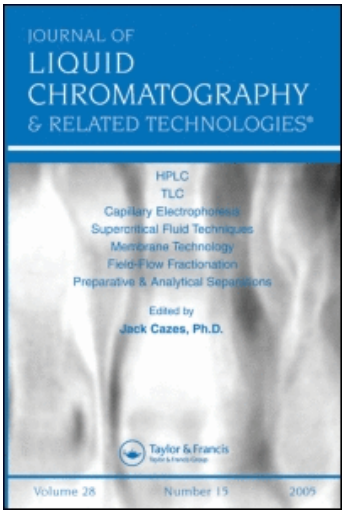

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MICROSCALE DETERMINATIONS USING SOLID PHASE ASSAYS: APPLICATIONS TO BIOCHEMICAL, CLINICAL AND BIOTECHNOLOGICAL SECTORS. A REVIEW

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MICROSCALE DETERMINATIONS USING SOLID PHASE ASSAYS: APPLICATIONS TO BIOCHEMICAL, CLINICAL AND BIOTECHNOLOGICAL SECTORS. A REVIEW

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ABSTRACT

The assays that have one of the reactant species immobilized onto a solid support are described as solid phase assays. During the last 20 years a large number of such assays has been developed, the majority of which are quantitative analytical methods known under the general term ELISA (Enzyme Linked ImmunoSorbent Assay). Solid phase assays, in general, have widely been used in Biochemistry, Clinical Chemistry, and Biotechnology, mainly for analytical purposes, and for the detection of specific macromolecules or the study of interactions between various molecules, as well.

INTRODUCTION

The term "solid phase assay" is used to define those types of assays in which one of the reactant species is immobilized onto a solid support. The support may be plastic (nylon, PVDF, but usually polystyrene), silica (SiO_2), alumina (Al_2O_3), etc.

For the last 20 years, polystyrene has been used in a wide range of solid phase assays, since this type of plastic is transparent and can be easily transformed to possess active sites. It has an additional advantage that it can be formed in various shapes, the most familiar being that of microtitre plate (ELISA plate), through which the results can be presented in a quantitative manner.

The use of microtitre plates in the laboratory provides a very useful tool in various studies of either qualitative or quantitative purposes. Microtitre plates have been initially used for the analysis of biologically active peptides, as an alternative to their quantitation by radioimmunochemical or chromatographic techniques, since these methods take a long time and are very dangerous for the analyst. After the onset of AIDS, where research had been directed to the development of assays that detect antibodies against the various forms of HIV, more scientists became familiar with ELISA. At the same time, scientists working with cancer used ELISA to establish tumor markers and to develop methods for the detection, the diagnosis of cancer, and the follow-up of patients after treatment. Scientists working with autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, have also tried to use ELISA to detect and quantitate autoantibodies in human serum in normal and pathological conditions. Scientists studying the various infectious diseases have used similar techniques to establish methods for the quantitation of antigens responsible for the diseases or the determination of antibodies produced.

The aim of all of these scientists is the same, i.e., the establishment of simple assays to answer in their respective question. The term simple is very comprehensive here and refers to the type of the sample (serum, plasma, or urine are the most easily obtained human samples), the quantity of the sample, the quality of the sample (heparinised serum, hemolysed serum, lipaemic serum, acidic or alkaline urine, e.t.c.), the convenience of the method, the frequency of the use of the assay, the fixed costs of the analyses, and the consumable.

ELISA has all the advantages required to be the technique in use to answer all these questions. ELISA, however, as initially described, is an enzyme linked immunosorbed assay. The way through which an immunosorption is obtained is mainly a practical and not a theoretical problem. Depending on the molecule to be analyzed, antigen or antibodies against a specific antigen, two different types of ELISA are well established.

According to the first type (Figure 1A), an antibody (usually monoclonal) is adsorbed onto the polystyrene microtitre plate wells, then the (human) sample, together with another monoclonal antibody conjugated with an enzyme, raised against a different epitope is added and left to interact with the adsorbed substance. Finally, peroxide and a specific color producing reagent are added; the latter being colored by the enzymic action on peroxide.

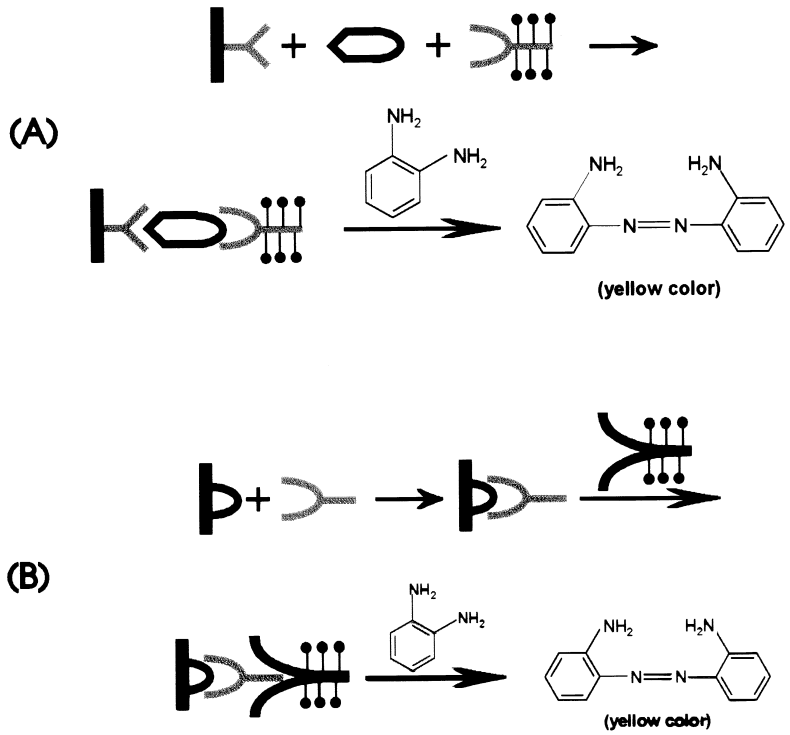

Figure 1. Schematic representation of the typical ELISA procedures. A. The antibody is immobilized: In this case a monoclonal antibody is immobilized, then reacted directly with the antigen and with another monoclonal antibody, conjugated with peroxidase, against a different region of the antigen. At the end, o-phenylenediamine and hydrogen peroxide are added and color is produced. B. The antigen is immobilized: In this case, after the immobilization of the antigen, an antibody (usually polyclonal) is added to react, followed by the second antibody, conjugated with peroxidase. Finally, o-phenylenediamine and hydrogen peroxide are added and color is produced.

Using this type of assay many antigens are quantitated in the clinical laboratory. The procedure used by LaRoche[1] for the quantitation of carcinoembryonic antigen, in which the enzyme is horseradish peroxidase and the substrate o-phenylenediamine, is one of the oldest well established methodologies. According to the second type (Figure 1, B), an antigen is adsorbed onto the polystyrene plate wells, then the (human) sample is added and left to interact with the adsorbed antigen. Finally, polyvalent anti-human IgG conjugated with an enzyme was added and the way of coloring the solution is the same as before. An example of this type of assay that has been applied in the clinical laboratory is presented by Clonatec[2] for the

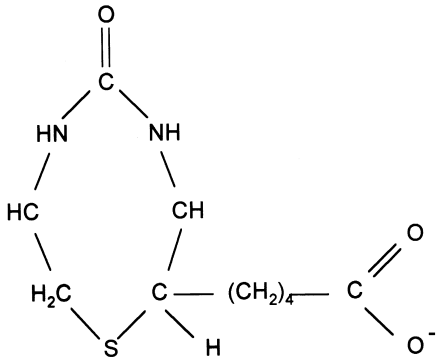

Figure 2. Chemical structure of biotin.

identification of antibodies against HIV in human serum, in which the enzyme is horseradish peroxidase and the substrate o-phenylenediamine. Similar procedures can also be used for the quantitation of any molecule, but only if antibodies against it can be obtained. This is not the case. The analytical chemist of the 21st century has a variety of tools that can be used alternatively to antibodies or in addition to them.

The discovery of the interaction between biotin (a vitamin) (Figure 2) and avidin (a 70-kd protein from egg white) has provided a very useful probe for less or non antigenic molecules. For the detection of a non antigenic molecule, biotin is first conjugated to it and then its presence is identified using avidin-enzyme conjugates. However, because of the patience and the persistence of the scientists, antibodies have been produced against even non antigenic molecules, such as carboxylic esters, aromatic hydrocarbons, etc., and used either for their detection or for their modification (catalytic antibodies).[3,4]

Another attempt to increase the sensitivity of these analytical methods is the optimization of the conditions used for the adsorption of the molecules to the plastic in order to have more amounts adsorbed. A different way to succeed is the use of ELISA plates in which active groups (-NH_2, -$NHNH_2$, -COOH, -SH, succinimide, etc.) are introduced to the polystyrene. In such active groups the molecule under investigation is bound via a covalent bond through its protein or carbohydrate part. An alternative way to immobilize huge amounts of molecules in ELISA plate wells involves an initial activation step with glutaraldehyde (OHC-$(CH_2)_3$-CHO), thus introducing -CHO groups onto the plastic surfaces. This methodology is mainly used for the immobilization of antibodies.[5]

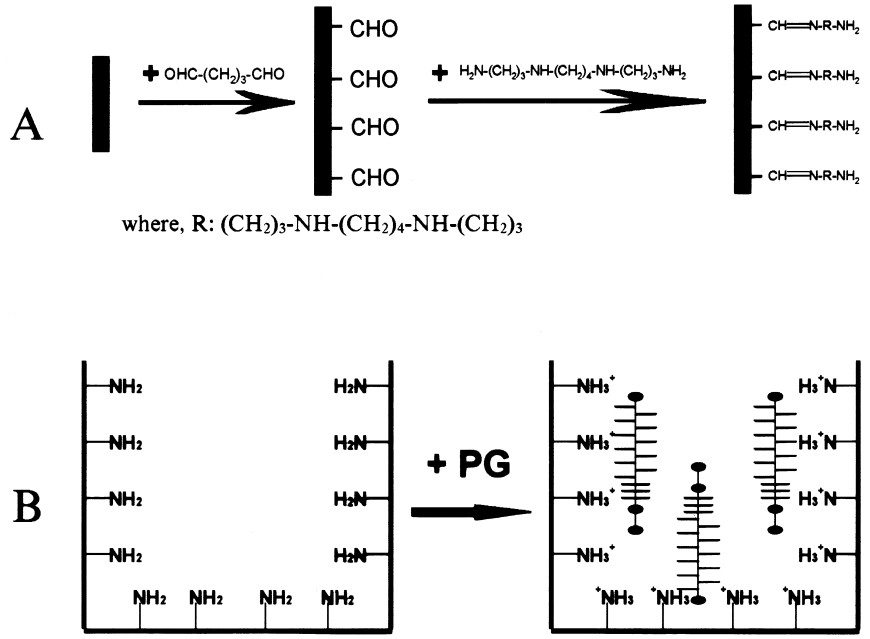

Figure 3. A. Activation of polystyrene by covalent immobilization of spermine: Onto the plastic wells, -CHO groups are introduced after incubation with glutaraldehyde, and then spermine is added to react with them, resulting to the introduction of amino groups on the wells. B. Interaction of spermine bound to plastic with aggrecan. The modified plastic wells of A can bind aggrecan via a simple electrostatic manner.

Starting from this activation step, and by the addition of a second one, a new procedure for the immobilization of negatively charged macromolecules was very recently presented.[6] It is used for the immobilization of connective tissue proteoglycans, for their quantitation and for studying their interactions with tissue proteins. According to this procedure, after the introduction of -CHO groups on the surface of a common ELISA plate using glutaraldehyde, spermine (H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2) is added which binds to the glutaraldehyde via a Schiff's base covalent bond (Figure 3, A). In such activated plates very high amounts of proteoglycans can be bound, in a third step, through their glycosaminoglycan chains either electrostatically (Figure 3, B) or covalently using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) as catalyst. The proteoglycans can then be measured by the use of antibodies against them. The sensitivity of this assay may be increased even more if biotinylated proteoglycans are used and their detection is performed using avidin-peroxidase conjugates. This type of microplate assay can be used for the study of the interactions between proteoglycans and proteins, against

which antibodies exist and for the sensitive analytical determination of the proteoglycans. Similar microplate assays can be applied for all the molecules that cannot be adsorbed directly onto plastic.

Other tools of the today's analytical chemist are a variety of microplate readers that can measure light or fluorescence emission, together with a variety of enzyme substrates to be used in such assays. These tools are not involved directly to the microplate assay and do not have any effect on it. They are used because of their very strong final signal.

Another very promising and also very attractive system for color amplification is the use of *in vitro* transcription of DNA and subsequent translation of the produced RNA, through which more enzyme molecules can be obtained than those initially conjugated with the antibodies.[7] According to this system the immobilized antigen interacts with a biotinylated antibody. Then, a streptavidin-DNA complex, where DNA is coding for luciferase, is added and left to interact with the immunocomplex and, finally, with an *in vitro* transcription and a translation system a lot of enzyme molecules are produced, and therefore, after the addition of the appropriate substrate, a very strong signal is obtained (expression immunoassay, Figure 4).

Solid phase assays may also be used for enzyme characterization, especially for enzymes that their substrate did not possess any physical property (radioactivity, fluorescence or light emission, strong absorptivity of the light, etc.).

There exist, however, additional types of quantitative or qualitative solid phase enzymic assays, that are more familiar to people working with enzymes. The most famous example of these assays is described as substrate electrophoresis or zymography, and means the initial electrophoretic separation of the enzyme(s) from any other protein on a gel, usually polyacrylamide, and the subsequent identification, on the same gel, of the enzymic band(s) by its (their) catalytic activity, after incubation in a suitable buffer. 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 the known analytical methods. It is rapid, reproducible, and largely quantitative.

The most usable type of zymography during the last years has the substrate copolymerized or entrapped within the polyacrylamide network. After the electrophoretic run, the gel is incubated in the appropriate buffer, and after the subsequent staining, the enzymic activity is recovered as white band(s) in a stained background. The results can be expressed in a quantitative way after scanning of the gel. The procedure has been extensively used for the characterization of metalloproteases (mainly collagenases), other proteases, and hyaluronidase.[8-11]

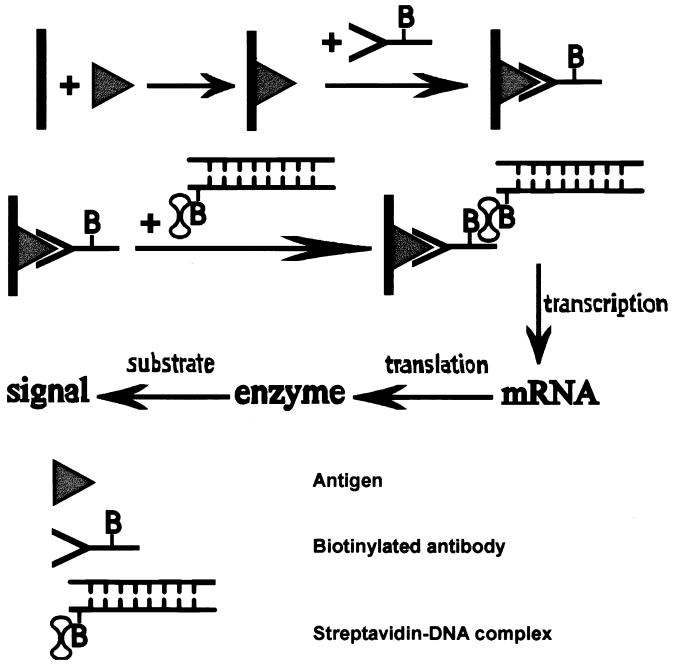

Figure 4. Schematic representation of the expression immunoassay: An antigen is immobilized onto a plastic surface, then a biotinylated antibody is added and left to react, followed by the addition of avidin complexed with a biotinylated DNA. Then the DNA is subjected to expression, a lot of enzyme molecules are produced and after the addition of a suitable substrate a strong signal is obtained.

Another type of solid phase assay is highly related to the affinity chromatography principles. It is mainly used for microseparations, but its use can be extended to analytical measurements or to studies of interactions between different macromolecules. It starts with the preparation of a suitable solid matrix containing one of the macromolecules under study. The matrix may be silica, starch, agarose, glass beads, cellulose, etc., in which active groups are introduced, and the macromolecule is covalently coupled to it via these active groups. The second macromolecule of interest, usually radioactive, either pure or as a mixture with other molecules that do not participate in the interaction under study, is added to a slurry of the solid matrix and incubated for the appropriate time. Then, the solid matrix is recovered by centrifugation with the macromolecule of interest bound to it. Using this procedure, it is possible to isolate a macromolecule from tissue extracts (purification of an enzyme), but it is also possible to determine the dissociation constant of the interaction between two macromolecules, as well as the strength of it.[12]

APPLICATIONS

Biochemistry

Interaction of Proteoglycans with Collagen

The collagens are the most abundant proteins in animals. They account for 25% of all proteins. The collagens form insoluble fibres, which are found as extracellular structures throughout the matrix of the connective tissue. The interactions of collagens with proteoglycans (aggrecan, biglycan, decorin, fibromodulin) have mainly been shown using histochemical and immunohistochemical methods. The difficulty of these methods is derived mainly from 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, can not be excluded, and it is very difficult to distinguish, in such methods, the direct and the indirect interactions. 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 exploit 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 4h the entirety of the collagen is under the form of fibrils. The molecule under study for its ability to interact with collagen is then added and its quantity is measured immunoenzymatically. Using this method the interaction of collagen type I and II with aggrecan, biglycan, decorin, the respective protein cores, link protein, fibromodulin, are performed, and the dissociation constant of the interactions is determined.[13-16] The assay is very simple, rapid and reproducible. It can be performed using whatever macromolecule, native or biotinylated.

Proteoglycan Quantitative Analysis

Proteoglycans are constituents of connective tissue extracellular matrix. They consist of a core protein onto which variable number of glycosaminoglycan and oligosaccharide chains are attached, thus the proteoglycan molecule becomes negatively charged. This property permits the electrostatic immobilization of proteoglycans either onto positively charged membranes or onto positively charged plate wells. Immobilization of proteoglycans onto positively charged nylon membranes either by dot blotting or

by electrophoretic transblotting and subsequent staining with alcian blue is proposed for the detection of small amounts of proteoglycans (10-50 ng).[17] However, for quantitative purposes, an ELISA procedure is needed. Such assay is described in the introduction section, and by using it, various quantitative analytical procedures can be performed.

Quantitation of Aggrecan Solutions

Aggrecan, the main proteoglycan of cartilage consists of a core protein of M_r of about 200,000 onto which about 100 chondroitin sulfate, 50 keratan sulfate, and 50 oligosaccharide chains are attached. Consequently, aggrecan is a high negatively charged molecule and this property is used to immobilize it electrostatically onto plate wells where spermine was introduced.

Microtitre plate wells activated with spermine can bind high amounts of aggrecan that are measured immunochemically. The absorbance obtained is linearly increased with the amounts of aggrecan added to the wells. The sensitivity of the assay permits the accurate quantitation of aggrecan amounts as low as 6 ng.[6,18]

Similar procedures can also be applied for the quantitation of whatever proteoglycan molecule. When antibodies against a particular proteoglycan are non existent, its quantitation may be performed after its labeling with biotin and addition of avidin conjugated with peroxidase.

Quantitation of Glycosaminoglycan Solutions

The analytical determination of free glycosaminoglycan chains is usually performed with simple chemical analyses (borate-carbazole reaction[19] or dye binding assay,[20] because the quantity of glycosaminoglycans in the various preparations is high compared with the sensitivity of these methods. Glycosaminoglycan analysis in samples derived of small tissue specimens or of cell cultures can be performed by HPLC or HPCE (for reviews, see ref. 21, 22) after degradation with selective enzymes. Solid phase assays are also presented for the quantitation of glycosaminoglycans down to nanogram level, the most applicable being those for hyaluronan and are based on the ability of hyaluronan to bind link protein[23] or G1 (N-terminal) domain of aggrecan.[24] For the quantitative analysis of small amounts of the other glycosaminoglycans, microtitre plates activated with spermine are used in a competitive solid phase assay.

The procedure is modified to be a competitive assay because antibodies against glycosaminoglycans have not yet been produced. Free glycosaminoglycan chains possess enough negative charge to be bound electrostatically to spermine and therefore to compete with aggrecan for spermine amino groups. The competition of the various glycosaminoglycans is influenced by their negative charge, the oversulphated chains of chondroitin and

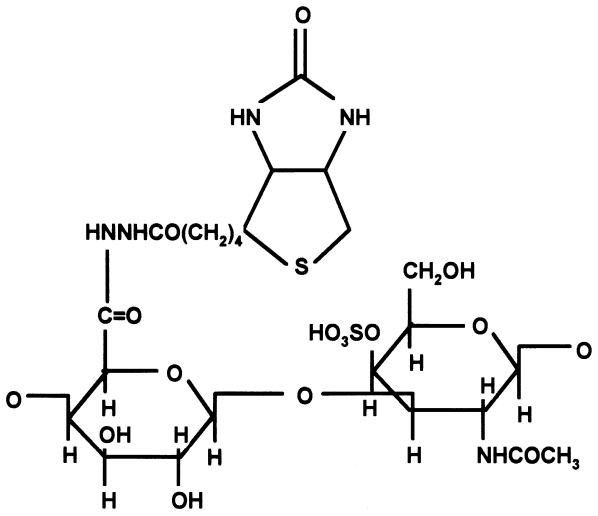

Figure 5. Chemical structure of biotinylated chondroitin sulphate disaccharide.

of heparan sulfate being the strongest competitors. With this method, glycosaminoglycan amounts as little as 10 ng (oversulphated chondroitin sulfate) or 100 ng (heparan sulfate) or 500 ng (chondroitin sulfate), can be accurately measured.

Glycosaminoglycans are also quantitated by a similar assay where aggrecan has been replaced by biotinylated chondroitin sulfate (Figure 5). This assay was more sensitive than that described above, and 4 ng of monosulfated glycosaminoglycans are accurately measured.[18] The linearity of the assay extends up to 80 ng. This analytical method is more advantageous than HPLC or HPCE in that a lot of samples can be measured at once.

Interaction of Lysozyme with Glycosaminoglycans

Cationic proteins can interact with the anionic glycosaminoglycans. When both are components of the same tissue, their interaction may contribute in tissue structure formation and stability. The interactions *in vitro* between such components may be examined either in solution or in solid phase, the latter being preferable, when very small amounts of one of the components can be isolated.

A small molecular weight cationic protein, lysozyme, exists in cartilage, where very high amounts of glycosaminoglycans, under the form of proteoglycans, are also present. The interactions of lysozyme with the various glycosaminoglycans are studied after immobilization of lysozyme onto

cyanogen bromide activated agarose. Once lysozyme is immobilized, the gel matrix is incubated in buffer solutions of increasing ionic strength with various amounts of radioactive glycosaminoglycans of different molecular weight. The gel is separated from the solutions after precipitation and its radioactivity is counted and plotted against the salt concentration.

Results from such experiments have shown that hyaluronan interacts very strongly with lysozyme. The interaction shows a maximum value at a salt concentration of 0.02 M NaCl and is abolished at about 0.04 M NaCl.[25] Its dissociation constant is similar to that of the interaction between hyaluronan and link protein, a cartilage protein that specifically binds to aggrecan and hyaluronan. Other glycosaminoglycans show either small (chondroitin sulfate) or no (heparin) interaction with lysozyme.[26]

Characterization of Oligosaccharide Structure

Various methods have been proposed for identification of the structure of oligosaccharides found in glycoproteins, including NMR, GC-MS, sequential degradation with enzymes and subsequent fluorescent labeling and electrophoretic separation of the digestion products, etc. A solid phase assay is also proposed, which involves covalent immobilization of the various monosaccharides in microtitre plate wells with the use of divinyl sulphone, their interaction with specific lectins and finally, identification of the bound lectins using antibodies against them.[27]

Based on these findings, the procedure can be applied for the quantitative analysis or the sequence determination of an oligosaccharide, after a suitable modification.

Quantitative Analysis

The assay described before, becomes quantitative when it is utilized as a competitive binding assay, where the competitor is always monosaccharide. The monosaccharide is added in the wells simultaneously with the respective lectin and, depending on its amount, different amounts of lectin are bound to it and not to that immobilized. Consequently, the determination of the monosaccharides present in an oligosaccharide chain can be performed after using the oligosaccharide as a competitor in the assay.

Oligosaccharide Sequence Determination

The same assay, as in case (i), can be used together with selective enzymic degradation of the oligosaccharide by exoglycosidases. The absence or the decrease of competition by the sequentially degraded oligosaccharide indicates the removal of concrete monosaccharide(s) derived of every enzymic treatment.

Clinical Chemistry

Quantitation of Autoantibodies

Autoantibodies are, as their name describes, antibodies produced by an organism against its own macromolecules. They are present in a lot of diseases concerning either a single organ or the whole organism. Diseases that are attributed to the whole organism are described as autoimmune connective tissue diseases, such as rheumatoid arthritis (antibodies against the Fc fragment of IgG), systemic lupus erythematosus (antibodies against nuclear proteins and against double stranded DNA), Sjögren syndrome (antibodies against acid nuclear and cytoplasmic proteins and against the Fc fragment of IgG).

Many analytical procedures have been presented for the detection and/or the quantitation of the autoantibodies produced by every type of disease, but in most of the cases, common autoantibodies exist, the distinction thus becoming difficult from the results of the clinical laboratory alone.

During the last decade, the scientists have introduced methodology to analyze the possible presence in serum (plasma) or urine of either autoantibodies against specific macromolecules or fragments of the macromolecules. Such methods are based on ELISA principles. The only demand of these assays is the adsorption of the maximum possible amount of the molecule under investigation in the ELISA plate well or, alternatively, to use a very sensitive detection system.

Recent results, derived from studies on the identification of autoantibodies against proteoglycans in autoimmune diseases, have revealed the presence, in the synovial fluid of patients with rheumatoid arthritis and psoriatic arthritis, of autoantibodies against the G1 domain of aggrecan. Such autoantibodies were absent from patients with other diseases. In these studies, a modified ELISA procedure was used. Aggrecan was first immobilized in nylon, then nylon was cut in small pieces that were put into the ELISA plate wells and the procedure was followed. However, using this assay, no autoantibodies against aggrecan were identified in serum[28] and, therefore, the method can not be routinely applied in the clinical laboratory. With the use of a procedure in which higher amounts of aggrecan are adsorbed onto the plastic wells, it is possible to identify autoantibodies against it. Such procedure is described above, and has been applied for the measurement of antibodies against aggrecan in the serum of autoimmune disease patients.[18]

Quantitation of Hyaluronidase in Serum

Hyaluronidase is an enzyme that cleaves the $\beta 1 \rightarrow 4$ glycosidic bond between β-D-N-acetylglucosamine and β-D-glucuronic acid of a hyaluronan chain.

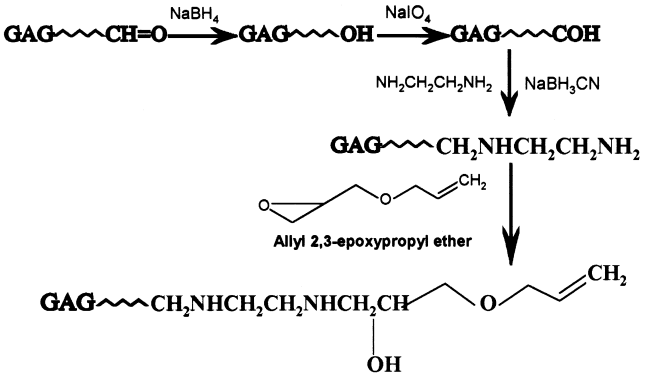

Figure 6. Modification of glycosaminoglycan to possess allyl groups at the reducing ends: The reducing ends of the glycosaminoglycan chain are modified by reduction, partial oxidation and reductive amination with ethylenediamine. The modified glycosaminoglycan reacts finally with allyl 2,3-epoxypropyl ether and thus allyl groups are introduced to it.

Hyaluronidase also cleaves the same bond between β-D-N-acetylgalactosamine and β-D-glucuronic acid of chondroitin sulphates A, B, and C. The minimum size of hyaluronidase substrate is a hexasaccharide and the major products are tetrasaccharides. Hyaluronidase activity can not be quantitated by conventional methods used in enzymic assays, because the enzyme acts on a poly- or oligosaccharide chains and the products derived of this degradation are of variable sizes. Their quantitation, therefore, from the generated reducing N-acetylglucosamine is not accurate, because reduction time is size dependent. During the last years, two different methods have been proposed for the quantitation of hyaluronidase units, both being solid phase assays.

The first method is a zymography and the substrate, hyaluronan, is entrapped within the acrylamide network. The samples are electrophoresed and at the end of the electrophoresis the gel is submerged in the suitable buffer and incubated at 37°C for the appropriate time. Then the gel is stained sequentially with alcian blue to stain undegraded hyaluronan and with coomassie blue to overstain the alcian blue stained hyaluronan and the non enzymic protein bands. The enzyme appears as white bands in a dark blue background.[9,11] Finally, the quantity of the enzyme and therefore the enzymic units can be quantitated after scanning of the gel at 500 nm. Another approach of zymography is also proposed and uses substrates of small molecular weight either chondroitin sulphate or fragmented hyaluronan. In this approach, the glycosaminoglycans are modified in their reducing end to contain allyl groups, via which are copolymerized with acrylamide (Figure 6).[29]

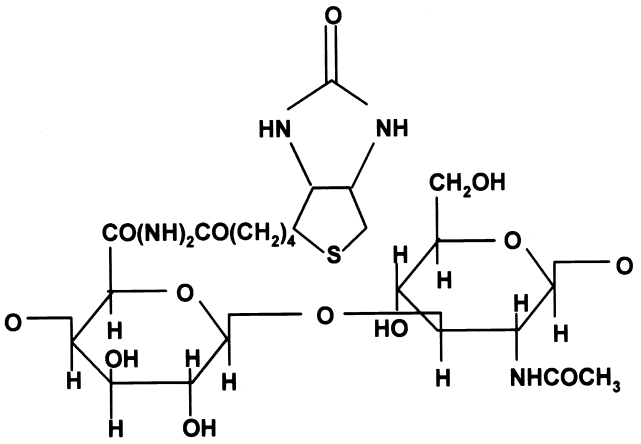

Figure 7. Chemical structure of biotinylated hyaluronan disaccharide.

The second and new method uses a microplate assay.[30] Hyaluronan is first conjugated with biotin using its carboxylate groups (Figure 7). Care is taken to have a sparse substitution of biotin in the hyaluronan chain in order to be susceptible by hyaluronidase and to have enough free carboxylate groups to be covalently bound to Covalink-NH microplate wells (1 molecule of biotin per 100 disaccharide repeating units of hyaluronan). After the covalent immobilization of hyaluronan, the samples diluted in the appropriate buffer are added and incubated with it. Hyaluronidase present in 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 color obtained is measured to give the units of hyaluronidase present in serum samples.

Identification of Metalloproteases

Metalloproteases are enzymes that degrade protein molecules in the presence of a metal cation (usually Ca^{2+}). They act in a variety of substrates (normal and synthetic) but the most important of the metalloproteases are those that degrade collagen. Collagen is the major constituent of basement membranes and any degradation of it leaves the respective organ susceptible to uncontrolled action of blood constituents. Penetration of the basement membranes via degradation of collagen network is thought to be one of the ways of tumors invasion, because tumors secrete metalloproteases in their environment. The identification and quantitation, if possible, of metalloproteases present in the serum of patients could be a useful tool for the diagnosis of early stages and the follow-up of cancer.

For the identification of serum metalloproteases, the classic zymography assay described in the introduction section, can be used. The substrate, collagen, is copolymerized with acrylamide, the serum samples are subjected to electrophoretic separation, and the gel is incubated in the appropriate buffer for a fixed time. Then, the gel is stained with coomassie blue. Metalloproteases appear as white bands in a dark blue background.

The most promising attempt for the identification of serum metalloproteases is a sandwich-zymography assay. Serum samples are first subjected to electrophoretic separation in polyacrylamide gel, then the gel is placed on a lighted and developed X-ray film (single coated), both submerged in the appropriate incubation buffer. The action of any metalloprotease present in the serum samples is directly seen by the degradation of gelatin (denatured collagen) of the X-ray film, as white band in a black background.[31] The procedure is very simple and rapid. Quantitative results can be obtained after scanning of the "degraded" X-ray film.

Whatever procedure is used, metalloproteases can be distinguished from other proteases, if a specific inhibitor (EDTA, TIMP, o-phenanthroline) is included.

Quantitation of mRNA From Malignant Cells

The detection and quantitative determination of a small number of tumor cells in the presence of a large number of normal cells, before the clinical appearance of tumor, is a difficult analytical problem in diagnosis and treatment of the various neoplasms. It requires analytical methodology with high sensitivity and specificity for the quantitation of specific DNA/RNA sequences characteristic of malignant cells.

Two similar procedures have been proposed to identify and quantitate small quantities of nucleic acids from malignant cells.[32,33] The tumor mRNA is first isolated and then reverse transcribed using the polymerase chain reaction (PCR). According to the first procedure, the product of PCR (DNA) is subjected to biotinylation and then immobilized onto microtitre plate wells, where streptavidin is incorporated. The immobilized DNA is denatured with NaOH and subjected to hybridization using specific probes conjugated with digoxygenin. The latter is finally detected with an anti-digoxygenin antibody conjugated with alkaline phosphatase. The sensitivity of the procedure is enhanced using 5-fluorosalicylate phosphate as substrate for the enzyme. The liberated 5-fluorosalicylic acid, after the enzymic action, forms complexes with Tb^{3+}-EDTA that are quantitated using time-resolved fluorimetry. The second procedure uses a biotinylated probe that is immobilized onto the microtitre plate wells, and the PCR product is labeled with digoxygenin. The protocol of the analysis is the same. Both procedures can quantitate mRNA from 1 malignant cell in the presence of 500,000-1,000,000 normal cells and they have already been used for the detection of leukemia cells[33] and prostate cancer cells.[34]

Biotechnology

Biosensors

The major, if not the unique, biotechnological applications of solid phase-type assays are the biosensors. They are devices of extremely small size that consist of a biological system immobilized onto a solid support (usually a plastic membrane) and a very sensitive detection system. The biological system may be a cell, a pure enzyme, or any other more or less sophisticated system. The detection system may be an amperometric, potentiometric, piezoelectric, optical, e.t.c. apparatus, or any other rapid and sensitive system.

Biosensors are used for either qualitative or quantitative measurements. They may be used for checking up of environmental pollution in chemical industries, for rapid clinical analyses of out patients and for a lot of other uses.

Biosensors become familiar because many types of them have been applied for routinely use as home tests. Examples are:

a) The determination of blood glucose of diabetic population: Glucose oxidase and peroxidase, together with a color producing chemical substance are immobilized onto a solid support. After the addition of a small quantity of blood, its glucose content is oxidized by glucose oxidase and produces peroxide that is reduced by peroxidase. The electrons are taken up by the color producing substance and its color is changed and compared with a reference.

b) The tests for urine constituent analysis that determine glucose, bilirubin, nitrite, ketone bodies, protein, urobilinogen concentrations, and additionally specific gravity, pH, blood and leukocytes. The reagents used for each analysis are immobilized in separate parts of a tape. After the addition of urine, variable colors are developed and they are compared with reference colors, from which the concentration of each constituent is determined.

c) The pregnancy tests that measure the amount (units) of the human chorionic gonadotropin (hCG) hormone in female urine. All of them are immunosensors, because the biological system is an antigen-antibody complex. Many types of pregnancy tests are offered commercially, depending on the company that produces each one. The last generation of these tests is a very simple, one-step procedure.

According to one of these tests, a sample of urine is poured onto a membrane where two different anti-hCG monoclonal antibodies (one conjugated with alkaline phosphatase) and indoxyl phosphate are present; the free monoclonal antibody is immobilized onto the membrane. If hCG

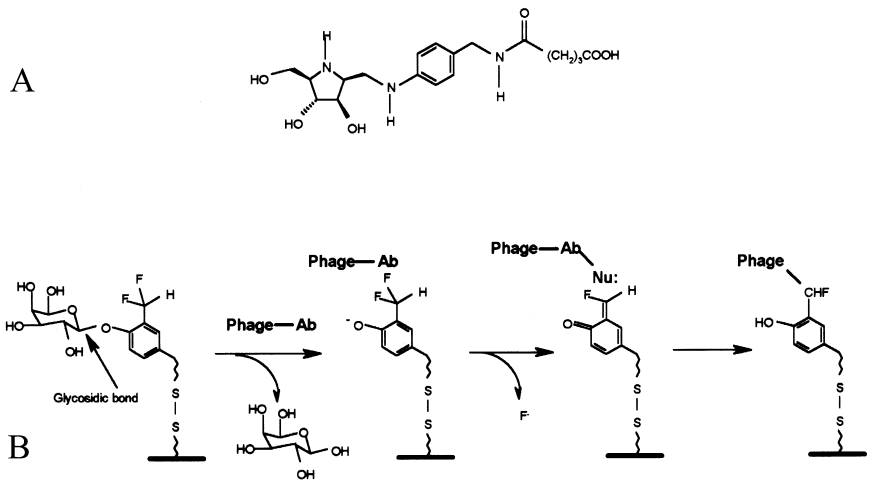

Figure 8. Representation of the catELISA. A. Structure of the hapten. B. Screening a library of antibodies for catalysts. The phage particles that produce catalytic antibodies cleave the glycosidic bond of the difluoromethyl aryl glycoside and the phenyl product is chemically active to form a covalent bond with any nucleophile in the combining site, and thus, the phage particle is entrapped covalently onto the plastic surface, from which it can be released after reduction of the disulfide bridge.

is present, the complex between the hormone and the two antibodies is immobilized on the membrane and alkaline phosphatase hydrolyses its substrate and produces the insoluble indigo dye (blue color). According to a second type of these tests, the urine sample is mixed with an anti-α-hCG monoclonal antibody labeled with colloidal selenium particles (red color). Any hCG in the sample combines with the selenium-labeled antibody and the mixture migrates to a region where a line of polyclonal anti-hCG antibody and an orthogonal line of anti-β-hCG:hCG complex have been immobilized. If hCG is present in the urine, the selenium-labeled anti-β-hCG:hCG complexes bind to the immobilized polyclonal anti-hCG and a plus sign is formed, denoting a positive result.

Preparation of Catalytic Antibodies

After the injection of a hapten to an animal, many antibodies are produced that bind to the antigen with a different dissociation constant. Scientists are in agreement on this difference and have shown that some of these antibodies may act, in an enzymatic manner, to a number of chemical substances. This property can be attributed to the diversity of the combining site, allowing

for recognition and chemical modification of a diverse set of substrate molecules. Unfortunately, the number of antibodies possessing catalytic properties (abzymes) is very small, compared to the number of antibodies produced, thus making the isolation of catalytic antibodies difficult or impossible with the known procedures.

A solid phase assay is developed during the last 5-6 years that seems to be very promising for the isolation of catalytic antibodies. This assay is termed catELISA (catalytic ELISA)[35] and it is quite different to the convenient ELISA. It is proposed as a method capable of screening a library of antibodies for catalysts. In a recently presented procedure[36] antibodies are raised against a hapten (Figure 8, A) to catalyze gycosidic bond cleavage. After subcloning into the appropriate vector, they are expressed as fusion proteins displayed on m13 phage particles. The substrate, difluoromethyl aryl glucoside, is immobilized onto plate wells via a sensitive covalent bond, a disulfide bridge. After the addition of phage particles onto the wells, the glycosidic bond is cut, leaving the phenyl product active to form a covalent bond with any nucleophile contained in the combining site. After removing the noncatalytic antibodies, the trapped phage particle is released by reduction, allowing for the recovery of the gene of the catalytic antibody and the subsequent expression of the antibody (Figure 8, B). Using this procedure, several catalytic antibodies are obtained and that having the greatest catalytic rate can be easily produced.

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