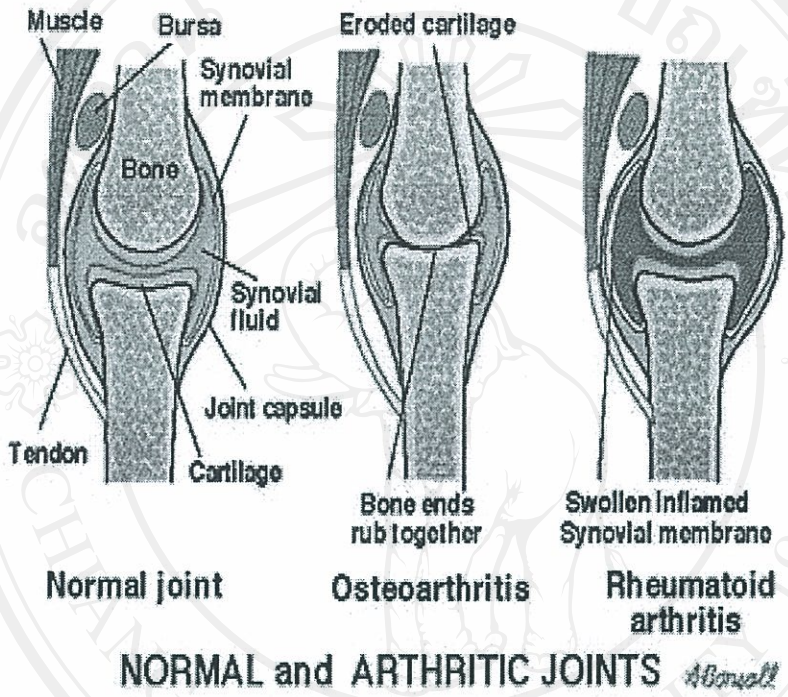


**Figure 10.** Joint tissues affected in osteoarthritis (OA) and rheumatoidarthritis (RA). In OA cartilage begins to degenerate by flaking or forming tiny crevasses. In advanced cases, there is a total loss of the cartilage cushion between the bones of the joints. Loss of cartilage cushion causes friction between the bones, leading to pain and limitation of joint mobility. Regarding RA, inflammation of the cartilage can also stimulate new bone outgrowths (spurs) to form around the joints.
(http://www.medicinenet.com/Osteoarthritis/article.htm)

### 1.2.9 Biochemical marker of cartilage metabolism

Biochemical or cartilage metabolism markers are increasingly used as helpful tools in the diagnosis and early detection of joint diseases. Their quantification may help to improve the ability of therapeutic monitoring and follow-up of patients affecting with rheumatic or degenerative cartilage disorder (29).

Under normal conditions, the balance between matrix catabolism and anabolism is well regulated, and the tissue integrity is maintained. However the pathological conditions, such as osteoarthritis (OA), rheumatoid arthritis (RA) or traumatic conditions changes in the tissue turnover occur and disturb the balance (30). Commonly, long-term consequences of these disease are large losses of proteoglycan and other matrix glycoproteins from the cartilage and severe disruption of macromolecular matrix interactions which eventually leads to the mechanical destruction of the tissue and complete removal of cartilage from joint surfaces. Destruction of the cartilage in joint disease involves several distinctive phases, the consequences of which have some potential for development of some diagnostic markers. For example, in the early phases of the development of OA, anabolic repair processes seem to occur prior to the later catabolic processes, which result in cartilage destruction. The early responses of this disease are largely due to changes in chondrocyte metabolism and not necessarily from external sources. Contrasting this, in inflammatory conditions such as RA or acute joint injury, lo of cartilage proteoglycans and other matrix macromolecules is an early event that usually precedes attempts to remodel and repair of the tissue. Degenerative enzymes from invading inflammatory cells or surrounding tissue such as the synovium are usually the initial source of catabolic enzyme that can degrade cartilage matrix in acute and inflammatory joint conditions. In addition, growth factors and cytokines which are released in the inflammatory response can induce metabolic changes in the cartilage chondrocytes which further compromises the tissue's integrity and its ability to function normally (31).

It is naive to believe that body fluids (especially, serum and urine) are a potential source joint disease markers. There are several intrinsic problems with body fluid markers. Serum or urine markers have the inherent problems that they contain systemic products resulting from connective tissue metabolism in all parts of the body, but some joint disease may involve different joints, each of which are different stage of diseases evolution at any time point. The alternative is

to use the synovial fluid to obtain a more direct readout of the process at one joint. But it is only regularly available from the knee joint, and the complexities of the variable clearance of markers from the synovial fluid, and the interaction between the blood and this body fluids, make it difficult to interpret findings (29;31) . Table 2 gives an overview of the most commonly used markers of cartilage metabolism.

### 1.2.10 Monoclonal antibodies directed against proteoglycan epitope

Immunological methods offer a number of advantages for the detection and quantification of all categories of markers of joint disease. They are more sensitive, more specific, large number of samples can be easily processed. They require small amount of sample or purification in order to avoid the effects of interference from other components already present in usually heterogeneous biological test samples. The use of monoclonal antibodies rather than polyclonal antibodies has several distinct advantages because the epitope specificity and biochemical characteristic of the antibody can be more easily to define.

Changes in chondrocyte metabolism produce compositional change in the newly synthesized proteoglycans and hyaluronans that occur in attempt to remodel or to repair the tissue response to altered mechanical environment. These subtle changes in cartilage biochemistry can be detected by antibody that recognize anabolic neoepitopes, a new epitope that is generated in a biological molecule as a result of either changes in anabolic or catabolic processes of metabolism, in these newly synthesized proteoglycans (32). Similarly, increases in proteoglycan degradation (catabolism) also occur in the early pathogenesis of OA During 1992-1995, Caterson and colleagues (33) have developed monoclonal antibodies that specifically detect newly formed N- or O-Terminal sequences (catabolic neoepitopes) on proteoglycan degradation products. In collaborative work with members of the Kenedy Institute of Rheumatology in London, Caterson and colleagues had described subtle biochemical differences in the chondroitin sulfate (CS) glycosaminoglycan chain of newly synthesized proteoglycan isolated from osteoarthritis cartilage. These differences were detected by two monoclonal antibodies; 3B3 and 7D4 (34).

Monoclonal antibody 3B3 recognizes its epitope, a non-reducing terminal unsaturated uronic acid residue adjacent to N-acetylgalactosamine-6-sulfate after the CS-chains have been digested with chondroitinase ABC. This epitope is denoted as 3B3(+) since one

requires that CS-proteoglycans be predigested with chondroitinase to generate its specific epitope. However, this antibody also recognizes a natural minotope, a biochemical structure that mimics the epitope recognized by a given antibody that occurs in CS chains isolated from osteoarthritic cartilage. When a mAb 3B3 is used without chondroitinase ABC pretreatment, immunoactivity with the native mimotope structure is designated 3B3(-) (33). The increase in 3B3 epitope expression on cartilage proteoglycans in experimental OA appears to reflect only increase the level of 3B3 expression, suggesting that it creates epitopes on many chains (35) .

### 1.2.11 Drug used in treatment of arthritis

The goals of arthritis therapy should be: (1) reduction of joint pain, (2) reduction of inflammation, (3) preservative of joint function, (4) prevention of disease progression, and (5) maintenance of lifestyle.

Pharmaceutical approaches are well known and consist largely of the use of nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. The principal pharmacological effect of aspirin and related NSAIDs is due to their ability to inhibit prostaglandin synthesis by blocking the cyclooxygenase activity of both COX-1 and COX-2. Their therapeutic effectiveness as analgesics, antipyretics, anti-inflammatories and anti-thrombogenics is due to their inhibition of prostanoid synthesis, which also accounts for their side effect profile. Toxic side effects of traditional NSAID's include stomach ulceration and/or bleeding, kidney damage, easy bruising because of loss of platelet function. Serious toxicity (leading to hospitalization or death) from NSAID use occurs for an estimated 100,000 to 200,000 people per year. Moreover, it is estimated that approximately 10,000 to 20,000 deaths per year are related to NSAID use (36). Problems associated with currently available therapies, along with the expanding knowledge of cartilage biochemistry and OA pathogenesis has focused research on slowering the progression of OA and promoting cartilage matrix synthesis. Recently, alternative treatments to relieve pain, symptom other than pain and less side effects than prescribed medications have been studied.

Hyaluronic acid (HA) is a glycosaminoglycan that is composed of glucuronic acid and N-acetylglucosamine. It differs from other glycosaminoglycans in that it is unsulfated; also, it does not bind covalently with proteins to form proteoglycan monomers, serving instead as the

backbone of proteoglycan aggregates. It is the only glycosaminoglycan that is not limited to animal tissues, being found also in bacteria. It serves as a lubricant and shock absorber in the synovial fluid, and is found in the vitreous humor of the eye. HA is not well absorbed orally, but has been widely used intraarticularly in the treatment of OA in animals and more recently, in humans (37) . Possible mechanisms by which HA may act therapeutically include 1) providing additional lubrication of the synovial membrane; 2) controlling permeability of the synovial membrane, thereby controlling effusions; and 3) directly blocking inflammation by scavenging free radicals. Other possible, though less certain, mechanisms include promotion of cartilage matrix synthesis and reaggregation of proteoglycans. HA is well tolerated with no demonstrable toxicity and few side effects (38).Because it is injected directly into the joint, its onset of action is rapid. Conversely, its route of administration does limit its therapeutic applications to some degree, and high cost is also a factor.

Glucosamine is an aminomonosaccharide that functions in the body as the precursor of the disaccharide unit in glycosaminoglycans. Normally, chondrocytes synthesize glucosamine from glucose. Supplying exogenous glucosamine provides the body with additional raw materials for matrix production. However, as a chondro-protective agent, glucosamine has a second function beyond its structural role. In contrast to HA, numerous *in vitro* studies have demonstrated that glucosamine stimulates the synthesis of proteoglycans and collagen by chondrocytes (39). Since OA results when cartilage breakdown exceeds the chondrocytes synthetic capacity, providing exogenous glucosamine increases matrix production and seems likely to alter the natural history of OA. Glucosamine also has a mild anti-inflammatory activity that is unrelated to prostaglandin metabolism, probably via a free-radical scavenging effect (40).

Chondroitin sulfate, as previously stated, is the most abundant glycosaminoglycan in articular cartilage. It is composed of repeating disaccharide units of glucuronic acid and galactosamine sulfate, and is a natural component of several tissues in the body in addition to cartilage, including tendon, bone, intervertebral disk, the cornea, and heart valve. As a glycosaminoglycan, chondroitin sulfate plays an important structural role in articular cartilage, notable for its role in binding with collagen fibrils. However, as a chondroprotective agent, it has a metabolic effect as well; its action is to competitively inhibit many of the degradative enzymes

that break down the cartilage matrix and synovial fluid in OA (41). An additional mechanism of action by which chondroitin sulfate may benefit joint tissues is via the prevention of fibrin clot in synovial or subchondral microvasculature. Platelets normally secrete chondroitin sulfate and other glycosaminoglycans (eg, heparin) as part of the body's normal control of thrombogenesis. With aging, chondroitin sulfate production by body cells decreases, to be replaced by less effective glycosaminoglycans like keratan sulfate, which predisposes to pathologic thrombus formation.

*Zingiber cassumunar* Roxb. is a medicinal plant belonging to the family Zingiberaceae. Its common names are Plai; Puu loi, Puu loei (Northern), Waan fai (Central), and Min-sa-laang (Maehongson). The rhizome part of the herb has a yellow to green color with fleshy thick texture containing multiple sessile tubers. Leaf stems 1 to 1.5 m tall. Leaves distichous, oblong-lanceolate, 20 to 30 cm long and 2 to 8 cm wide, pubescent below; ligule very short, bilobed, pubescent; sheath glabrous or hairy. Inflorescences scapose; peduncle 8 to 30 cm long, with pubescent sheaths. Spike ovoid-ellipsoid; bracts greenish red, narrowly obovate or rhomboid, 2.5 to 3.5 cm long; bracteole shorter than bract, ovate, 3-dentate. Calyx truncate, glabrous. Corolla tube ca. 2.5 cm long, pale yellow, dorsal lobe cymbiform, lateral lobe linear-lanceolate. Labellum pale yellow, suborbicular, apex emarginate, lateral lobe ovate-oblong, append-age slightly longer than anther; stamen pale yellow. Ovary inferior 3-celled. Fruit, small, globose capsule ca. 0.5-1 cm (Figureure 11). It requires fertile clay soil with sand, good drainage and moderate sunlight. It is cultivated only in tropical Asian countries. The odor is reported a strong and reminiscent of a mixture of ginger, camphor and turmeric, the taste as hot and camphoraceous. The rhizome part of the herb has a yellow to green color with fleshy thick texture containing multiple sessile tubers (42;43). Essential oil of *Plai* is steam distilled from the rhizome and has a pale amber color. The scent is a cool, green peppery one with a touch of a bite. Active chemicals in *Plai* are sabinene (27-34%), -terpinene (6-8%), -terpinene (4-5%), terpinen-4-ol (30-35%), and (E)-1-(3',4'- dimethoxyphenyl) butadiene (12-19%) (44).

The rhizome of *Zingiber cassumunar* Roxb. is widely used in Thai tradition medicine for topical treatment of sprains, contusions, joint inflammations, muscular pain, abscesses and similar inflammation-related disorders. The topical anti-inflammation activity of the five major components of the essential oil demonstrated that (E)-1-(3',4'-

dimethoxyphenyl) butadiene(DMPB), terpinen-4-ol and -pinene significantly inhibited edema formation, whereas sabinene and -terpinene were inactive up to 6 mg/paw. The most active compound, DMPBD, was found to be an anti-inflammatory agent twice as potent as the reference drug diclofenac ( =3 vs 6 mg/paw, respectively)(45;46). Interestingly, the five compounds isolated from hexane extract of the rhizome were all found to be equally or more potent anti-inflammatory agents than the reference drug diclofenac as shown in Table 4 (47).

*In vivo* preliminary study revealed the effect of DMPBD on both cyclooxygenase (COX) and lipoxygenase (LOX) in arachidonic acid (AA) metabolism pathways (4). Acute toxicity test of Plai revealed no evidence of toxicity in mice when given 10 g/kg body weight(48). The 50% alcohol extract administered per os or subcutaneous was more than 20 g/kg and intraperitoneal was 14.8 g/kg. Chronic toxicity test during 12 months in 192 Wister rats showed that Plai forced fed 3.0 g/kg/day male rats consumed less food than the control by 12%, therefore less body weight gained. Hematological examination also suggested all groups were normal (49;50). The detailed of preliminary studies revealed a new pharmacological agent for the treatment of inflammatory disorders.

A

B



**Figure 11.** Plai and its rhizomes. Plai is a perennial herb which has bright yellow underground rhizome.The rhizome of Plai is widely used in Thai tradition medicine for topical treatment of sprains, contusions, joint inflammations, muscular pain, abscesses and similar inflammation-related disorders.

A= http:// www.gpo.or.th/herbal/ phlai/phlai.htm

B=n http:// www.thaifitway.com/.../ n2db/question.asp?QID=22

**Table 4.** The five compounds isolated from hexane extract of the rhizome were all found to be equally or more potent anti-inflammatory agents than the reference drug diclofenac (47).

| Sample | $ID_{50}$ (μg/ear) |
|---|---|
| Hexane extract | 854 |
| (E)-4-(3',4'-Dimethoxyphenyl)but-3-enyl acetate (4) | 62 |
| cis-3-(3',4'-Dimethoxyphenyl)-4-[(E)-3'''',4''''-dimethoxystyryl]cyclohex-1-ene(5) | 21 |
| cis-3-(3',4'-Dimethoxyphenyl)-4-[(E)-2'''',4'''',5''''-trimethoxystyryl]cyclohex-1-ene (6) | 20 |
| cis-3-(2',4',5'-Trimethoxyphenyl)-4-[(E)-2'''',4'''',5''''-trimethoxystyryl]cyclohex-1-ene (7) | 2 |
| (E)-4-(3',4'-Dimethoxyphenyl)but-3-en-1-ol (8) | 47 |
| Diclofenac | 61 |

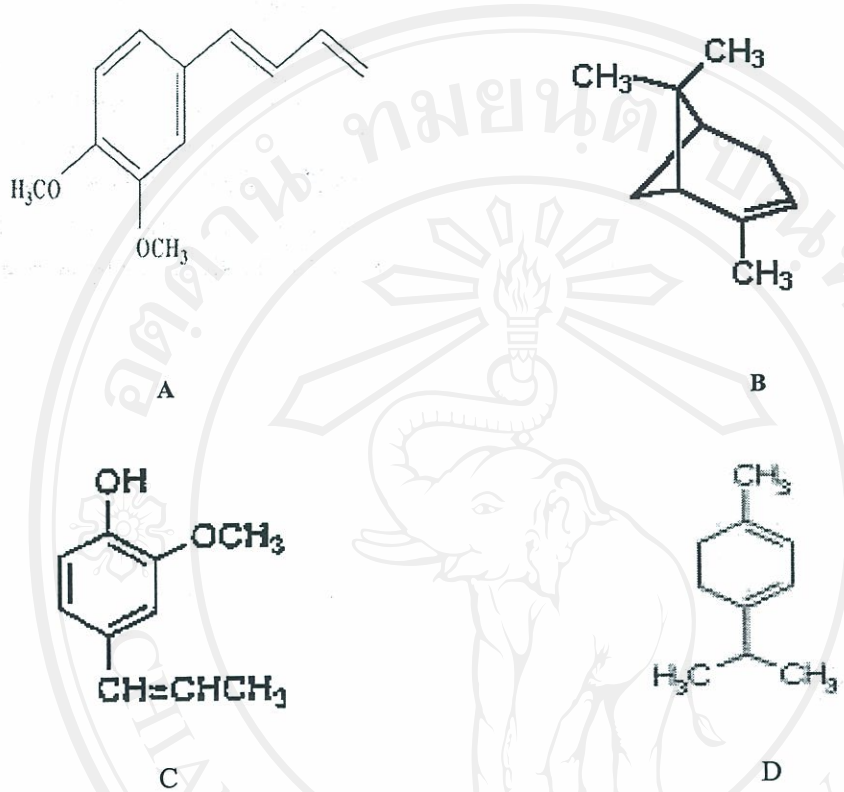


**Figure 12.** Structure of active compounds of Plai.

A= (E)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD) (4).

B= pinene(http:// www.uah.edu/colleges/science/chemistry/setzer/Monteverde%20Costa%20Rica.htm)

C= saninene (http:// www.fao.org/docrep/ v4084e/v4084e07.html)

D= terpinene (http://www.phys-chemie.uni-wuerzburg.de/kiefer/Web/ramandb/data/alpha_terpinene514.html)

### 1.2.12 Objective

To investigate the effect of *Zingiber cassumunar* (Roxb.) on cartilage degradation induced by interleukin-1β (IL-1β) or retinoic acid (RetA) and also analyzed the enzymatic activities of the matrix metalloproteinase (MMPs) in cultured media.