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Solid phase assays in glycoconjugate research: applications to the analysis of proteoglycans, glycosaminoglycans and metalloproteinases

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Abstract

Glycoconjugates are a class of macromolecules consisting of different constituents, one of which is sugar moieties. Glycoconjugates comprise the majority of tissue constituents, both intracellular and extracellular. Extracellular glycoconjugates (glycoproteins and proteoglycans) participate in a wide variety of interactions, through which they maintain tissue integrity. Therefore, their analysis or the study of their possible interactions would give evidence for the state of tissues. Since the amounts of some of the extracellular glycoconjugates are usually low or the amounts of tissue to be examined come from biopsies, specific analytical systems are developed for their study, the most familiar being solid phase assays, which have the advantages of analysis of multiple samples on the same time, cheap instrumentation and high specificity.

Keywords: ELISA; Zymography; Glycosaminoglycans; Proteoglycans; Enzymes; Systemic diseases; Cancer; Arthritis

1. Introduction

The term glycoconjugates describes a large family of biologic macromolecules composed of two moieties, an oligosaccharide and a protein (glycoprotein or proteoglycan) or lipid (glycosphingolipid). The oligosaccharide portion is covalently linked to the protein or lipid. The building blocks of the oligosaccharide portion of the glycoconjugate are monosaccharides. These include glucose, galactose, *N*-acetylglucosamine, etc. These compounds share a similar structure (i.e. polyalcohols), but can be distinguished in several ways. For example, some sugars are neutral (e.g. glucose), some other are charged (sialic acid), some have amino groups (hexosamines) and some are deoxy sugars. Most of the sugars are D-sugars, but there are some exceptions such as L-fucose. Nucleic acids, even they contain carbohydrate (ribose) residues, are not considered as glycoconjugates.

The oligosaccharide portion of the glycoconjugate is structurally complex due in part to the structural variations of its building blocks. This is similar to the situation of other macromolecules such as proteins,

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which derive structural diversity from the variation in the structure of amino acids that are used as building blocks. Thus, there are several different types of sugars that can be linked together. However, the oligosaccharides of glycoconjugates have additional structural features not found in other macromolecules. Sugars can be linked in an alpha or beta anomerity and the linkage can occur between different hydroxyls (e.g. 1, 3 or 1, 4). In addition, oligosaccharides can be branched (i.e. having multiple non-reducing terminal sugars). In fact, many glycoconjugates contain branched oligosaccharides.

It is important to look at a sugar or oligosaccharide in terms of its 3D structure. Most interactions between glycoconjugates and proteins occur via non-covalent interactions such as hydrogen bonds, van der Waals and non-polar interactions. Often the interactions between the oligosaccharides of a glycoconjugate and another protein involve hydrogen bonds between the R group of particular amino acids and so called key polar groups (often the hydroxyl groups) of individual sugars, plus hydrophobic interactions. Different proteins (e.g. different antibodies or lectins) can link to the same oligosaccharide by binding to different faces of the sugar rings and utilising different key polar groups. Oligosaccharides undergo dynamic motion in solution, but adopt a limited number of minimum energy conformations that are recognised by their binding proteins.

1.1. Glycoproteins

Many proteins on the outer surface of the plasma membrane and most secreted proteins carry covalently linked oligosaccharide residues, post-translationally added in the endoplasmic reticulum and Golgi complex. In contrast, cytoplasmic proteins are rarely glycosylated. Glycoproteins may contain more than 50% carbohydrate, but, in general, the protein fraction predominates. The carbohydrate part may be composed from one or more chains sized from one ore more sugar residues. Carbohydrates are linked to the protein via mainly two types of bonds (Fig. 1), although some others are observed to occur.

Glycosylation of proteins is a normal event, through which they exhibit their various characteristic properties, i.e., movement to a distinctive cell compartment, antigenicity of blood group substances, cell adhesion. However, many pathological conditions are characterised by abnormal glycosylation of specific proteins, such as haemoglobin, PrP, among others.

1.2. Glycolipids

They constitute a complex population found on the outer surface of the plasma membrane. The common constituent is sphingosine, an amino alcohol, which is linked with a fatty acid via its amino group and with a sugar or oligosaccharide chain to form a ceramide. Typical representatives include galactosylceramides and glucosylceramides. In some cases, the sugar moiety of a ceramide is esterified with sulphuric acid and referred as sulphatide. The most complex glycolipids are gangliosides. They constitute a large family of membrane lipids with receptor function. A characteristic component of the gangliosides is *N*-acetylneuraminic acid.

1.3. Glycosaminoglycans

They are a specific class of polysaccharides made up of repeating disaccharide units, which are routinely linked to glycoproteins to give whole proteoglycan structures. They exhibit a large variety of functions, such as, joint lubrication and shock absorption, cell adhesion, binding to cellular growth factors and extracellular proteases, signal transduction. Binding of specific proteoglycan molecules, such as aggrecan and versican, to hyaluronan provides the embryonic or the malignant tissues with the suitable milieu for cell proliferation and migration or differentiation. In addition, normal and malignant tissue remodelling advances through an activation of extracellular proteases made by the glycosaminoglycans.

Five different glycosaminoglycan structures are recognised (Fig. 2). Hyaluronan is the glycosaminoglycan of the simplest structure. It is composed by β-D-glucuronic acid (GlcA) and β-D-*N*-acetylglucosamine (GlcNAc) residues linked via β1 → 3 glycosidic bond. The disaccharides in the polymer are linked via β1 → 4 glycosidic bonds to form a polysaccharide of a molecular mass of more than 1000 kDa. It is found in almost all tissues in minor amounts and in relatively large amounts in vitreous body, synovial fluid, many embryonic tissues and tumour stroma [1]. It is a polysaccharide with high

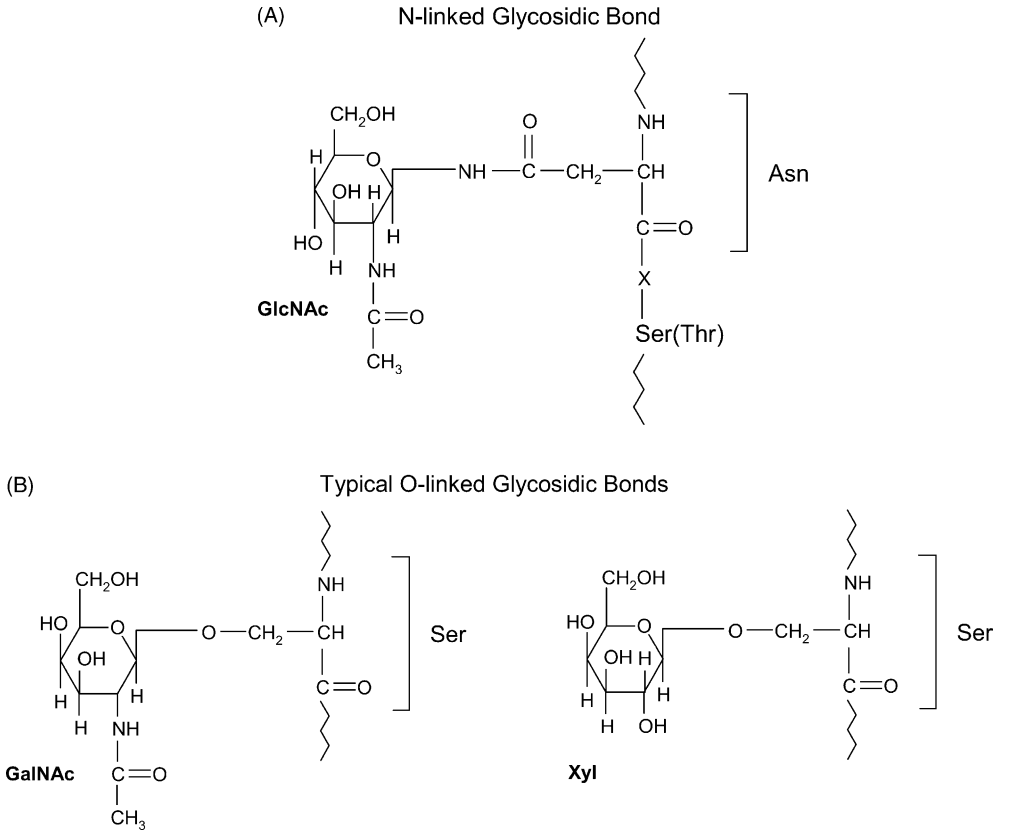

Fig. 1. The main types of glycosidic bonds occurred in proteins. (A) *N*-glycosidic bond, where *N*-acetyl-D-glucosamine is linked to asparagine of the protein. (B) Typical *O*-glycosidic bonds, where *N*-acetyl-D-galactosamine or D-xylose is linked to serine of the protein.

biologic significance and various properties. Its ability to maintain high amounts of water, more than one litre per gram hyaluronan, is mainly responsible for its special lubricating properties of synovial fluid and maintenance of the swelling pressure of the vitreous humour in the eye [1]. In embryonic and malignant tissues it is responsible for the hydration of the matrix, through which the cells proliferate and migrate [2]. Removal of the hyaluronan from the matrix leads the cells to differentiate. This is a normal phenomenon in embryonic tissues and has been proposed to be a therapeutic process in many cancers. Hyaluronic acid is mainly produced by fibroblasts and other specialised connective tissue cells and can be found as a free molecule in plasma and synovial fluid. In plasma, the half-life of the HA molecule has been estimated to be about 5–6 min [3,4]. HA is found in synovial fluid in high concentrations. Synovial HA may pass into plasma via the lymphatic system [5]. In circulation, HA levels ($\leq$100 ng/ml) are maintained by an efficient receptor-dependent removal mechanism present in sinusoidal endothelial cells of the liver and by the enzymatic action of hyaluronidase [6–11].

Serum HA levels can be elevated during synovial inflammation as seen in rheumatoid arthritis (RA), due to increased production and passage into circulation. Inflammation soluble factors (IL-1, TNFα) enhance synovial production of HA [12]. In RA patients, elevated serum HA levels (>100 ng/ml) have been reported and found to correlate with disease activity and degree of synovial involvement [13–20]. Elevated serum levels of HA have also been found in some patients with more advanced or active osteoarthritis (OA), progressive systemic sclerosis (PSS) and systemic lupus erythematosus (SLE), and are believed to result from growth factor activity in connective tissue cells and

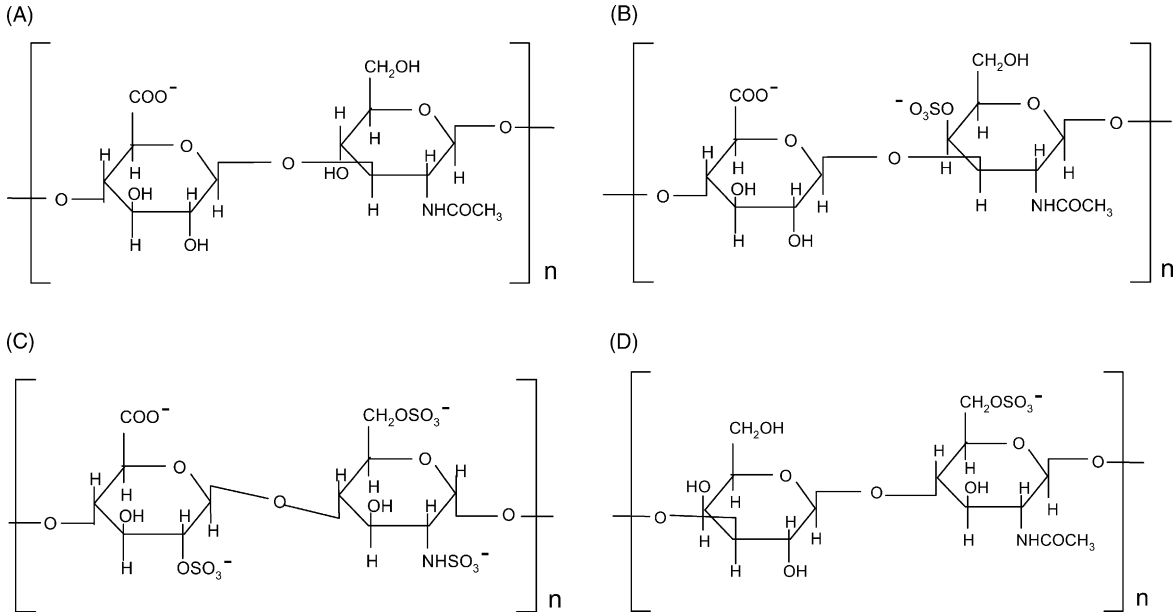

Fig. 2. Structure of the main vertebrate glycosaminoglycans: (A) hyaluronan, (B) chondroitin/dermatan sulphate, (C), heparan sulphate/heparin and (D) keratan sulphate. Dermatan sulphate and heparin are hybrid structures containing GlcA and IdoA. Hexosamine in heparin/heparan sulphate may be either *N*–acetylated or *N*–sulphonylated. The number of disaccharide units (n) may be from 5–6 (keratan sulphate) up to more than 1000 (hyaluronan).

synovial involvement [21–23]. The role of HA in disease activity or severity has been demonstrated in experimental animal models of arthritis [24,25].

Serum HA levels can also be found elevated in various liver diseases characterised by liver fibrosis and cirrhosis, which may decrease hepatic clearance and/or increase hepatic production of HA during liver inflammation [26]. Increased HA levels have shown better correlation with the degree of liver damage than conventional liver function tests including ALT/GOT, alkaline phosphatase and bilirubin [27]. It has been proposed that determination of serum HA levels may be useful in distinguishing cirrhotic from non-cirrhotic liver, for assessing the degree of liver fibrosis, and for monitoring liver function [28–31]. It has also been shown that HA levels reflect the extent of hepatic fibrosis in patients with chronic hepatitis C and may be useful in predicting the response to interferon alpha treatment [30]. Similar correlation has been found in patients with alcoholic cirrhosis [32] and primary biliary cirrhosis [29]. Increased amounts of HA are also found in the affected tissues/fluids in many malignant cases, especially in malignant mesothelioma [33–35].

Chondroitin sulphate (CS) and dermatan sulphate (DS) chains are constructed by the repeating unit [→ 4GlcAβ1 → 3GalNAcβ1 →], which is then sulphated at the C-4 or C-6 positions of GalNAc. Some, but little, of these positions remain unsulphated. In the case of DS, further enzymic modifications complete the final structure, such as C-5 epimerization of GlcA to IdoA, and *O*-sulphation at C-2 of IdoA. L-Iduronic acid imparts conformational flexibility to the dermatan sulphate chain. It further alters the shape and spatial orientation of sulphate residues, endowing the chain with higher negative charge content than the GlcA [36,37]. Although the principles of the biosynthetic process are not yet fully elucidated, it is well known that this process results in the generation of highly modified oligosaccharide domains within the polymer chain which are separated by regions of relatively low-degree structural modifications. Thus, the DS chain has a hybrid copolymeric structure consisting of low modified (CS) and highly modified (DS) domains [38]. The IdoA-containing units are often sulphated at C-4 of the GalN, while sulphation at C-6 is frequently associated with GlcA-containing

disaccharides [39]. Twenty-three different CS/DS disaccharides have been identified so far [39]. The detailed structure of this GAG is modified during certain diseases, such as all types of arthritis, atherosclerosis and cancer. The well-described structural modifications involve changes in ratios of IdoA to GlcA and of four-sulphated disaccharides to six- and non-sulphated disaccharides [39]. Changes in the size of chains are also described. Many of these alterations can be observed after analysis of CS/DS in urine of patients.

Heparin and HS have a distinctly different repeating disaccharide structure than the previous GAGs, that is [→ 4GlcAβ1 → 4GlcNAcα1 →]. The distinction of these GAGs is confused, due to their structural similarity, however heparin is more negatively charged than HS. They follow different biosynthetic steps in different cells and different core proteins. The glycosidic linkage between uronic acid and GlcN is β1 → 4 instead of β1 → 3, and that between GlcN and uronic acid is α1 → 4 instead of β1 → 4. The growing GAG polymer chain is *N*-deacetylated and *N*-sulphonylated at the glucosamine residues, yielding regions in the chain particularly available to further structural changes: C-5 epimerization of GlcA and *O*-sulphation mainly at C-2 of IdoA and C-6 of glucosamine [40]. Other more infrequent *O*-sulphations occur at C-2 of GlcA and C-3 of *N*-sulphonylated glucosamine, while a few of the glucosamine amino groups remain unsubstituted. This process yields hybrid structures with hypervariable, highly sulphated domains and poorly modified ones. Thirteen different disaccharide structures have been recognised. Heparin has the highest charge density of any known biological macromolecule, while HS is generally less sulphated and with lower IdoA content and, thus, presents greater structural variability [41,42]. Both GAGs are highly polydisperse molecules, depending on tissue origin and status [43–45]. Heparin exhibits diverse biologic functions, its halmark being the antithrombotic activity. HS participates in a large number of interactions with other effective extracellular and cell membrane molecules, such as growth factors, integrins and thrombin/antithrombin [46–50]. Therefore, the analysis of heparin/HS, which may identify structural variations due to differential expression of the corresponding PGs and/or of the enzymes responsible for their biosynthesis, may provide evidence for tissue and cell status. Such structural changes are observed in cancer and correspond mainly to size changes and degree of epimerisation and of *O*- and *N*-sulphonylation of glucosamine. HS of various sizes is observed in the urine of patients of many of the known mucopolysaccharidoses (MPS), due to deficiency in specific degradative enzymes.

The repeating disaccharide unit of KS [→ 3Galβ1 → 4GlcNAcβ1 →] contains a galactose residue instead of uronic acid and the glycosidic bonds are reversed, compared to those in HA and CS/DS. Sulphate esters are present at the C-6 of one or both of the component monosaccharides, but any other hydroxyl group may carry an esterified sulphate group [51]. This GAG is synthesised on two different linkage oligosaccharide precursors, the structure of which depends on the type of glycosidic linkage on the protein core. *N*-glycosidic linkage, usually found in the cell-bound glycoproteins, is characteristic of the corneal KS (KS I) and *O*-glycosidic linkage, usually found in mucins, a specific type of glycoproteins, is characteristic of the cartilage KS (KS II). Sialic acids attached to this O-linked oligosaccharide regulate the synthesis of KS; KS elongation can occur by glycosyl-transferases and sulphotransferases only when sialic acids are not present in the non-reducing terminal of the chain. Once the sialic acids are added, the elongation of KS chains is terminated. The molecular weight of KS is usually small, and it increases with ageing [52]. A third type of KS (KS III) has been isolated from brain tissue, which has an *O*-glycosidic linkage between mannose and serine or threonine [53,54]. The main tissue containing KS is cartilage and thus the determination of KS in serum, urine or synovial fluid is proposed as a marker for the measurement of cartilage damage in joint diseases [55–61].

1.4. Proteoglycans

Proteoglycans are a specialised family of glycoproteins composed of a core protein onto which variable number of glycosaminoglycan chains is attached, mainly through an *O*-glycosidic between serine or threonine and xylose or *N*-acetylgalactosamine bond (Fig. 1). However, a large number of exceptions have been proposed to occur, the most known is that of skeletal keratan sulphate, were the glycosaminoglycan is attached onto the core protein through

Table 1
Proteoglycan categories, their structures and functions

Category	Name	GAGs	Core protein (kDa)
Extracellular			
Large aggregating proteoglycans	Aggrecan	100 CS, 50 KS	220
	Brevican	1–3 CS	100
	Neurocan	3–7 CS	136
	Versican	13–30 CS/DS	265–370
Small and leucine-rich proteoglycans	Biglycan	2 CS/DS	40
	Decorin	1 CS/DS	40
	Epiphycan	2–3 CS/DS	35
	Fibromodulin	2–3 KS	42
	Keratocan	3–5 KS	38
	Lumican	3–4 KS	38
	Osteoglycin	2–3 KS	35
	PRELP	2–3 KS	44
Basement membrane PGs	Agrin	3 CS	250
	Bamacan	3 HS	138
	Perlecan	3 HS/CS	400–467
Cell membrane			
SLIPS	Syndecan 1	HS/CS	80
	Syndecan 2	HS	48
	Syndecan 3	HS	125
	Syndecan 4	HS	35
GRIPS	Glypican	1–4 HS	62
Assorted	Betaglycan	CS/HS	120
	CD44	1–3 CS/HS	25/39
	Thrombomodulin	1 CS	57
Intracellular	Serglycin	CS/DS/HS/HP	19

N-glycosidic linkage, common in many glycoproteins (Fig. 1). Proteoglycans are present in the extracellular matrix and the cell surface (Table 1). Proteoglycans in the extracellular matrix, due to their high negative charge, are responsible for the hydration of the tissues. In addition, they participate in a lot of interactions with other tissue or cell components and thus offer in tissue integrity. The most known and well-examined interaction is that of hyaluronan with aggrecan and link protein in cartilage [62–65]. Similarly, the interaction of versican with hyaluronan in embryonic tissues is responsible for tissue development and morphogenesis [66–68]. The interactions of proteoglycans with collagen, the main extracellular component of connective tissue, are responsible for the fine formation of collagen fibrils and finally the integrity of the tissue [69–75]. Proteoglycans, in addition, participate in specific interactions with cytokines and growth factors and thus regulate expression and biosynthesis of macromolecules [66,76–81]. Proteoglycans in cell surface interact with a variety of extracellular components mainly acting as their receptors [82–84] thus playing specific roles in biosynthetic events.

Proteoglycans alterations in their composition and structure are observed in many pathologic conditions, due either to altered biosynthesis or degradation. Among these, the substantial increases of both decorin and versican in gastrointestinal cancers are notable [66,85], and the alterations of syndecan in various cancers has been proposed to be a biochemical marker for the diagnosis and follow-up of the patients [86,87]. The alterations of the proteoglycans are detected in the affected tissue and rarely in body fluids. The most characteristic example is the detection of aggrecan degradation products in the sera and synovial fluid of patients of various types of arthritis and systemic diseases [56–59].

1.5. Enzymes

Enzymes are normally located intracellularly, where they participate in the various metabolic steps. In addition, under normal conditions, many enzymes are found in the extracellular matrix, the activity of which is altered according to the state of the tissues or diseases. The majority of these enzymes belong to the metalloproteinases (MMPs) family, but some others such as heparanases, hyaluronidases or aggrecanase also exist. The role of these catabolic enzymes is the degradation of extracellular macromolecules during the normal turnover of the tissues and, at least in the case of metalloproteinases, this is regulated by the concomitant presence of their tissue-specific inhibitors. In many diseases, increase of extracellular enzymatic activity is observed, and in many cases reflects the activity of a specific enzyme, together with no alteration or decrease of the respective tissue inhibitor.

MMPs comprise a large family of secreted or transmembrane enzymes that degrade extracellular matrix and they are categorised into five groups, based on their substrate specificities as collagenases, gelatinases, stromelysins, matrilysin, metalloelastases and the membrane-type MMP (Fig. 3). MMPs have been implicated in cancer metastasis, bone remodelling, embryogenesis, angiogenesis, various types of arthritis, periodontal disease, various pulmonary diseases and exfoliated syndrome [88–97]. Most of the MMPs are produced in the form of biologically inactive proenzymes and need to become activated by proteolytic cleavage (Fig. 3) or in vitro by treatment with various reagents as amino phenyl mercuric acid (APMA) or sodium lauryl sulphate (SDS). The activity of MMPs is inhibited by α_2-macroglobulin in serum or tissue inhibitors of metalloproteinases (TIMPs) in body fluids that are secreted by the same cells that secrete MMPs. The activity of MMPs is thus under multiple control through the processes of secretion, activation and inhibition. This means that there are various difficulties in estimating the activity of MMPs in crude samples, because they must be activated at the same time as inhibitors are eliminated.

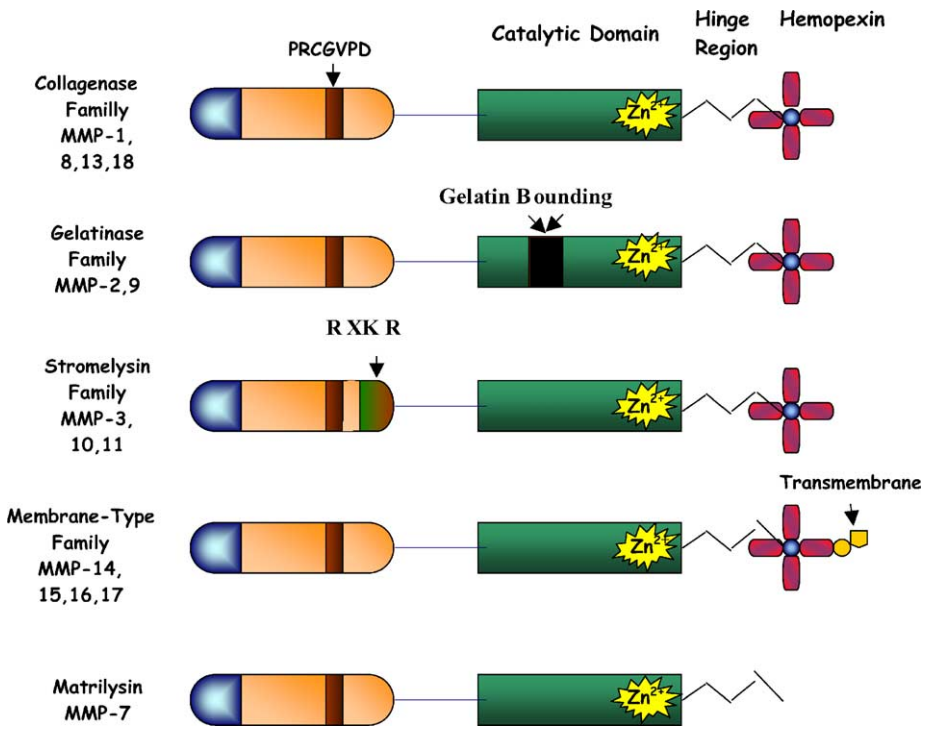

Fig. 3. The main matrix metalloproteinase categories. The structural domains are shown.

Hyaluronidase is an enzyme that cleaves the β(1 → 4)glycosidic bond between β-D-*N*-acetyloglycosamine and β-D-glucuronic acid of a hyaluronan molecule. It also cleaves the same bond between β-D-*N*-acetylogalactosamine and β-D-glucuronic acid of chondroitin sulphate. The minimum size of hyaluronidase substrate is a hexasaccharide and the major products are tetrasaccharides. Hyaluronidase is present mainly intracellularly or in the cellular membrane, but in many cases it is known that it acts extracellularly. Six hyaluronidase-like sequences have been recognised in the human genome [98] clustered in groups of three at two chromosomal sites. Two of these enzymes seem to be mainly responsible for the normal degradation of hyaluronan. High levels of hyaluronan are present normally in the joint capsule, in the vitreous of the eye, in Warton's jelly of the umbilical cord, in amniotic fluid and foetal tissues, and in all tissues undergoing rapid proliferation or repair. Enhanced levels of hyaluronan occur in inflammation, edema, the swelling following organ transplantation, stroke, or myocardial infarction, in sepsis, wound repair, and in carcinogenesis. Hyaluronidases and their attendant control mechanisms are apparently of critical importance in normal and abnormal biology. It is the modulation of catabolic turnover, the modulation of hyaluronidase activities that provides rapid response mechanisms for changing levels of hyaluronan, rather than the synthetic reactions. Hyaluronan has also been invoked as mechanisms for tumour invasion and metastatic spread and the levels of hyaluronan surrounding tumour cells often correlate with tumour aggressiveness and poor outcome [99]. Loss of hyaluronidase activity, permitting accumulation of hyaluronan, may be one of the several steps required by cells in the multi-step process of carcinogenesis. Hyaluronidases would then qualify as candidate tumour suppressor gene products [100,101].

Another recently described extracellular enzyme is aggrecanase. Two structurally related enzymes have been identified from bovine cartilage culture that are highly effective at cleaving aggrecan at the Glu 373/Ala 374 site [102,103]. These two enzymes represent a new family of metalloproteinases, referred to as ADAMTS (a disintegrin and metalloproteinase with a thrombospondin motif) enzymes and are collectively referred to as aggrecanases [102,103]. Aggrecanase activity has been suggested to be well correlated with cartilage destruction in arthritic diseases. Hence, identification of the enzymes responsible for this activity, and the identification of selective inhibitors of these enzymes, has been major goals of arthritis research.

2. Solid phase assays

The analysis of extracellular components may give significant information concerning the state of the tissue or organ and, subsequently the state of the donor. Direct analysis of tissue components is difficult to be applied in many cases, since the material contained in biopsy specimens or biologic fluids is limited. Therefore, the development of analytical techniques, requiring very small amounts of a specific macromolecule, and possessing specificity and accuracy, must be regarded as a prerequisite for the determination of any differentiation of tissue components concentration. In addition, and since the result of the analysis is best to be given in a few hours, any pre-treatment of the samples under analysis should be avoided or limited. For these reasons, analysis of macromolecules is better to be performed via immunologic assays. In addition, by employing monoclonal antibodies, specific structural alterations can be identified. An alternative to common ELISA techniques, in which the macromolecule under study or an antibody against it is immobilised, is the development of activated microplate wells to be used for the immobilization of molecules lacking hydrophobic nature. A lot of such activated microplates appeared commercially and groups, such as –COOH, $–NH_2$ among others are introduced. However, scientists prefer to use traditional methodology, i.e., microplate wells activated with streptavidine for the immobilization of biotinylated molecules or glutaraldehyde-coated wells for the huge immobilization of protein components. Activation with glutaraldehyde followed by covalent immobilization on it of spermine has been proposed for the introduction of strong positive charges onto the microplate wells and the plates can be used for the electrostatic immobilization of negatively charged macromolecules, such as glycosaminoglycans and proteoglycans [104].

In the case of enzymatic constituents of extracellular matrix, due to limited amounts of enzymes and the

concomitant presence of specific tissue inhibitors, additional to immunologic assays, the zymographic assays are also developed. This type of assays combines the electrophoretic separation of the enzyme(s) from any other protein(s) of the sample with the detection of catalytic activity on the same gel. The molecular mass of the active enzyme molecule can be easily defined by the comparison of the mobility of the enzyme with that of reference proteins [105]. This procedure is more valuable for the determination of the activity of enzymes that degrade high molecular weight macromolecules to a small extent or to products unidentified by known analytical methods.

3. Immunologic assays

3.1. Analytical procedures

ELISA-based procedures have widely been applied for the analysis of the extracellular components. These assays are mainly of competitive nature, although monoclonal antibodies have been prepared for most of the macromolecules. The assays usually start from the direct immobilization of the macromolecule under analysis, although indirect immobilization is also applied [104,106–108], followed by their detection using two antibodies system. In many cases and to increase the sensitivity of the proposed assays, labelling of the antibodies with specific reagents is applied.

3.2. Glycosaminoglycans

3.2.1. Hyaluronan

Because of the significance of hyaluronan in many human diseases, a lot of analytical methods, such as HPLC [109–112], HPCE [113–116] and various electrophoretic assays, the most recent being FACE [117–120] have been applied for its quantitation. In addition, some indirect ELISA procedures have also been developed for hyaluronan quantitation, since it is a non-immunogenic glycosaminoglycan, although the production of antibodies against it has been reported [121,122]. Hyaluronan immobilisation onto the microplate wells varies according to the method [123–126]. All these ELISA procedures are based on the ability of hyaluronan to interact with a variety of proteoglycans/proteins, which are finally detected immunochemically or, when the proteoglycans/proteins are labelled with biotin, their detection follows after interaction with streptavidine–enzyme conjugates [123,127,128] (Table 2). Another attempt to detect immobilised hyaluronan uses peroxidase-labelled hyaluronan-binding protein [129,130]. The strength of the interactions between hyaluronan and proteoglycans/proteins depends on the size of hyaluronan and therefore, this type of procedures overestimates small sized hyaluronan [131]. However, this overestimation does not seem to be very critical for the commercial application of ELISA procedures to analyse hyaluronan [130]. Overestimation of small size hyaluronan is the only one disadvantage of ELISA procedures and their ability to analyse a large number of samples accurately and with great sensitivity makes them preferable, when compared with other assays. In addition, the detection of hyaluronan using ELISA requires a simple pre-treatment of the sample with a proteolytic enzyme, contrary to other assays, which require an additional digestion step with chondroitinases. The sensitivity of the various ELISA procedures for the quantitation of hyaluronan depends on the detection system used, which in most of the cases is down to the picogram level.

3.2.2. Chondroitin sulphate

Intact chondroitin sulphate is also a non-immunogenic glycosaminoglycan, however the chondroitin sulphate stubs linked to the core protein of aggrecan have been used for the preparation of monoclonal antibodies, which are able to detect specific structures within the chain [132–134]. Most of these antibodies appeared commercially and have been widely used for the characterisation of chondroitin sulphate chains under normal and pathologic conditions [135–140]. The analysis of chondroitin sulphate is usually performed via chromogenic reactions for its simple quantitation or via HPLC and HPCE assays [141–145], when the relative concentration of constituents disaccharides is required. ELISA procedures have also been developed (Table 2) for the analysis of specific disaccharides structures of chondroitin sulphate, and they have almost entirely been applied for the quantitation of structural alterations observed in cartilage, synovial fluid or serum of patients with various joint diseases [146–152]. However, HPLC and HPCE procedures exceed ELISA for the analysis

Table 2
Preferable solid phase assays for the analysis of extracellular glycoconjugates

Extracellular glycoconjugate	Immunologic assay	ELISA-based solid phase assay
Glycosaminoglycans		
Hyaluronan	Interaction with a hyaluronan-binding protein that is finally detected	Interaction with a biotin- or peroxidase-labelled hyaluronan-binding protein
Chondroitin sulphate	Direct detection with specific MAb. Structural alterations usually give confusing results	Competitive assay using biotinylated chondroitin sulphate as reference compound. Strongly negatively charged glycosaminoglycans compete and must be specifically degraded
Keratan sulphate	Direct detection with specific MAb	–
Heparan sulphate	–	Similarly as chondroitin sulphate
Proteoglycans		
Aggrecan	All proteoglycans can be directly detected with specific MAb	–
Versican	Purification is not a prerequisite	–
Decorin	False results may be obtained when structural alterations occurred	–
Syndecan		–

of chondroitin sulphate, due to the complexity of its structure.

3.2.3. *Keratan sulphate*

Keratan sulphate appeared to be an immunogenic glycosaminoglycan and monoclonal antibodies against it have been prepared and characterised [153–158]. The antibodies react with specific keratan sulphate structures due to their size and/or sulphation pattern. Most of these antibodies appeared commercially and have been widely used for the quantitative analysis of keratan sulphate (Table 2), especially in serum and synovial fluid of patients with joint diseases [55–61,159–163]. Since a well-documented alternative procedure, possessing the sensitivity and specificity of ELISA, for the quantitation of keratan sulphate does not appear in literature, ELISA is the only one procedure for its quantitation and hence the detection of cartilage destruction.

3.3. *Proteoglycans*

Antibodies against proteoglycans have been produced from the early 1960s, and they have been used in structural comparison and/or characterisation studies. The development of monoclonal antibodies and the establishment of ELISA at the onset of 1980s make easier the detection of structural differences of proteoglycans and hence the detection of new proteoglycan populations which are present in minor amounts in tissues and body fluids. In addition, the sensitivity and specificity of the immunologic assays facilitated the detection of structural alterations observed in various diseases. Since proteoglycans are complex macromolecules, the antibodies produced against them have the ability to recognise epitopes in either the core protein or the glycosaminoglycan chains (see above). Both types of antibodies have been widely used but especially in immunohistochemical studies for the qualitative and/or quantitative analysis of structural variations. Results from these studies suggest that they can be used for diagnostic purposes in various types of cancer [86,87,164–168]. Similarly, competitive ELISA procedures are developed for quantitative purposes, which are based either on the core protein [169–172] or the glycosaminoglycan chains [132–140,146–152] but the complexity of proteoglycan structure does not allow the wide application of ELISA methodology for the quantitative analysis of these macromolecules. However, in the case of the quantitation of the proteoglycan core protein, the competitive ELISA offers the specificity and sensitivity required, and it can be applied to samples directly or after a brief purification step (Table 2).

3.4. Enzymes

3.4.1. Metalloproteinases

The catalytic activity of MMPs can be detected by a variety of assays, including in vitro incubation with radioactive collagen, reverse phase high-performance liquid chromatography (RP-HPLC), ELISA and zymography. In in vitro incubation experiments, MMP solubilizes collagen partly and the soluble products are obtained in the supernatant after trichloroacetic acid precipitation and counted. In RP-HPLC, the procedure starts with the reaction between the MMP and a synthetic polypeptide substrate, which varies according to the detecting enzyme. MMP hydrolyses a specific bond of the substrate, so a new polypeptide with different retention time from the initial substrate is produced. The samples are then subjected to RP-HPLC and their absorbance is consecutively measured, from which the amount of enzymatic units is obtained. The high cost procedure can accommodate only one sample each time. The assay time is about 30 min and requires a lot of attention and consecutive surveillance. Additionally, it can identify only one form of MMP per sample, denatured or not.

A common solid phase assay for the identification and quantification of MMPs is the commercially available ELISA kits for MMPs. The analytical sensitivity of the kits is comparable to zymography (see below) and the assay time varies from 2 to 4 h [173]. One disadvantage of ELISA kits is the extravagant cost of the 96-well plates, which can actually accommodate 48 samples per plate, including controls, since the high accuracy of the detection is based on the average absorbance of the doubled accommodated samples. In addition, in most of the cases, this type of procedures cannot distinguish between the various forms of each one MMP and they are usually used for the detection of pro-MMPs (Table 3).

3.4.2. Aggrecanase

The activity of this enzyme is difficult to be identified in biologic samples, due to the concomitant presence of MMPs. A major advance in aggrecanase assays has been the development of an antibody, referred to as BC-3 that selectively recognises the N-terminal neoepitope $^{+}$NH3-Ala-Arg-Gly-Ser-Val, produced by aggrecanase-mediated proteolysis of aggrecan [174]. However, BC-3-based Western blot assays using full-length aggrecan as substrate are cumbersome, difficult to quantify, and thus difficult to use for routine tasks, such as screening column fractions for the presence of aggrecanase or screening compound libraries for potential inhibitors of the enzyme. A simple assay of aggrecanase activity using a peptide containing 41 amino acids as a specific substrate for these enzymes has been developed (Table 3). The biotinylated peptide was immobilised onto streptavidin-coated microplate wells and the biologic samples to be analysed were added to the wells, incubated, and the production of neoepitopes was quantitated immunochemically [175]. The assay is not affected by the other known proteases and in addition it can be used for the identification of selective inhibitors of aggrecanase [176].

3.5. Solid phase assays to study biological interactions

3.5.1. Collagen and proteoglycans/proteins

The interactions of collagens with proteoglycans (aggrecan, biglycan, decorin, fibromodulin) have mainly been shown using histochemical and

Table 3
Disadvantages of the various solid phase assays for the analysis of extracellular enzymes

Extracellular enzyme	Immunologic assays	ELISA-based solid phase assay	Zymography
Metalloproteinases	Detection mainly of the latent enzyme. Confusion between latent and activated forms	Detection of the activated forms of the enzyme. Latent enzymes or enzymes complexed with TIMPs need activation	Long time of incubation
Aggrecanase	The only one proposed detects the final product of the enzyme	–	–
Hyaluronidase	–	Confusion between enzymatic forms. Decreased values in the presence of inhibitor(s)	Long time of incubation and staining

immunohistochemical methods. The difficulty of these methods is derived mainly of the preparation of the sample to be used in such studies. Decalcification alone is not sufficient, because connective tissues contain additionally a lot of aggregated molecules. On the other hand, indirect interactions between two different macromolecules with the participation of a possible third, of very small size, cannot be excluded, and it is very difficult to distinguish the direct and the indirect interactions by using such methods. The best way to perform these studies is to have highly purified molecules and to mix them and to measure one of the physicochemical parameters, i.e., molecular weight, that will change. However, this is not the case of collagen, because of its insolubility in conventional buffers. The scientists have used exactly this property of collagen to perform studies in solid phase assays.

Various types of solid phase assay to study the interactions of collagen with other molecules have been established. The majority of them exploits the insolubility of collagen to adsorb it onto ELISA plate wells. Collagen, in 0.1 M acetic acid, is mixed with the suitable buffer and put onto the plate wells. Fibrillogenesis starts rapidly and after about 4 h the entirety of the collagen is under the form of fibrils. The macromolecule under study for its ability to interact with collagen is then added and its quantity is measured immunoenzymatically. Using this method, the interaction of various types of collagen with aggrecan, biglycan, decorin, the respective core proteins, link protein, fibromodulin are performed, and the dissociation constant of the interactions is determined [75,177–179]. The assay is very simple, rapid and reproducible. It can be performed using whatever macromolecule, native or biotinylated.

3.5.2. Proteins and proteoglycans/glycosaminoglycans

The interactions between the extracellular macromolecules are critical in tissue organisation and cell behaviour. The identification of the possible interactions in which a macromolecule participates may be performed via a variety of assays, ELISA being the simplest. ELISA is used for the examination of the interactions of proteoglycans with growth factors [180–184] and with other extracellular, cell surface or intracellular macromolecules [185–191]. The specific interaction of heparan sulphate proteoglycans with lipoprotein lipase can also be studied with ELISA and in addition the same technique can be applied for the quantitation of heparan sulphate proteoglycans [192,193]. In all the above assays, the first of the macromolecules under study is directly immobilised onto the microplate wells and then left to react with the second one, which is detected immunochemically.

A different approach of the ELISA techniques usually applied has been proposed for the study of specific interactions between hyaluronan and proteoglycans/proteins. In microplate wells activated with protamine, hyaluronan is immobilised and then the molecule under study is added and identified immunochemically [106].

Another different approach of the ELISA methodology for the study of the specific interactions of proteoglycans/glycosaminoglycans and proteins starts from the electrostatic immobilization of the negatively charged macromolecules in activated wells, addition of the protein, the interactions of which are studied and detection of the protein immunochemically. The assay was applied for cartilage link protein [104] and normal prion protein (Triantaphyllidou et al., manuscript in preparation).

3.6. Identification of autoantibodies

ELISA is an immunological technique and thus its application for the examination of autoantibodies is obvious, although the titre of the autoantibodies is usually low and/or cross-reactivity of the autoantibodies is observed. In most of the cases, and especially in systemic diseases, autoantibodies against specific macromolecules are produced. Among these, the most characteristic and referring to extracellular components are the autoantibodies against: (a) the optic nerve head glycosaminoglycan in patients with glaucoma and endocrine ophthalmopathy [194,195], (b) heparan sulphate proteoglycan in systemic lupus erythematosus [196,197], (c) aggrecan present in various joint diseases and systemic diseases [198–200], (d) matrilin 1, type II collagen and COMP in patients with relapsing polychondritis [201], and (e) chondroitin sulphate C in neurological diseases [202].

4. ELISA-based solid phase assays

In the case of some of the extracellular macromolecules, which are not immunogenic, their labelling with specific reagents was proposed for their identification in small amounts. Among the labelling reagents, biotin is the commonest and simplest, offering high sensitivity and specificity to the detection system and has widely been used in solid phase assays. Biotin has also been applied to label macromolecular substrates to be used for the quantitation of extracellular enzymes.

4.1. Analytical procedures

4.1.1. Glycosaminoglycans

The quantitative analysis of glycosaminoglycans may be performed through a variety of assays (see above). Simple quantitative analysis may also be performed in microplate wells activated to contain positive groups onto which they are immobilised electrostatically together with reference biotinylated chondroitin sulphate. The extent of the competition of the immobilisation of reference compound, which is identified with avidine-peroxidase, gives the concentration of the unknown glycosaminoglycan [203]. Since the competition is due to the negative charge of the glycosaminoglycan, specific enzymatic treatment is required for the quantitation of the various glycosaminoglycans in a sample [204]. The procedure is simple and uses limited amount of the sample, however, it can be applied for the quantitation of the whole glycosaminoglycan and not for its individual disaccharides.

4.2. Enzymes

4.2.1. Metalloproteinases

Gelatinase and collagenase activity can be determined by using biotinylated collagen. There are commercially available kits for this determination. According to the procedures usually applied [173], the substrate is mixed with the sample under analysis and incubated. The solubilized biotinylated collagen or gelatin peptides are transferred to biotin-binding microplate wells and quantitated using streptavidine-peroxidase conjugates. The procedures are quite simple, require small volumes of sample and can be used for the determination of either the active form of the enzymes or total enzyme after activation with APMA. The sensitivity of the assays is down to 5 ng/ml. The same assay can also be applied for inhibitor screening. The disadvantages of this type of assays is the extravagant cost of the 96-well plates, compared with zymography, and their inability to identify the type of collagenase or gelatinase present in the sample.

The presence of specific enzyme(s) in a tissue specimen can also be detected by analysing its RNA content after amplification and specific solid phase assays are established and commercialised [173]. These procedures cannot quantitate the enzymatic activity(ies), however, they are useful in identifying the type of the enzymes present in a biopsy sample.

4.2.2. Hyaluronidase

Hyaluronidase was detected and its activity expressed in quantitative terms using common enzymatic assays, i.e., by mixing the enzyme with the polysaccharide substrate, incubating and measuring the decrease of the turbidity of the solution [205] or by analysing the reducing ends produced by the action of the enzyme [206]. The method is very simple, however, due to the variations in the size of the products, it is of decreased accuracy. In addition, the possible presence of hyaluronidase inhibitors in the samples makes the results questionable. A different approach of this assay, but using a solid phase assay is presented in the last decade. The substrate (hyaluronan) is first conjugated with biotin using its carboxylated groups (Fig. 4). Care is taken to have a sparse substitution of

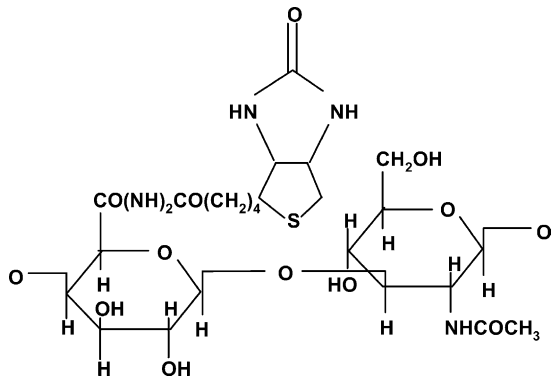

Fig. 4. Chemical structure of biotinylated hyaluronan disaccharide.

biotin in the hyaluronan chain (one molecule of biotin per 100 disaccharide units of hyaluronan) in order to be susceptible by hyaluronidase and to have enough free carboxylate groups to be covalently bound to Covalink-NH microplate wells. After the covalent immobilization of hyaluronan, the samples diluted in the appropriate buffer are added and incubated with it. Hyaluronidase present in the samples degrades hyaluronan, which is removed from the microplate by subsequent washings. Then, avidin-peroxidase conjugates are added to introduce a very sensitive enzyme in sites where biotinylated hyaluronan is not removed. Finally, *O*-phenylenediamine is added together with peroxide and the colour obtained is measured to give the units of hyaluronidase present in the sample [207].

The microplate assay is still on researching stage; in any event, it will detect neither all isoforms of hyaluronidase at the same time nor the simultaneous activity of free and inhibitor-complexed enzyme.

4.3. Study of interactions

An alternative procedure with high sensitivity is proposed for detection of the carbohydrate-binding activity of proteins [208]. The method is based on interactions of carbohydrate-binding proteins, immobilised on a solid phase, with an enzyme-labelled soluble polysaccharide (peroxidase conjugated glycosaminoglycans—heparin, chondroitin sulphate or hyaluronic acid). Binding capacity is measured spectrophotometrically after enzymatic reaction with chromogenic substrate. The assay is believed to be applicable for the identification and characterisation of a variety of carbohydrate(glycosaminoglycan)-binding proteins, especially, when traditional methods can not be applied (e.g., when proteins are water-insoluble). However, the method has not been extensively applied.

5. Zymography

5.1. Metalloproteinases

The most usable type of zymography during the last years has the substrate copolymerised or entrapped within the polyacrylamide network (substrate electrophoresis). After the electrophoretic run, the gel is incubated in the appropriate buffer, and after the subsequent staining, the enzyme activity is recovered as white band(s) in a stained background. This procedure has been extensively used for the characterisation of all metalloproteinases (MMPs) and hyaluronidase, in either native or denaturating polyacrylamide gels, with a detection limit of less than 5 ng of the enzyme [88–97,105]. Enzymes, such as β-glucuronidase, acid or alkaline phosphatase among others, acting to a small substrate and split it in well-defined site(s), have been characterised after their electrophoretic separation from extra proteins in non-denaturating polyacrylamide gels and incubation of the gels in a solution of a chromogenic substrate, producing insoluble chromogen at the migation site(s) of active enzymic band(s).

Zymography with collagen as substrate is considered as a practical method for identification of serum MMPs. The substrate is copolymerised with acrylamide, the (crude or not) samples are subjected to electrophoretic separation and the gel is incubated in the appropriate buffer for a fixed time. Then the gel is stained and MMPs appear as white bands in a stained background (Fig. 5).

Zymography with gelatin as substrate overcomes these difficulties and therefore is considered the best method for detection of gelatinases, with proteins in

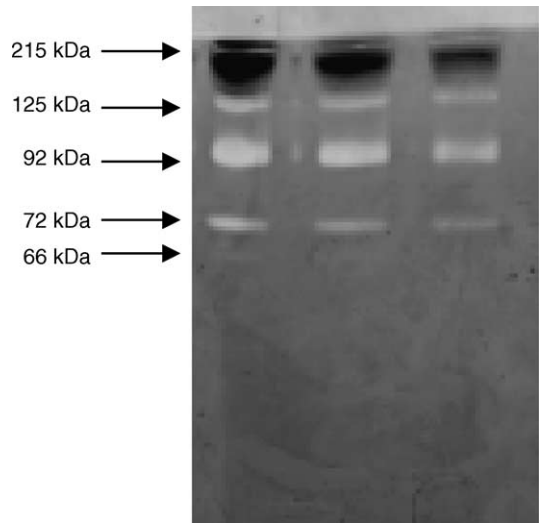

Fig. 5. Zymography of serum MMPs. One microliter of normal serum was diluted to 20 μl with water and 7.5, 5 and 2.5 μl (lanes 1–3, respectively) were subjected to zymography (Ziouti and Vynios, unpublished data).

the samples of them (Fig. 5). After electrophoresis, the TIMPs and the molecular species of gelatinases are migrated according to their molecular sizes. The inactive forms of gelatinases, zymogens, are activated by SDS in the process, and after renaturating with Triton X-100 or Brij-35, all of the molecular species of gelatinases can be easily detected without further treatment. The results can be expressed in a quantitative way after scanning of the gel.

The sensitivity of zymography reaches down to 1 pg of protein with a required volume of only 1–10 μl. This procedure can accommodate many samples per gel, depending to its size, with a short time preparation of 30 min. It may require long-lasting incubations, but observation is superfluous. Additionally, it can detect all possible active forms of MMPs, even as inhibitor-complexed MMPs, with α2-microglobulin or TIMPs.

5.2. Hyaluronidase

According to the zymography procedure, the substrate, hyaluronan (HA), is entrapped within the acrylamide network, the samples are subjected to electrophoretic separation and the gel is incubated in the suitable buffer for the appropriate time. Then the gel is double stained with Alcian blue, followed by Coomassie blue that enhances colour formation and thus sensitivity [209]. The HA-substrate gel procedures have the advantage that hyaluronidase and its inhibitors are separated, making thus possible to detect enzyme activity in crude biological preparations that would not otherwise be possible. A more recent modification is the incorporation of additional glycosaminoglycans (GAGs), such as chondroitin sulphate, into the polyacrylamide gels. This low molecular weight GAG is chemically modified so that it becomes immobilised in the gel, making it possible to examine substrate specificity of individual hyaluronidases [210,211].

The advantages and disadvantages of the zymography method for the detection of hyaluronidase are the same with that for MMPs, as far as it concerns the same experimental procedure. Moreover, this assay may determine the isoforms of hyaluronidase and the variety of their products, as much as the enzyme's catalytic behaviour at a range area of pH values. With an appropriate processing of hyaluronidase, inhibitors are removed, so the activity of the inhibitor-free enzyme can be analysed. Another advantage of zymography is the use of crude serum samples; since the isolation and purification of the enzyme from serum samples is arduous, because of its small concentration. Zymography of hyaluronidase permits, in addition, the study of its possible inhibitors of protein nature. In this case, which is termed reverse zymography, the sample under analysis is electrophoresed in a hyaluronan-containing polyacrylamide gel, which is incubated in the presence of hyaluronidase in a suitable buffer. After incubation, the gel is stained as described and the hyaluronidase inhibitors are detected as blue bands in a light background, since degraded hyaluronan is removed from the gel during incubation and staining process.

6. Concluding remarks

ELISA methodology, in general, provides simple, sensitive, specific and accurate assays for the detection and quantitation of macromolecules and the study of their interactions. In the case of extracellular glycoconjugates, a lot of assays has been established and commercialised. They exceed other techniques in specificity and simplicity and by the application of specific detection systems increased sensitivity is obtained and thus they may substitute many other assays applied in the analytical laboratory. These assays are still used for research only, but they may also be applied for diagnostic purposes, since the alterations of extracellular macromolecules reflect the state of the tissues. The complexity of the extracellular macromolecules and the rather limited knowledge of their alterations in tissues of patients do not allow now the application of the assays in clinical diagnosis. Additional studies are required to recover the key extracellular macromolecular altered in a given disease and the optimum tissue specimen or biologic fluid for its analysis.

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